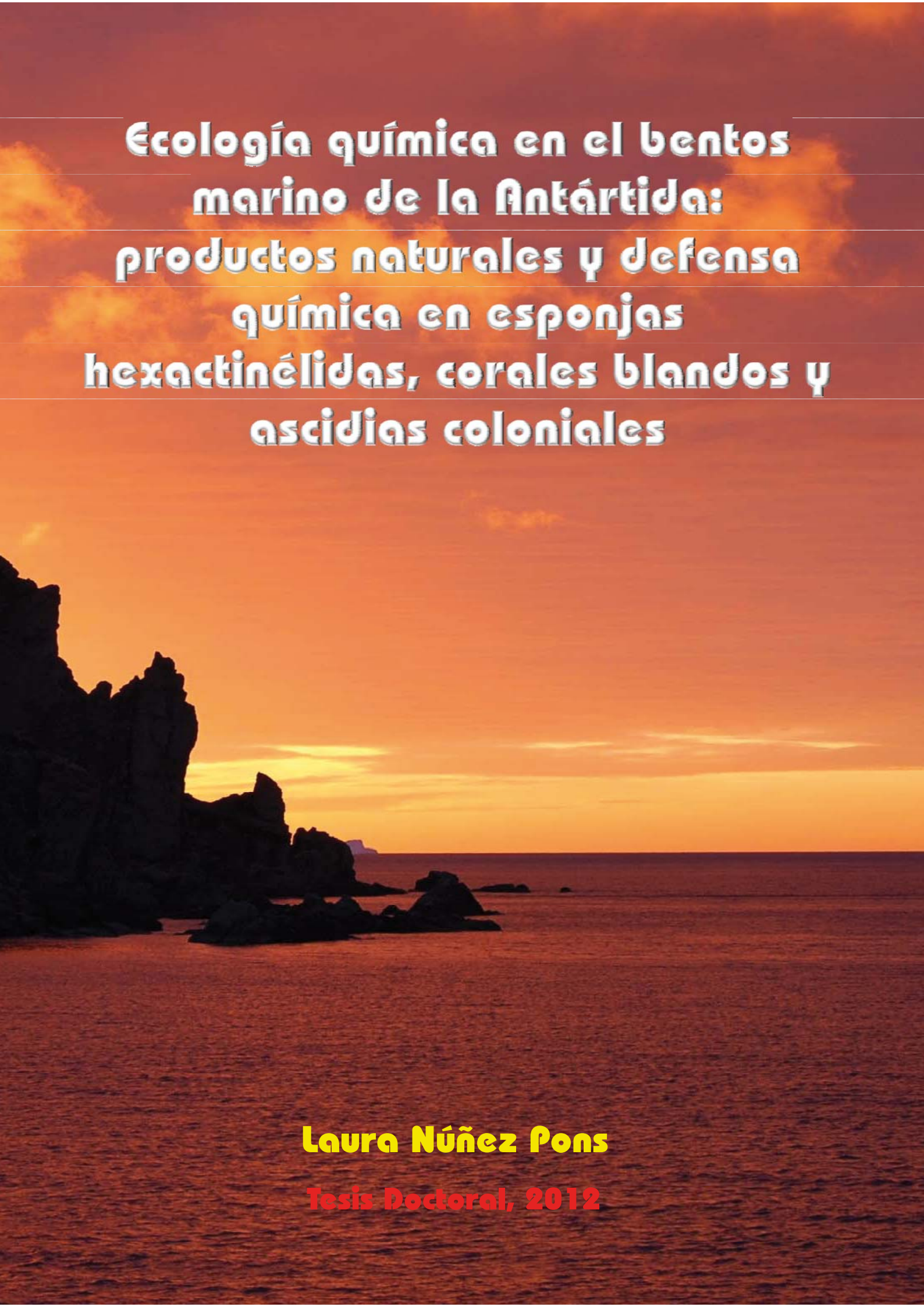
Ecología química en el bentos marino de la Antártida: productos naturales y defensa química en esponjas hexactinélidas, corales blandos y ascidias coloniales

Laura Núñez Pons



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Tesis Doctoral, 2012

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CHEMICAL ECOLOGY IN THE MARINE BENTHOS FROM ANTARCTICA: NATURAL PRODUCTS AND CHEMICAL DEFENSE IN HEXACTINELLID SPONGES, SOFT CORALS AND COLONIAL ASCIDIANS

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Julio 2012

Foto de Portada:

“Atardecer en las Shetland del Sur”

Foto de Javier Cristobo (Cálico Bentónico)

TESIS DOCTORAL

Facultat Biologia - Departament Biologia Animal (Invertebrats)

Programa de Doctorado: Ciencias del Mar

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CHEMICAL ECOLOGY IN THE MARINE BENTHOS FROM ANTARCTICA: NATURAL PRODUCTS AND CHEMICAL DEFENSE IN HEXACTINELLID SPONGES, SOFT CORALS AND COLONIAL ASCIDIANS

Memoria presentada por

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para a optar al título de

Doctora por la Universitat de Barcelona

Barcelona, Julio del 2012

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“Why can't man be more like animals?"

(¿Por qué el hombre no puede ser más como los animales?).


La Pantera Rosa.

AGRADECIMIENTOS… y otros relatos.....

Una pequeña referencia a la Antártida desde Valencia con amor:

"L'Antártida no està lluny…, ni molt lluny…, està a fer la ma!!!...

On mengen els pingüins."

En un lugar de la Antártida, de cuyo nombre, seguro consigo que acabéis acordándoos… existen unos fondos marinos misteriosos habitados por maravillosos seres… adaptados a condiciones extremas y duras. Es casi como pasar un invierno en Teruel, que sí que existe, y verte a esas abuelillas comiendo sopas de ajo. Igualmente, estos sorprendentes animales polares llegan a ser totalmente desconocidos para muchos, con esponjas que pueden vivir más que Chavela Vargas dopada con antioxidantes. Sin ellos nada de esto hubiera pasado, quizás la tesis hubiera sido mucho más sencilla, pero seguro no tan divertida ni intensa. A estos seres les debo todo lo que me han dejado descubrirles… Puede ser que en algo me engañen… (nunca hay que fiarse, ni con seres sésiles y blanditos como ellos, en esto de la ciencia hay que ser un poco gallego) pero han definitivamente contribuido a enriquecer mi pasión por la biología marina, todo dicho, a veces no me hubiera importado calentarme un poco más estudiando especies caribeñas... Pero en nada me arrepiento vestirme de cazafantasmas con nuestro traje seco DUI y bajar a visitar a estos curiosos organismos. Este lugar del que os aprenderéis el nombre sin quererlo, es el Mar de Weddell y el Archipiélago de las Shetland del Sur, con la Isla Decepción como punto de referencia, experimentación y residencia secundaria… De hecho, si hubiera

continuado lo del ladrillo, yo me compraba un iglú adosado con lago, y un leoncito marino de mascota… Y por supuesto me apuntaba al comité de fiestas locales, que no son pocas.

Mi padre, al igual que mis animales de estudio, no ha sobrevivido a mi tesis… , la Antártida es dura, y todo ocurre con mucha lentitud… pero cada uno ha tenido sus causas. Ahora, que me registren, no hice ningún extracto con mi padre. Si alguien tuvo la posibilidad de conocerlo físicamente, habría pensado que se pasó media vida buscando a la Pantera Rosa (era igual que Peter Sellers)… No, en realidad arreglaba corazones, pulmones y otras cosas… también rompía unas cuantas pero de esas sin vida conocida. Me hubiera encantado que estuviese aquí, aunque no hubiera aguantado una hora y media de tesis sin fumar… con lo cuál una cosa que se ha ahorrado. Me he dado cuenta que de pequeña detestaba a los enfermos porque me quitaban el tiempo que podía estar con mis padres, que trabajaban como mulas… Mi padre adoraba a los enfermos, pero detestaba a los médicos, me decía que eran todos unos gilipollas, y que nunca se me ocurriera estudiar medicina como él. Sin embargo, cuando le dije a mi padre al acabar el instituto que de estudiar algo, quería estudiar Biología o Filosofía, mi padre echó el grito en el cielo…. ¡te morirás de hambre! decía… Ahora se arrepentía de haberme inculcado ese odio hacia los médicos, a lo que añadía: pues hazte forense, tratarás con policías, detectives y asesinos, y los enfermos no se te quejarán… pero hija mía, biología, filosofía… yo no voy a vivir para mantenerte. A lo que yo respondía, bueno, al menos o estoy con locos o con animales, pero no con enfermos y gilipollas… Ahora, me gustaría que viera que, aunque no está dicho que no me vaya a morir de hambre, al menos he hecho algo dejando mucho esfuerzo y dedicación, y de lo que estoy orgullosa. Lo de librarme de enfermos y gilipollas, casi me lo he ahorrado, lo de locos ya… menos mal que, por si acaso, siempre tenemos a los animales.

Una persona paciente y parsimoniosa es lo que debió ver mi Jefa en mí al conocerme… nada más equivocado para casi todo, pero por el camino yo me entretengo, y eso engaña, y en algunos aspectos, estos años de ciencia me han enseñado. Un buen día de cumpleaños, cuando hacía menos años que una esponja de cristal, pero más que un anfípodo, me llamaron (una tal Conxita Avila a la que intentaba venderle mis servicios de buza, bióloga, y potencial doctoranda como fuese), y me hicieron el mejor regalo de cumpleaños que me podían dar… ¡¡PharmaMar me contrataba y me pagaba una campaña a la Antártida!!!... Impresionaaaaaante… considerando que yo me encontraba enterrando un tejón atropellado medio descompuesto por el norte de Cataluña, la noticia hacía tronar mi cabeza y las Campanas de San Juan del centro cerebral del "¡eso hay que celebrarlo!". La noticia me vino tan a gusto, que ya ni me olía el cadáver del mustélido ese, y fue un entierro bastante alegre, con aperitivo de celebración… Así, poco más o menos, empezó el contacto con-tacto con la que se convertiría mi directora de tesis, Conxita. Ella se ha convertido en mi "madre científica", hemos discutido muuucho, reído y nos hemos abrazado, la verdad que la quiero, me sigue sorprendiendo en cierta cosas, y eso me

gusta… y considero que tiene unos ovarios que ya quisieran algunas avestruces a veces. En particular valoro que durante su tutela he aprendido a ser independiente y a no encogerme en lo que haga, y a tirar pa' alante… como ejemplo de cosas que no se aprenden en los libros… Y por supuesto valoro en los últimos meses el intenso tiempo que ha dedicado haciendo puzzles en su agenda para a corregir esta tesis. Aquí también incluyo a su familia, a Rafa y a las niñas y al perri, y madres hermanos et al., por esos momentos que nos das a nosotros y por esas barbacoas y calçotaes que no acabin mai!!!... Nada Jefa ¡por muchos años y que corra el Mont Ferràn!... Peeeeero, la paella ya la pongo yo…

A mi Jefa la conocí gracias a Manolo Ballesteros, al cuál le agradezco muchas de sus acciones primeras, como salvarme de una mariscada que tenía demasiado orujo, pero no tanto algunas posteriores. Otras personas que en lo profesional han estado desde el inicio son Cristobo (ves, estuviste desde el principio de la gestación… y hasta nos pusiste delfines de ría), Creu Palacín (contigo descubrí, a parte de esa emoción por animales pequeños y feos, también que en la meiofauna viven ositos de gominola, ¡los tardígrados!), Xavier Turón (me rechazaste como becaria, pero luego me has ayudado siempre que te lo he pedido), Eduardo (empeñado en buscar sanguijuelas cuando todos queríamos bucear, ¡sólo tenías que ir al parlamento!). Y también mi co-director, Manuel Maldonado, que desde que un día me sacó de dudas de que las esponjas carnívoras no tenían siete dientes (ni uno), le vi como un hombre sabio, aunque ahora me vuelven a salir las dudas con Bob Esponja. En el fondo me gustaría formar un grupo con él de Héroes del Silicio… yo creo que nos iría muy bien. Los primeros coletazos empezaron en el CEAB, donde destaco a Carmela (secretaria de voz sexy y amante de nuestras rosquilletas como buena valenciana), Ángel (el Schwarzenegger del centro y brazo de hierro, creo que era él a pulso el que conseguía organizar que cupiesen todos los coches en el parking), Gemma (qué eficiencia al teléfono)… Y de becarios, pues éramos unos cuantos, formando una secta bastante endémica de la que me salí porque empecé a tener apariciones "ceabianas". De los que más me acuerdo son de Begoña Ezcurría (nos vemos en Laurel, con o sin trompa, o con snow), Carmen (la reina de El Húmedo), Charlotte (mi querida compañera de piso francesa con su habitación llena de duendes, y como casi todos los franceses que vengo conociendo, ¡no le gusta el queso!), Marc (mi otro gran compañero fantástico, verdadero amante de la ciencia y del marxismo, pero no del orden, ni ninguno de nuestro piso, vaya…), Óscar (¡oscarminífero!), Paula Risas (huertico), Guillermo (¡esquiadas y gintonics cuando quieras ya!), Javi, Romero, David (pues casi que quedamos en Poo), Diana, la otra Bego, Johan (ser belga nadie dijo que fuera fácil), JeanCris, Ariadna, Adri (ya sea por construcción de adosados alicantinos, que por terremotos Emilia Romagnos siempre acabas yéndote donde la tierra se remueve), Joao (que ya sé que a los poliquetos no les gusta M80), Anna Hievas (how is the playground!), Johana la alemana, Ivone, Michelina (que no sé si considerarte blandengue o napoletana… ma che sei?...

me encantaría volver a recoger rovellons de mogollons!!!... pero sin dejarse el ojo en el intento… y la de ploreras con mails tontos…, y lo bien que me sienta como somnífero que hables por teléfono con tu madre… verameeeeente!... ¡guapa!), Ana Riesgo (nuestra maestra de ceremonias, directora de cortos, y consejera real del reino), Carmen la pirata, Estel, Patricia, Rafa, Miquel, Raffaelle, Alicia, Ester, Sonia y un etcétera…

Del CEAB como garrapatas, o como ratas al Fautista de Hamelin, seguimos a mi Jefa sus becarios y técnicos a su nuevo trabajo, y nos montamos nuestro hueco (a veces invasión…) en el Departament de Biologia Animal (Invertebrats) de la segunda planta de la UB. Allí las cosas no eran tan nuevas como en el CSIC, pero tenían más historia, más romanticismo, y el ambiente estudiantil siempre rejuvenece, y lo que he estado allí me he sentido muy bien. Entre las personas que han contribuido a ello están las Super Secres (de mis amores… menos mal que existís, pues el departamento sería un lugar de monótono de científicos locos, habéis sido sin quererlo mis confesionistas de dudas burocráticas, que después de lo existencial, es casi lo más incomprensible), Joan (el cartero, recadero, arregla marrones, dicharachero y chico para todo), Los Guasos (donde estén ellos, que se quiten todos los MacGivers del mundo, si es que yo creo que les das un corcho y te hacen un Belén giratorio, ¡por vosotros y alguna cervecita fría que nos debemos!), los chicos y chicas de los acuarios y mantenimiento de animales (esas nocturnidades para cambiarles el agua a los erizos y otros… a veces me arrepiento de no haberme pegado una erizada con vosotros… nos la merecíamos), algún que otro segurata de la puerta (por todas aquellas veces que mi tarjeta no funcionaba y me abríais sin contraseña), secretarias de abajo (siempre atentas y eficientes, ya podríais ir a hacerles un cursillo a las de la Universitat de València), la gente del Servei Cientificotécnics (impresionada me tenéis con vuestra competencia, vaya fotos… si es que hasta las diatomeas posan para vosotros). Ahora hablaremos de profes, empezando por Marina Blas (empezamos mal, pues yo pensaba que tus firmas eran como autógrafos de Marilyn Monroe, cuando eran mucho más que eso… luego mejoramos), Miguel Ángel Arnedo (mi colega pirata de mac), Humbert (aún no he probado el vino de tu tierra prometida), Marta Goula (siempre alegre con su tupper). Fuera de nuestro apartamento compartido quisiera mencionar a Santi Mañosa (siempre disponible, me has cautivado con tu carácter humilde), Pedro Moral (no te conocía hasta hace unos días… pero ha sido algo intenso, creo que en poco has conocido hasta mis gustos sobre arte, y yo algunos trucos… no sé si es tu nombre o qué, pero me has subido la moral en todas mis 192358 llamadas, y me has hecho de psiquiatra, gracias), Jacob (cómo olvidar ese inolvidable viaje con Karaoke en el Las Palmas… menos mal que te duchaste después de Bayers… y la canción Bienvenidos ya no es lo mismo, por cierto ¡tienes tomas delicadas que hemos de ver!), Marta Pérez (viste mis inicios más prematuros como directora de DEA, y estuvo bien mientras duró, no me hubiera importado quedarme en tu departamento, pero a parte de problemas de dineros,

siempre fui más animalesca que fanerógama), Susana (como estás de tribunal, te pondré bien… y sino también, en el fondo sé que te gustaban mis canciones y charloteos departamentales a volumen ambiental…), las algólogas Amelia y Toña (mis más íntimas algólogas, si algo tenéis es que sois majísimas y unas currantas, ¡enhorabuena por el artículo!). Bueno, y ahora voy con becarios y técnicos y estudiantes: Isa (asombrada me tenías de todas las cosas que conseguías hacer… y de ese acento en inglés que no aprendiste en un curso CCC), Chica Checa (nunca pensé que tendría ojos en la espalda… y eran sólo para mirarte por si me lanzabas una araña mortal, y tienes una nuca muy sexy), Leti (que lo sé, que lo sé que sos uruguaya, peeero, ¿por qué hablas como una argentina ché?... al principio pensaba que te pasabas el día jugando a sopas de letras uruguayas, luego descubrí que eso eran secuencias), Oriol (oye, que a ver si hacemos de una vez la merendola de becarios que trabajan en agosto como si de marzo se tratase…), Oriol Posilargo (desde esas alturas siempre me hiciste reír con tus locuras), Massimo (il siciliano!!!... entre cacas de tortuga nos conocimos, y luego vas y te sacas una FPU antes que yo… es que los genios suelen esconderse), Alberto Maceda (el pezonero de agua dulce que se cuela en congresos de agua salada… gracias a tus enormes conocimientos en piensos, conseguimos alimentar a bichos antárticos, ¡eso merece un gallifante!), Luigi el sardo (me encantaría volver a probar tu pan sardo a modo de tacos de ostias gigantes…), Kiku y su pareja de egagrópilos (vaya veranito, me divertí mucho con vosotros… ni el del 69 de Bruce Springsteen, vosotros venga a traer regurgitados de rapaces, y a buscar pelos de roedores, y nosotros venga a machacar bichos marinos… no si eso olía como un tanatorio, sobretodo si se quemaba algo), Mari Carmen (y sus muñecos, y sus ascidias invasoras), Rocío (al principio me acojonabas… luego hasta me enseñaste el SigmaPlot y sus secretos, el cambio fue verte disfrazada con trenzas en un cumple de Sergi).

Prossima fermata... Pozzuoli, ICB-CNR. Lì ho fatto praticamente la metà de la mia tesi, tutta quella parte chimica che ha riempito questo libro di cacchette di mosche, e di alveare di formule molecolari. Infatti, mia mamma dice che non sono più valenciana, ma totalmente puteolana, non solo per il tempo che sono stata là, senno anche perche veramente mi sento a casa!... Iniziando per il lavoro al laboratorio di prodotti naturali marini sono andata inizialmente per Guido Cimino, che era il co-direttore di tesi del mio capo (grande scientifico e saggio del mundo della chimica dei molluschi, non ho avuto il piacere da conoscerti molto, dato che, como se dice varie volte in questa sezzione non ho avuto il piacere di potere lavorare con i molluschi, ma non siamo razisti con gli altri animali marini, loro anche meritano lo studio, e pur'e un giorno mi faccio anche io una saggia di altre bestie invertebrate… ti tengo molto rispetto per i lavori che ho letto e dal inizio mi hai sembrato una modesta persona, sopratutto dopo sapere che vivi con tartarughe). Lá al lavoratorio mo troviamo al Capo, a Margherita Gavagnin (del primo giorno mi ha fatto sentire bene là, sempre ha stato per le cose importanti, ed ancora ho l'esperanza che

qualche giorno mi lasci la sua casa di Procida per un fino di settimana... hai visto che sono diventata brava e non rompo tante cose più…). Prima persona che ho conosciuto al suo laboratorio è stato Franco (che belle colonne abbiamo fatto e ballato per fare separare i composti, e quella estrazione mitica della placenta di bufala-*Aplidium*!... tutto sempre con un po' d'aiuto da qualche goccino di limoncelo ogni tanto), dopo tra qualche giorno ho avuto il piacere da conoscere a una delle persone più importanti di questa tesi, a Marianna Carbone. Che posso dire della mia strutturatrice favorita intima e personale!... Al inizio ha stato un po' fredda, si la fa un po' tirare, ma dopo si è trasformata in una delle donne che più bene voglio, anche essendo chimica!... e non è che solo abbiamo chimica tra di noi, ma anche altro, biologia ed ecologia. Lo so che li ho fatto lavorare con bestie che non voleva, e mai molluschi (io li dicevo: il capo mi ha punito senza molluschi), cara, ma le mie bestie sono anche carine... alla fine abbiamo fatto cose belle, anche se dovendo lavorare con metaboliti primari, che neanche impazzivano al personale... la desidero tutte le bone cose che si merita!. Al laboratorio da fianco ho conosciuto a la Letizia Ciavatta (Letty, che tremolava ogni volta chiedeva materiale di vetro...), Emiliano Manzo (il re delle metanolisi, e calcistico magrissimo che io pensava puliva paccheri per dentro), Guido Villani (o Willow, con la sua bella casa piccola e blu, le cene fantastiche con suoi licore, é veramente un saggio della natura, e un mio eroe, voglio essere come te di grande e viaggiare per fare immersioni sempre!), e anche sta le gente di altri laboratori, come Domi (carissima, che professionale del NMR, e tutto quello che mi hai tentato da insegnare su le cacche di mosca), Lella (tutta una soldata antartica e regina della spettrometria di masse), Debora (ti devo in brindisi!), Enzo (ancora non abbiamo fatto gara a correre), Maurizio (ti cambio la camper per il mio Peugeot), Alessandro (abbiamo riuscito a fare qualche spettro senza distruggere la macchina), Eduardo (ed il suo mercato di metanolo adultero deuterato), la Signiora Lina (il Dj di lavastoviglie), Angelo Fontana (tu neanche mi davi retta perche no lavoro con molluschi). Anche al ICB ho conosciuto al grande Aniello (il autista camorrista, che primo giorno mi ha tolto pa preoccupazione da perdere tutto il mio materiale d'immersione portandomi a mangiare gelato di pistacchio e guardando come le copie facevano le sue cose nel Laco Averno, dopo anche mi ha fatto conosciere alle bufale che fano il latte delle mie favorite in assoluto mozzarelle!, grazie!!!), a Genni (bravissima e precisa come un orologio svizzero), Giovanna (quella bionda stravagante), Antonella (lo so che ti piaceva mia tortilla liquida, prossima volta porto cannuccie), Francesca (quando assaggiamo più vino di casa tua?), Markus (tutte quelle cene a casa, e mai hai fatto la lasagna di batteri marine), Yosur (you are a danger with the mortar!!!), Juang (so cute our little Chinese beer drinker at home, we miss you!), Ian (Chiny-Tinny), Fatima (My great roommate Miss Cous Cous and Miss Collone Silica gel!!!), Dolores (¡qué findes más llenos de actividad!!!... a veces hacía falta trabajar para descansar...), Javi Fallero (menos mal que nos teníamos para cervecear entre tanto mozzareleo), Gregorix (dobbiamo organizare una immersione a Ischia, conosco un posto dove...), Estela y Claudia (no

coincidimos casi, pero estuvo bien mientras durò... cuidado con las muelas Este!)..., e non dimentico una sera lavorando alcune di queste persone per preparare miei campioni per Antartide... grazie mile!!!. Molto importante, sta la gente che mi fatto di assistenti nel sterno con i miei campioni, come Miriam (mia assistente personale della chimica paranormale... che brava, tu e io di copia potevamo rivoluzionare la chimica molto di più che i radicali libri!!!), la cugina Olga (la ballarina del Cheese Steak), Chica Boom! (devi venire a Valencia, là ci piace fare fuoco a tutto!). Finalmente, nominarò a gente come Paolo il guardiano di giù, Bruno (non è il suo nome, ma è un bruno buono e simpatico) che mi hanno dato una mano in sentirmi a casa.

Existe también gente mundana de congresos y cursos, que conoces y te llaman la atención y también te ayudan de alguna manera, así puedo nombrar a los griegos Vassilioss (you invited us to your lab in Athens at a wonderful local temperature of 45ºC … I really appreciate your conciousness for making us go sampling to Santorini to survive!... behind that Black dressing there is a nice big heart, and thanks for teaching me dance in Crete!!!), Fei (Hei! Fei, we have a lot of celebrations to make!), Kostas (wonderful guy, miss you…) et al. Y también hay otros variopintos como Peter Schupp (you conquered me with your work, hopefully I will catch you as an adviser…), Covadonga Orejas (me encantó compartir unas palabrillas contigo de corales profundos, fueron además bastante profundas como palabras…), Paco e Irene (en cuanto vaya por Santander os visito… a ver si me enseñáis el Cachucho en directo), Anna Adano (va que a la próxima conseguimos otra copa de la Chouffe), Carmen Cuevas (es que te comes el micrófono en los congresos… claro luego me toca detrás tuyo y me tengo que inventar algún chiste bueno…), Julijana (thank you also for sending those little tiny sponges which I though were earrings!), Alaa (let's see if I ever get to visit your Pharaon palace with *Aplysia* in the swimming pool) etc... Y antárticos, como Manuel Berrocoso (tierno lobo marino…), Rasgul (primo, que sí que bajaré a Caí y caerán unos pescaitos), LuisMi (¡el nuevo corretón!... y Rey Gaspar chungo…), Teresa y Pepe (¡qué pareja de cómic!), Inma (la andaluza más curranta y de peso más pluma, ¡olé esa Vulcanologa cañera!), Benito (una balsa de agua que da un buen rollete a las campañas…), Jose Manuer (el morenazo), Andrés Barbosa (ese gran antártico que me intentó enseñar que era más sabio valorar una planta superior con forma de matojo de césped chungo, que elefantes marinos, focas, leones marinos o pingüinos… pues a veces la ignorancia mola…), Paco (el pingüinólogo de pico más dicharachero, y amante del chocolate sustitutivo…), Ana la mejicana (hay que hasta compartimos ropa interior… pero no olvidaré esos "pollotes" de los pingüinos, cuando te parecía que empollar era malsonante…), Manolo (¿soy yo una de tus rubias?...), Hilo (que se ha hecho un hijo de Utah), Cris (un alemán afincado a Barcelona y a Juan Carlos I), Ana Ramos, Álvaro Peña, Ignacio Olaso, Fran Ramil etc… O personas que han contribuido aportando material, como por ejemplo preciadas hexactinélidas, así tenemos a Thierry Pérez (beautiful night at Sharm el Sheikh and wonderful dinner!!!... your

Oopsacas is in good hands, or in God's hands…), Sally Leys (I ate all your coockies *Aphrocallistes*… they were very good), Dorte Jannussen (I have to visit your glass sponge Collection, after discovering these beauties). Están además aquellos que me han ayudado en ciertos aspectos del trabajo, y ahí remarco a Jaime Rodríguez que ha trabajado varias veces con las meridianinas de nuestras muestras (esas meridianinas no sé si nos darán de comer, pero tú te tienes ganada una cena!), y junto con él Carlos Jiménez y Rosa Mª Nieto, Mecedes Varela mi ascidóloga particular (esa Merchita qué lejos se te quedan las ascidias coloniales ahora…) en combinación con Alfonso Ramos (el Lucky Luck del desierto de Alicante), y Pilar Ríos (esa esponjóloga que no deja espícula sin definir). Extrañamente otra persona que me ha ayudado indirectamente con sus inventos es Ferrán Adrià, pues su kit para hacer caviar es el que usamos en algunos de nuestros experimentos. Con este cocinero estuve hablando en una ocasión, se interesó mucho por el experimento, pero no conseguí que me invitara su restaurante.

No en la Antártida, no hay morsas, ni osos polares (morsas porque no hay buenos dentistas y osos porque en el agua se disuelven…), pero tenemos militares, que nos traen Rioja Reserva, Bombay Sapphire y hasta jamón de pata negra. El primer año mi madre en una conversación desde la base me preguntó que si comía bien… yo mirando fijamente a los 3 cochinillos que teníamos en la mesa le respondí que más o menos… Allí nos dan apoyo, y algunos buen rollo, y me gustaría destacar a algunos terrícolas especiales como Jose cocinero de Parla (¡qué boquita de esparto!… para retransmitirte en horas fuera de horario infantil, peeero la mar de majete), Jose cocinero valenciano (tierno como tus platos, y elaborado…), Juanjo (qué puedo decir de todo un señor que no se le caen los anillos ni para revisar la junta de la trócola…), Javi Franco (sin pegas), Juanjo Monje (casi te come un págalo), César (con toda la cobertura matinal), Pedro el electricista (que arroces mallorquines maaare), Bea (la comunicadora con las galaxias… y compi de habitación, a ver si te veo por el Río de correteos, y hacemos estiramientos de esos), Aitor (si me estiras como a Bea, me convierto en la mujer de Boomer), Carmen (vaya peligrín con los colorantes alimentarios) etc... Y ahora a los marineros, como el gran Santi (ya quisiera Fernando Alonso hacer lo que este ejemplar con la zodiac… que parece eso el Dragón Kaan en tempestad, pero tú sin despeinarte), Gerardo de la campaña del 2006 Tembleque (el Joaquín Sabina de los mares del sur), Contramaestre murciano Cálico (¡esos asiáticos que te dan superpoderes!), los fantásticos zodiaqueros junior, grandes promesas Fernando, Adrián, etc… y en general a toda la dotación del Las Palmas de este año. El Ruiseños Xente (esa vocecita linda que te caracteriza, qué alegría tenerte berreando mientras achicabas agua de nuestros pies con días de falta de sueño, ¡viva la ternera gallega!), y como no el Jefe de Máquinas (de nombres variables, según la ocasión… pero de amistad noble y cálida, pues no te reíste tú poco cuando intentaba marisquear algas en la Isla Snow y las elefantas marinas querían jugar al waterpolo conmigo… ¡¡esas nocheviejas en Ushuaia no nos las quita nadie quillo!!!). También nos dan

apoyo civil invalorable la UTM, con personajes como Miki (tienes una capacidad de tranquilidad y sosiego que me da hasta risa, ¡eres un campeón!... y ahora además Jefe de personal…), y toda su trup.

Por supuesto todo esto hubiera sido harto complicado sin financiación, y eso se lo debemos a nuestros proyectos con Conxita como IP y becas personales disfrutadas. Entre los proyectos, todos financiados por el Ministerio, bien de Educación y Ciencia, bien de Ciencia y Tecnología, o bien como lo rebauticen pero siempre nacionales, están los proyectos ECOQUIM (REN2003-00545, REN2002-12006-E ANT) y ECOQUIM-2 (CGL2004-03356/ANT. 2005-2007) y los ACTIQUIM-I (CGL2007-65453/ANT. 2008-2010) y -II (CTM2010-17415/ANT. 2011-2013). Dentro de las becas destaco mi FPU predoctoral de 4 años maravillosos concedida por el Ministerio, mi primera beca para ir de campaña antártica, que fue de PharmaMar, y una I3P de postgrado del CSIC que disfruté poco tiempo hasta que llegó la FPU. Por último están las ayudas para hacer estancias, a parte de las estancias incluidas dentro de la FPU, como la concedida dentro del proyecto REDES (CTM2009-06185/E. 2010), una acción integrada (HG-2005-0027), y bolsas de viaje varias para participar en congresos y simposios concedidas por la Universitat de Barcelona en su mayoría, o por los propios comités organizadores, además de la IAS (International Association of Sedimentologists) y PSE (Phytochemical Society of Europe).

Creo que ha llegado la hora de introducir a mi grupo de trabajo… Somos los "Conxitos", y tenemos hasta nuestra canción. Famosos en el Polo Sur, en el Departament de Biologia Animal de la UB, pero sobretodo nuestro nombre rompe en Argentina. Ahora entiendo por qué mi Jefa, con lo entera que es ella y lo ella misma que es, al llegar a tierras patagónicas se cambia el nombre por Concepción, y es que es todo un concepto. Los Conxitos chicos son, en primer lugar por horas de vuelo está el Sergi (se convirtió por un tiempo en mi conciencia, y siempre ha estado receptivo y servicial, luego hemos tenido nuestros más y nuestros menos, pero es que somos esencialmente de caracteres distintos, y de la variedad salen los grupos especiales), luego vendría Cristobo (ya te he nombrado, pero desde que entraste a ser Conxito recibiste un hechizo de una meiga polar y te convertiste en Super Héroe… y ahora has ya pasado a ser Cálico Bentónico. Sin ti creo que estaríamos todos aún decidiendo si meternos en el Antártico o no… es lo que tienen los superhéroes, que consiguen conferir superpoderes a otros simples mortales, y ahora semos todos buzos polares. A parte de esto, creo que no hay tantos especialistas para reunir todo lo que tú resuelves, menos llevar la carretilla que se te da regular… Y ya para acabar haces unas fotos cojonudas, a veces hasta me sacas buenorra y todo, y por ello he decidido usar una de tus instantáneas de portada). Ahora cabría empezar la nueva remesa de Conxitos machos, y es que en el último tiempo la Jefa se ha decidido a incorporar chicos, pero además morenazos y guapos, que si no fuese porque además trabajan bien y son majos y listos, pensaría que estaba pensando montar un grupo tipo Back Street Boys… uno de ellos es Carlos (el de los truños

largos, y el Mortadelo del Grupo… va, que este año nos llevamos una caja sólo de disfraces… En fin, qué puedo decir, ¡la de momentazos compartidos en los casquetes polares, y de vuelta!, y me has hecho descubrir Hoyo, que no es algo que se aprende a lo largo de una vida normal…), y Juan (el fervor de Pakistán, me encantas, soy tu fan… pero me caes un poco mal porque nada más llegar la Jefa te ha dejado tocar sus moluscos… menos mal que la naturaleza es sabia y yo ya me he enamorado de otros tres… Has sido toda una sorpresa, y tengo toda la confianza en que llegarás lejos, ¡por lo menos hasta el fin del Mundo!). Bueno, y ahora vienen mis queridas queridísimas Conxitas… ¡nosotras sí que la rompemos en Argentina!... y no veas cuando decimos que no estamos todas, que falta la Conxa mayor… En fin, por orden de socias del Club, empiezo por la Jennnnnny, o la Je (mare meuaaaaaaa… la de cosas que hemos pasado juntas… tanto de rubia como de morena, como de café con leche como de moreno negro zumbón, ¡eres un bombón!... Por fuera y por dentro ehhh… de esos rellenos de cosa jugosas, o de licor cuando estamos por ahí abajo… no sé qué haríamos sin ti, pero seguro que nada mejor. Lo tuyo sí que es eficacia aprobada y no lo de Cucal, ¡ay mi mulata!), la Blanke (de Reus, aunque ahora igual se hace un poco de Nueva Zelinda… pero veo más probable que lleve allí a los Juanchis que, que ella se convierta al guirismo… Briozorrona sin frustraciones ni nematofrustraciones, espero aún una calçotada en tus queridas tierras…), Neus (nos conocimos poco, pero me das buena espina…), María (no te conozco, pero si te gustan los poliquetos y los huesos de ballena, creo que estás aceptada). En general este variopinto grupo de los Conxitos es una piña, y da gusto trabajar en este ambientillo. Aunque creo que nos debemos bastantes barbacoas, calçotaes, y ¡sobretodo una paella en tierras de paella!... A casa de la Rubia se ha dicho…

Como todo en la vida, sin unos desagradecimientos no se valoraría lo bueno que has recibido, y aquí también hay una sección para esto. Se lo desagradezco al algunos pocos animales antárticos, que se negaban a comer las deliciosas y trabajadas dietas que con todo mi amor les preparaba, frustrando así mi dedicación como cocinera de invertebrados polares… ni aún haciéndoles el "avión"… Y ahora entiendo los que de pequeña tuvieron que soportarme a la hora de comer… y por qué mi padre decía: "de pequeña estabas para comerte…. Y ahora me arrepiento de no haberte comido". Pero, bueno, aquellos animales quizás sabiendo que nosotros a veces comíamos centollos, pues se revelaban a comer pienso deshidratado, aunque fuera con caviar de Ferràn Adrià. Bueno, a Ferrán en cierto modo le desagradezco no invitarme al Bully. Pero a quien sobretodo se lo desagradezco es a algunas personas que por no quererme conocer mejor y no comprenderme han desconfiado de mí, y poniendo sus intereses propios como primera moneda, han conseguido llegar a esta sección. A diferencia de mucha gente, me considero bastante más noble que mis partes, pero como a todos hay que conocerme, y las bromas no impiden ser trabajador, ni leal, ni fiable. En mi opinión esto es como en la naturaleza, hay que tener el coraje de los animales de probar alimentos nuevos para poder

determinar si son comestibles o no, y la mayoría algo tienen, por eso no es bueno comer demasiado siempre de lo mismo, sino variar. Algunas de estas personas están también entre las agradecidas, pero el tiempo, o algunas situaciones les han vuelto egoístas, lo cuál no quiere decir que sigan siendo unos desagradecibles, lo serían en determinado momento, otros en cambio sí. Luego en concreto sí que existe un ser que yo conozca que creo fue siempre malo, y de eso nadie teníamos ni idea, y es que para ser malo hay que valer...

Bueno, ahora creo que toca un poco de ocio y vicio, y empezaré hablando de mis novios o amigos con los que he mantenido algo más íntimo íntimo. Con David compartí una de esas muchas formas de relación entre dos, ¡durante cinco años!, la más larga… no llegó a vivir la tesis esta en sí, pero sí que vio los primeros coletazos y mis deseos por dedicarme a la biología marina. Daviki expele buen rollo por todos los poros y eso siempre se agradece. Antes he hablado de un entierro de un tejón atropellado, sí esas cosas y otras muchas las hacía con Guille, mi compañero de todo durante más de tres años, aunque en varias fases. Me ha enseñado muchas cosas sin darnos cuenta, menos una perfección en la ejecución de la lengua catalana, y gracias a él hay un toque catalán en esta tesis ¡me he divertido mucho contigo y espero así siga morenàs, pelut, morrut!... Ximo fue novio del instituto, pero en realidad siempre fue más amigo que otra cosa, sólo que fue el único rubiales que me cautivó… es que es más simpático que las pesetas… Desde que llegué a Barcelona para llevar a cabo mi carrera de bióloga marina viví en muchas casas y con varias personas, empezando por el principio, en Escudellers. Allí me encontré con Tania (tuvimos vidas paralelas durante varios años, ella es uno de esos seres celestes que hacen falta en todas las casas, o al menos de vez en cuando para tomar una cerveza), y con Xavi y Cris (genial pareja inseparable y nuestros papis de Barcelona). Luego me mudé al Raval, con Blanes de por medio, y allí viví con Sachais (dulce brasileña gemela de Miss Culo, pero que ella misma podría ser Miss de tantas otras cosas). Del Raval pasé al Poble Sec, donde estuve con Meri (divertidísima y noble, a ver si volvemos a Ibiza…) y Tanya la Tocha (la suiza más divertida que he conocido). Luego me pasé a un pisazo con barra de bar setentera que tal era lujoso que se llamaba PDL (Pis De Luxe)… donde habitaban Rut (mi consejera de cosas de zonas bajas) y Loic (un francés más alto que la Torre Eiffel), y ocupaba la cama de luxe, casi más grande que la propia habitación de Vanessa Truños (Che! Que majeta y fantástica… Nápoles nos unió, y Barna nos reunió). Fue el único piso que mi madre no consideró un agujero. Y en mi estancia en Barcelona muchas personas se han convertido en amigas, a veces imprescindibles, y me han alegrado al vida, entre ellas Jacob (mi orco perfumado favorito), Martita (ojos de antiniebla), Vanessa mami (la más leal de lo mon), Mitxel (el Dj que más reparte), Franky (el terror de las nenas), Lluc (el misterioso Lluc Skywalker), Pepa (va que te alquilo la casa de mi madre para otro cumple), Jose (me sorprendiste con tu paella casera) y Maite (la Olivia del Poble Sec), La Bodega Saltó en general, el Quimet Quimet,

el Bar Tomás (siempre hay que tener un barista, que es más barato que un psicoanalista)… En Valencia me gustaría mencionar a mis amigas del instituto Aida (lo que semos capaces de hacer con un chubasquero y un bocata de tortilla de patatas… un Vega Sicilia chorizao), María (como te pille en martes, ni te casas ni te me escapes, ¡pendón!), y los de mi ex-grupo de música Laboratorio Funk, todos tenemos varios pasados y yo fui cantante. Ese grupo lo abandoné por la biología, lo cuál dice mucho, no es que fuéramos nefastos y aburridos y espantáramos a los pájaros en primavera, al contrario, nos lo pasábamos teta y hasta teníamos un grupo de fans y pipas… y estaba compuesto aparte de por mi hermano y Daviki de Xaume, Paco, Nachete, y ahora Luis, más otros que fueron pasando. También quiero recalcar a Nandito (uno de los pipas, y un alma alegre donde las haya, amigo de sus amigos), Carlos (otro pipa con risa explosiva) y Rocío (mola el C3 ehhh…), y a Irenilla (mi amiga del alma desde el cole). Existe otro personaje muy especial con el que hemos hecho tantas cosas, que es casi como un alma gemela, ese es Carlos, pero Carlos el de los cojones largos… lo nuestro no tiene nombre, pero tampoco apellidos, con lo que podemos respirar, no es un hijo… es una amistad indestructible.

Se está haciendo larguito este apartado de agradecimientos, pero es que son otros relatos también… y además, creo que a parte de agradecer a la gente que ha podido contribuir activamente a una tesis, hay gente que, aguantando tus innumerables ausencias, también merece mención. Aquí entran familia y amigos, pero empiezo por la familia. Mi Primo Mariano, que en realidad no es sangre de mi sangre, pero es mi primo antártico. Primo eres de las personas más especiales que conozco, me has ayudado siempre a todo en las campañas, y me haces reír de continuo, qué más se puede pedir… tenemos un artículo juntos, pero eso no representa ni la mitad de las cosas que hemos compartido y espero que sigamos haciendo tanto juntos… La familia de mi padre que es en esencia castellana y de Valladolid no se ven mucho, y es que como bien dice mi sabio tío Bernardito, del cuál soy una eterna fan, nuestra familia se quiere mucho, pero de lejos. La madre de mi padre, mi abuela Ascensión murió hace lo suyo, pero todavía la recuerdo con cariño extremo, y con ella estaba MariTere, que me vio nacer prácticamente y sufrió mi inapetencia por la comida hasta aburrir, a MariTere la quiero como a una madre y desde hace años no he sido capaz de ir a verla, sin ti no me hubiera acabado ningún filete. Mi padre tiene dos hermanos, Antonio, casi maño que con tía Pili tuvo cinco retoños ahora ya casi seniors y señores: Antoñito (padrazo), Amparo (con dotes de bailarina de barra como una chimpancé), Patricia (la que me roba los ramos en las bodas), Carolina (mi primitis compañera de sueños de Alicia), e Iván (el guapo bibliotecario). Y Fernandito, que con tía Margarita engendraron a Tanya (mi guapísisma prima morenaza con marido que es le más buscado por Valencia como un George Clooney, y con dos hijos que son sexsymbols, Luiy y MariFer) y Fernandito (a ver si conozco a tu enana). La familia de mi madre en cambio son casi sicilianos, bueno valencianos pero se reúnen hasta para cortarse las uñas. Aquí quiero destacar a

mi abuela Pilareta (que intenta enormemente en entenderme, aunque le esconda al Niño Jesús), mis tíos Sandro y Reme (qué puedo decir de mis otros padres adoptivos, con vosotros la vida tiene más luz… sois fantásticos), y sus hijas Clara (Clarix, mi doble con ojos azules y melenamen de Timotei) y Bárbara (como bien dice su nombre Bárbara), mis tíos Ramón y Ana y sus hijas María y África (no olvidaré nuestros veranos en Xàbia), y mis tíos Piluca y Octavio (con los manjares que nos hacéis en navidad, y las charlas gastronómicas con mi tío) y sus hijos Octavio, Piluquita y Elisa (con vosotros siempre aprendo modales a la mesa). Hay otros tíos más lejanos, como Mar, tío Eduardito (que siempre me resuelve cosas de mi coche) pero eso, quedan más a la lejanía. Luego en esta tesis tengo un primo consorte y con suerte, quizás, que ha sido el animador y creador de las viñetas… ese es Ricar, y es un artista que sabe plasmar una idea y mejorarla… gracias a ti esta tesis tiene una animación de nivel que muy pocas tendrán.

Y ahora hablaré brevemente, si sé, de la rubia más despampanante que habita sobre la faz de la Tierra, y esa es mi madre. No es que sea sólo increíblemente bella por fuera… es que, como decía mi padre, por dentro es aún más hermosa. La Rubia es un ser celeste, de algún lugar de la galaxia, que te hace sentir bien. Desde luego si no hubiera existido yo no existiría, pero si ahora no existiera, el mundo sería mucho más duro. Junto a ella, vaya desde que tengo conocimiento mis primeros hermanos de convivencia fueron mis perris, siempre todos ellos mastines españoles. Hemos tenido infinidad de ellos, pues es lo que tiene, se mueren… ellos me enseñaron a jugar, a ser un poco más animal y no perder del todo la conexión con la naturaleza salvaje, y hasta a hablar en cierto modo (quizá por eso no vocalice mucho). Entre mis perros puedo nombrar a Lara, Patón, Pons, Sultán, Babia, Pola, Melosa, Sintrón, Diana, Tara, Nubia, Elsa, Moa, Trufa… y ahora nuestra loca Coca. Les he querido mucho, pero, desde luego si alguien me dijera que mi mejor amigo me está haciendo una putada, no me iría a matar a mi perro, ellos sí que no me levantarían ese tipo de sospechas… El amor por los perros es especial, y sólo he conseguido querer a alguien como a un perro, y creo que es el amor más sano que he tenido. Bueno, y mi germanet querido… Joseán, estuve profundamente enamorada de él de pequeña, y es que mi hermano es un genio, pero no de esos que sale por una lámpara, no, aunque le siente bien el turbante como pudimos comprobar en una boda medieval, no, es que no soporta los sitios cerrados y pequeños… Mi hermano me ha dado grandes consejos, y sí es un genio de la creatividad y un líder con una presencia impresionante, junto con Trufi, Silvana, son unos pioneros del arte. Silvana me ha ayudado mucho en darle personalidad a unos cuantos de mis gráficos feos en inicio, convirtiéndolos en unos señores gráficos con armonía. Y qué puedo decir de Valentina, valiente y femenina… mi sobri favorita, con sus dotes de mono de circo pero ese terrible miedo a la oscuridad y a quedarse sola, y es que es tanta ternura junta… Una de las razones importantes de acabar esta tesis ya, es para conocerte mejor, como el lobo...

Luego hay siempre gente dispersa que te ayuda en lo estético y lo médico, en lo feliz y en lo doméstico... Sí como mi peluquera, que con cuatro pelos era capaz de hacer una "melenita", como en la postguerra cunado hacían pucheros con restos de los restos… sí Angelina la fantástica… o mi dermatólogo Manel, que ha confiado tanto en mis folículos a base de corticoides, como yo en mis esponjas, y me ha dejado la cabeza como un colador… O Josele, que es casi familia y un gran hombre donde los haya, como Pepito, además de la misma profesión, radiólogo remendón… O mi amiga Diana, que me ha puesto un trozo de diente ya cuatro veces… creo que no aprenderé a no morder cosas, ¿no hay de esos de muérdeme y no te rompas?... Y ahora, y desde donde escribo, desde una deliciosa casa con vista a la Patja de la Marbella de Barcelona me acuerdo de Cuca y Mariano… ¡me habéis dejado de okupa en una casa con terraza y palmeras!... qué sueño exótico lúdico-festivo si no tuviera que acabar de corregir esta tesis… haría un guateque tropical… ¿se repetirá la oferta, o es sólo para enfermas de tesis aguda?... Sois unos soletes.

A todas estas personas nombradas las he echado más que más, menos que menos, mucho de menos… y es que para escribir esto decidí recluirme del mundo de una manera absoluta, que ha venido a durar casi un año y medio… Y como si de una Doña Quijota me tratara, errante y caballera, aunque con poca cabellera por el estrés y mis rebeldes folículos, me dispuse a recorrer el mundo con una tesis itinerante… Primera parada, Benicasim. Allí descubrí lo fantásticos que pueden ser unos porteros sin o con siendo cotillas (oye de las cosas hay que hablar)… Se han convertido en una familia y me han sacado de casa, de fiesta y descubierto varios gin tonic's bares que hacen historia, os adorooo… ellos son Ximo y Merche. Entre otros también está Mamen, mi segunda comandante que ahora es además pitbullera… a ver quién se mete con ella… o Ester, que hace reír hasta a las ranas… o Gema la cartera, Víctor el peluquero, o Anacarda la gintoniquera mayor…

Don Quijote llegó a una tierra de molinos, que parecían gigantes… yo llegué a Cádiz, de la cuál los abuelos se quejan que en su día los gobiernos con el afán de promover las energías renovables, empezaron a poner "ventiladores" por todo Tarifa… -Quillo, con la de aire que hase aquí, y encima nos meten ventiladoreh-… Maravillosa tierra, con sus manzanillitas, su pescaito, la manteca colorá de La Señora Manuela, o su rabo de toro indigerible sin varios kilos de bicarbonato y chupitos de Fairy. Pero Cádiz, sobretodo maravillosa por sus gaditanos. Ahí he de agradecer a Domingo y a la Chari abrirme su casa y su simpatía, además de a sus colegas como el buenorro de David, etc (no es que sólo me acuerde de los buenorros, peeero, una es humana).

De ahí me dirigí ni corta ni perezosa a Asturias, a la casa de mis tíos Fernando y Margarita, que siempre me han abierto su casa, y con ello han contribuido a la ciencia (o eso creen), o al menos a que su sobrina escribiera a gusto entre sidras y cabrales, ¡os lo agradezco tíos!... Allí en

Llanes conocí a personas, que siempre marcan tu camino, como el de las tapas XXL, que me invitaba a sidritas y a platines… el lechero que me regalaba leche fresca sin hervir todos los días recién ordeñada y que me ayudó a subir mi colesterol a niveles históricos en mi historia, a los dominicanos del lugar, que acabaron conmigo entre Presidentes y salchichas de madrugada, las nutrias de la ría que no paraban de sorprenderme, los de la Casa del Mar y su papagayo que comía centollo y zamburiñas (así yo también insulto a la peña posado en una vara de madera)… Jacinto, de El Cabañón que me daba a probar de todo y luego pa casa a cuestas… Bueno, y por supuesto al Bar Roxin por esas fabadas con pantruque a la hora que fuese, y con doble ración…

Última pero no para menos parada… Napoli… Dicen que cuando ves Nápoles mueres… Yo al parecer me salvé, meno male!.. peeero no de su encanto. Nunca pensé que me enamorara de un lugar en absoluto, pues hace años que me considero nómada y polígama. Pues me he enamorado de Nápoles, y en varios sentidos, pero quizás porque allí he aprendido a enamorarme bien, es como lo de querer a un perro. La amo, pero no la pido responsabilidades, ni correspondencias, ni siquiera que se limpie o sea más organizada, puedo hasta vivir separada de ella… simplemente la amo. Cuando llegué por vez primera en el 2007, sin ningún interés por Italia, perdí mi equipaje de buceo, por rincoglionita que dirían ellos, vaya, la azafata no puso el destino bien, y yo no puse en nombre a mi equipaje. Pero al ver lo fantástica que era la gente, todo tenía mucha menos importancia de lo que ya en realidad le damos a casi todo. Empezando por Aniello que me recibió por primera vez, hasta el mismísimo Luigi. He pasado en Nápoles muuucho más de un año en total, y dicen que el enamoramiento dura un año científicamente… si es así lo nuestro ha de ser una excepción, o un caso a estudiar, y de ser así, tendré que volver a estudiarlo, quiero tomar esos datos por mí misma… Bueno, en estas estancias de escritura, donde he concluido cuatro capítulos y he hecho dos enteritos, he vivido con cierta gente realmente especial… Empecemos con Amleto… cuando le conocí y descubrí que su nombre venía de Hamlet no me lo podía creer, con lo que le dije que yo era Doña Quijota. Nos llevamos siempre muy bien, aunque decidiésemos discutir por una taza, entre otras… Si (mi sono rincoglionita con lo spagnolo) abbiamo discusso per una taza, ma alla fine la sua o la tua nobiltà come persona ti fa meritare anche quel nome strano da novella, come già sai, andiamo a farsi tante birrete e tante altre…e stai sempre invitato quà… Amleto era un uomo solitario e tranquillo fin che è arrivata Valeria, arrivata mi riferisco a casa, lei porta sempre un lato feminile che sempre ci vuole in qualche casa, ma aparte, quelle mozzarelle che sfidano la legge della gravità e quelli tiramisú che veramente tirano sú!... con tutto sei riuscita a temperare un po' alla bestia… E mo… chi viene… Ah si!!!... Anche Vittorio (un uomo di mare in montagna con un carattere bello che fa parlare anche alle pietre), e Nadia (porto ancora la tua catena, ma la chitarra non sona… mo ti vieni ad assagiare i nostri vini), e Sidra (la mastina spagnola piú bella e brava del Apenino!) e Cinci (che ha presso i Ricci da qualche umano vicino… tutti in sieme,

tutta una famiglia in Abruzzo)… e come no il grande zio Piero… Ma anche la mia famiglia cara in absoluto di Arco Felice, la Famiglia Cuomo, ma con Franca al capo… Franca (la cuoca pazza e con poteri sopranaturali), Pino (il avocato delle barzelette) e Mimmo (mio cugino tour oporrero). Dopo… E come no come persona di bar devo parlare dei bar… bravi quelli di Guanxi, Franco, Rachele, Umberto et al., del Vineapolis, con Salvatore, Stella e clienti vari, e la pizzeria Carboni come ultimo scoprimento!!!... ¡Ah! Hay una persona pequeña que a la vez es tan grande… Il piccolino Luigi, il patrone di casa, che un po' controlla alcuni ritmi di questo cuore. Un piccolo che mi insegna tante cose, con cui ho visciuto tanto, che amo come un cane, e con te voglio ancora vivere intensamente tante cose… Grazie in generale a tutti per sopportarmi in questa scrittura del casso!... Vi voglio tropo bene!!!...

Con esto no acaban los relatos ni mucho menos… pero creo que estarán muchos deseando empezar a leer mi tesis en sí… con lo que daré esta parte por acabada. Cuando a veces no tengo tiempo de leerme el periódico en su totalidad, lo que suelo hacer es mirarme la viñeta de Forges, que creo me da una idea bastante global de lo que sucede con el mundo… Para aquellos a los que esta lectura les haya parecido extensa, y necesiten un receso, o directamente dejarlo para otra tesis, les ofrezco una viñeta muy explicativa de lo que consiste esta tesis doctoral, que junto con la contraportada puede dar una idea general, si pararse a leer en detalle… ¡A disfrutar!.

¡¡Kesos!!!

LAU


A TRAVÉS DEL SISTEMA SE HA FILTRADO EL RUMOR...
...DE QUE VORACES DEPREDADORES PRETENDEN ATACAR NUESTRO **BENTOS ANTÁRTICO**
¡COMPAÑEROS! ¡CAMARADAS!
ROSELLIDAE
ALCYONIUM
ALCYONIUM
¡NUESTRAS COLONIAS ESTÁN ABASTECIDAS DE **PRODUCTOS NATURALES**, MI COMANDANTE!
APLIDIUM
SYNOICUM
BIEN...
...VAMOS A COMENZAR UNA
GUERRA QUÍMICA

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CHAPTER 1.

GENERAL INTRODUCTION

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1.1. Southern Ocean ecosystems: composition, ecological threats and adaptations

Most of the Antarctic marine benthic fauna evolved during the Cretaceous break up of Gondwana about 140 million years ago and the relative movement and separation of the forming continents, including the Antarctic continent (Clarke and Crame, 1989; Crame, 1992). The climate remained temperate to sub-tropical until 22 million years ago, when the establishment of a circumpolar current led to a hydrographical isolation of the continent, promoting a high proportion of endemisms (Arntz, 1999; Crame, 1999; Gili et al., 2000). Antarctic biota are therefore derived from relict autochthonous fauna, plus an eurybathic fauna from deeper waters, and also some cool-temperate species, mostly arrived from South America (Arntz et al., 1994; Brey et al., 1996; McClintock and Baker, 1997a; Barnes et al., 2006; Brandt et al., 2007). Despite some taxonomic connectivity that remains with South America through the Scotia Arc, acting as biogeographic bridge between Antarctica and the Magellanic region (Arntz et al., 2005; Primo and Vazquez, 2009; Demarchi et al., 2010), the current benthic marine invertebrate fauna is largely an ancient, endemic one (Aronson et al., 2007). As such, the benthos has had ample opportunity to evolve ecological interactions (Amsler et al., 2000a).

Antarctic marine ecosystems are characterized by low temperatures and pronounced seasonality, with broad periodic limitations of food resources. Despite some coastal shallow regions (less than 33 m) exposed to periodic disruption of the benthos by iceberg scour and anchor ice (Smale, 2007), the benthic communities appear to be somewhat stable environmentally and 'biologically accommodated' (Gutt, 2000). Hence, they are supposed to be largely regulated in distribution and abundance by predatory and competitive interactions (Dayton et al., 1974). The continental-shelf communities of Antarctica are markedly diverse (Burton, 1932; Koltun, 1970; Dayton et al., 1974; Dayton, 1979; Sirenko et al., 1997; Dayton, 1989; Blunt et al., 1990; Arntz et al., 1997; Brandt et al., 2007). They harbor rich suspension-feeder macroinvertebrate assemblages (see Fig. 1), dominated by sponges, soft corals, bryozoans, hydroids, and ascidians, as well as abundant macroalgae in the photic zone (Arntz et al., 1997; Gutt et al., 2000; Wiencke et al., 2007). Higher trophic levels include mostly high densities of crustaceans (De Broyer and Jazdzewski, 1996; Huang et al., 2007), as well as macroinvertebrates, typically nemertean worms, sea stars, sea urchins, sea cucumbers and brittle stars (DeLaca and Lipps, 1976; Dearborn, 1977; Gutt et al., 2000; Obermuller et al., 2010), and fish (Richardson, 1975; Eastman, 1993). Indeed, sessile and sluggish organisms are subject to intense pressure, especially through the generalist foraging activities of abundant nemerteans,

sea stars, and populations of mesograzer amphipods (0.2 to 20 mm length consumers; Hay, 1991). Moreover there are known specialized spongivores, such as the nudibranch *Austrodoris kerguelenensis* preying on hexactinellids of the genus *Rossella*, or the asteroid *Perknaster fuscus* consuming primarily *Mycale acerata.* The feeding activity of *P. fuscus* complements that of *Acodontaster conspicuus* in regulating the abundance of *M. acerata*, a rapidly growing and potentially space dominating sponge (Dayton et al., 1974). These biological factors, along with the slow growth rates and long lifespans that characterize this fauna (Clarke, 1983; Dayton et al., 1994; Linse et al., 2006), suggest opportunities for the selection for chemical defensive adaptations (Amsler et al., 2000a).

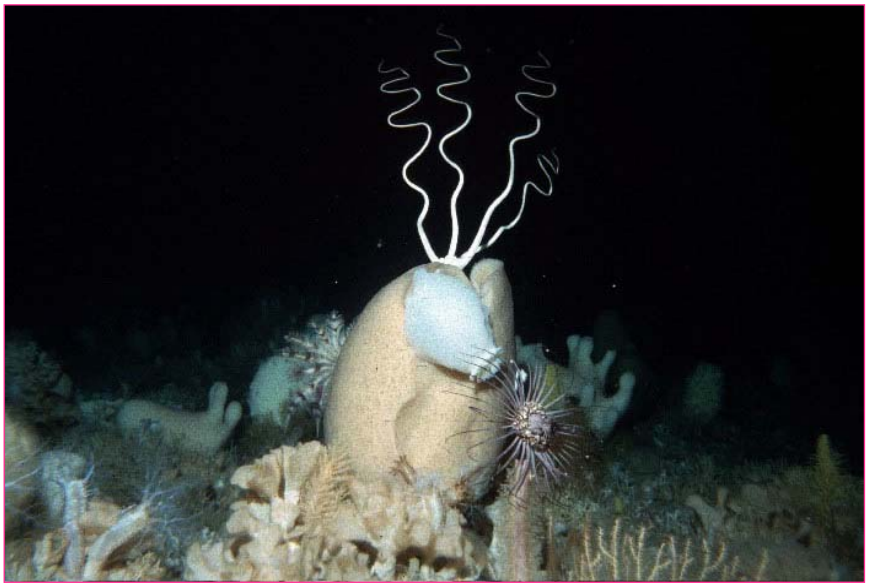


Fig. 1 Antarctic benthic community (J. Gutt)

In general, there was a long-held prediction that species interactions and predation pressure, linked with potent chemical defenses and reduced palatability, are gradually stronger at lower latitudes. But the few studies that allowed the formulation of this hypothesis were concerned only with tropical and temperate systems, leaving the poles out of their general scheme (Ruzicka and Gleason, 2008; Freestone et al., 2011). Moreover, early biogeographic models that proposed this latitudinal hypothesis were largely based on patterns of fish predation (Bakus and Green, 1974). While preying fish occur in Antarctic waters (Eastman, 1993), predation by invertebrate generalists is far more intense, and the incidence of bioactivity detected in feeding deterrence and toxicity bioassays is elevated, even if compared to temperate and tropical systems (McClintock, 1989; Baker et al., 1993; Amsler et al., 2000a; McClintock and Baker, 2001; Avila, 2006). In addition, protection by structural or skeletal devices, such as spicules in sponges or sclerites in cnidarians may not have the same relevance as in tropical sessile

organisms subject to intense fish grazing. This is because sea stars, often referred to as Antarctic keystone predators, practice external pre-digestion of their prey (Hyman, 1955; Sloan, 1980), but also other influencing invertebrate consumers may not be as affected by mechanical defenses. Indeed, chemical defenses are now known to be common in Antarctic organisms (Taboada et al., 2012; and reviewed by Amsler et al., 2000a; Avila et al., 2008; McClintock et al., 2010), and our results do agree with that (Chapters 3.1 and 3.2).

The abundance of Antarctic benthic fauna in a fluctuant plankton depauperate system is somehow a paradox. Suspension feeders from the Southern Ocean must adapt to an apparently intermittent food supply, with high summer primary productivity, reduced to almost zero in winter. Yet resuspension processes enable constant water renewal close to the bottom. Actually, seasonal zooplankton, along with the less seasonal fine fraction of seston (phytoplankton, pico- and nanoplakton) via resuspension, seem to comprise the diet of most suspensivores throughout the year (Orejas, 2001; Orejas et al., 2001, 2003; Tatian et al., 2002, 2004). In addition, lipidic energy reserves, in the form of wax esters and triglycerides, play a key role in polar marine organisms (Sargent et al., 1977). Another phenomenon observed in Antarctica is the association of diatoms with sponges, which provide these filter feeders with nutritional inputs, and maybe with other substances. These associations are more common here than in other latitudes (Gaino et al., 1994; Cattaneo-Vietti et al., 1996; Hamilton et al., 1997; Cerrano et al., 2004a; Cerrano et al., 2004b), equalling that with symbiotic bacteria (Hentschel et al., 2006; Taylor et al., 2007). This outcome was observed and indirectly analyzed for metabolic purposes in our glass sponge samples (Chapter 3.3). Moreover, sinking microalgae are crucial in pelagic-benthic coupling, providing the main source of hydrocarbons to benthic filter-feeding communities, and probably an additional silica source for siliceous sponges to use (Hayakawa et al., 1996). Most vagile species of the Antarctic benthos have likewise developed flexible opportunistic omnivorous/necrophagous foraging strategies, probably forced by this discontinuous phytoplankton cycle and unpredictable food availability (Arnaud, 1977). Among these opportunistic omnivorous are the principal keystone predators, which include voracious sea stars, such as *Odontaster validus* (Dayton et al., 1974; McClintock, 1994) and abundant amphipods like *Cheirimedon femoratus* (Bregazzi, 1972; De Broyer et al., 2007). Most of our samples came from deep bottoms of the Weddell Sea, incorporating additionally the difficulties inherent to any ecological studies in waters not accessible by diving. Nevertheless, most of the Antarctic benthic communities are described to lack a marked depth zonation, and there is a predominant circumpolar and eurybathic distribution of many of the dominant benthic organisms (Dell, 1972; Arnaud, 1977; White, 1984), coupled with that of the keystone nemertean and asteroid predators, that feed in mass aggregations (Dayton et al., 1974; McClintock, 1994; Barnes et al., 2006). Also extremely dense populations of amphipods with

diversified diets are found in a wide range of depths in association with biosubstrata, which represent often their prey too (Coleman, 1989b; Coleman, 1989a; Coleman, 1990; Kunzmann, 1996; Graeve et al., 2001; Nyssen et al., 2005; De Broyer et al., 2007; Huang et al., 2007; McClintock et al., 2009; Zamzow et al., 2010). Hence, influencing Antarctic predators and potential prey basically share both shallow and deep habitats (Dayton et al., 1974; Gutt et al., 2000). This in part facilitates investigation in these fairly unapproachable communities, since predator species collectable by diving maybe used to assess defenses in deep specimens. Considering that Antarctic scientists are constrained to develop their experiments in the available bases or vessels provided, these facts represent an advantage. In our case, most of the studies were performed at the Spanish Antarctic Base (BAE) Gabriel de Castilla, at Deception Island (Fig. 2), South Shetland Archipelago (62º 59.369' S, 60º 33.424' W).

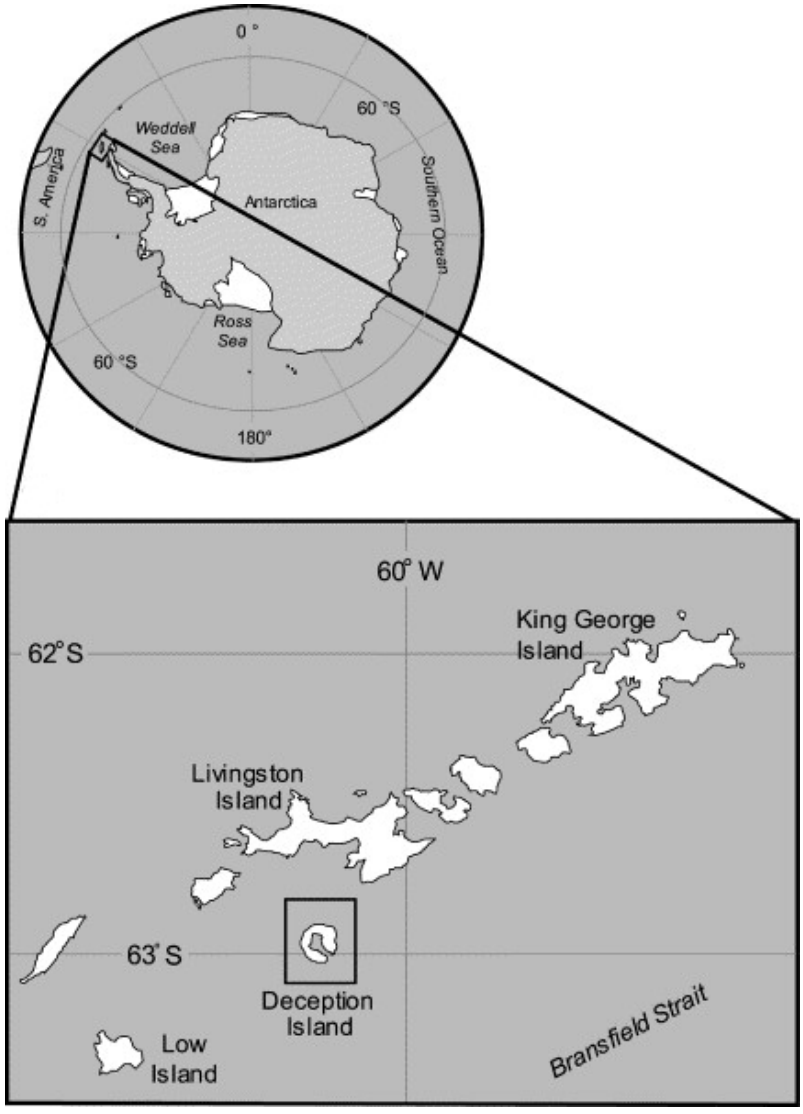


Fig. 2 Map of Antarctica and in detail the South Shetland Archipelago and Deception Island

1.2. Chemical ecology of marine organisms

In the marine environment, ecological pressures, mainly driven through interactions such as predation and competition for space and food resources, represent constant challenges for co-existing species (Barnes and Hughes, 1988). Sessile and sluggish invertebrates for instance are extremely vulnerable to mobile predators. Moreover, they are exposed to overgrowth by settling propagules of other invertebrates and algae, diatoms and microorganisms. Therefore, the ability of these organisms to evolve strategies to defend themselves plays a significant role in structuring marine communities (Barnes and Hughes, 1988; Paul, 1992; Pawlik, 1993; Hay, 1996).

Biological defenses include ecological (e.g., niche selection), behavioral (e.g., nocturnal habits), and or physiological adaptations (*i.e.* growth and/or reproductive rate optimization), while physical means include elaboration of external and/or internal skeletons (*i.e*., shells or spines, spicules and sclerites), sloughing of surface tissue, mucus, etc. Chemical defenses consist on toxic, noxious or distasteful agents, these compounds being mostly derived from the secondary metabolism (Paul, 1992; Eisner and Meinwald, 1995; McClintock and Baker, 2001). Nonetheless, there are examples of primary metabolites possessing defensive properties (Bobzin and Faulkner, 1992; Tomaschko, 1994; Slattery et al., 1997a; Fleury et al., 2008; Moran and Woods, 2009; Núñez-Pons et al., 2012a). Actually, in our investigations, we found both types of metabolites displaying repellency (Chapter 3.3. and 3.4.). The most studied activity is the ability of some metabolites to act as deterrents, and usually generalist consumers are deterred by secondary metabolites, mostly of lipid-soluble nature (Paul, 1992; Eisner and Meinwald, 1995; McClintock and Baker, 2001; Sotka et al., 2009). For this reason, we decided to focus first on those bioactivities and chemicals present in the most apolar lipophilic fractions of our specimens of study (diethyl ether soluble), leaving the remaining fractions for future investigations. Alternatively, specialist grazers, such as some opisthobranchs, target their diet on chemically defended organisms, which provide them with deterrents, or with precursors to synthesize their own chemical defenses (Avila et al., 1991; Fontana et al., 1994; Cimino et al., 1999; Paul et al., 2007). In benthic communities, soft-bodied, sessile, clonal invertebrates such as sponges, octocorals, and ascidians, are effectively defended from diverse types of predators by repellent metabolites (see reviews by Paul, 1992; Pawlik, 1993; Hay, 1996; McClintock and Baker, 2001; Paul et al., 2011). Clonal growth likely facilitates the evolution of chemical defenses because distasteful individuals can survive bouts of partial predation, while promoting learned aversion by co-occurring predators. In contrast, solitary organisms are less likely to recover from a significant loss of tissue (Jackson and Coates, 1986).

Presumably, protective mechanisms are energetically expensive and organisms must balance the costs of defense versus those of growth and reproduction (Coley et al., 1985; Cronin, 2001). For this reason, the production of defensive secondary metabolites has to be managed efficiently. Most resource allocation models that address patterns of secondary metabolite distributions are based on observations made in terrestrial plant systems (Cronin, 2001), which indeed parallel with sessile or sluggish marine invertebrates (*i.e.,* Hay, 1996). The most comprehensive model is the optimal defense theory (ODT; Rhoades and Gates, 1976), which examines within-organism variations in defensive chemistry, assuming that there is some metabolic expense for the production of defensive compounds. ODT predicts that defenses should be directly correlated to the risk of attack and inversely correlated to the energy cost of a particular defense. Furthermore, the theory proposes that within an organism, defenses should be differentially allocated to those tissues or structures most valuable in terms of fitness, or more vulnerable to co-existing predators. As in many existing studies on chemical defenses, most of our assays are integrated with the predictions of the ODT. Allocation of defensive compounds to particular organs and/or tissues has been observed in higher plants (McKey, 1979; Cronin, 2001), as well as in sponges (Furrow et al., 2003), gorgonians (Harvell and Fenical, 1989), opisthobranch molluscs (Faulkner and Ghiselin, 1983; Avila, 1995; Waegele et al., 2006), tunicates (Pisut and Pawlik, 2002) etc. Similarly, the optimality theory (OT) states that common defensive traits should be made effective for a variety of "enemies" to save energy (Herms and Mattson, 1992). And the inducible defense model (IDM; Harvell, 1990) predicts that defense production should be directly correlated with the risk of attack. In relation with this, chemical induction is another mechanism that permits organisms producing defensive compounds, or increasing their concentration, only when under attack by a consumer or aggressor. Chemical defense induction is likely most effective towards small, relatively immobile consumers (Hay, 1996), such as small crustaceans and gastropods, which, over short time intervals, cause only partial damage to their prey (McClintock and Baker, 2001). But sometimes, larger consumers can also prey on an individual for long periods of time (*i.e.* Antarctic sea stars that can be >30 cm in diameter and prey on a sponge individual for periods of days to months; Dayton et al., 1974). Inducible chemical defense is prevalent in marine organisms provoking increased levels of repellents, such as phlorotannins or terpene alcohols in algae (Cronin and Hay, 1996; Toth et al., 2007), alkaloids in poriferans (Thoms et al., 2006; Thoms and Schupp, 2008), terpenoids in soft corals (Slattery et al., 2001; Hoover et al., 2008), or dithiocarbamates in hydrozoans (Lindquist, 2002), yet it has not been still proved in Antarctic waters (Avila et al., 2008; McClintock et al., 2010).

Even if anti-predatory chemistry has been largely measured, the mechanisms by which defensive metabolites promote predation avoidance are still unknown, since many of these

compounds are usually not highly poisonous. Actually, distastefulness, rather than toxicity, is the most common strategy against predators (Paul, 1992; McClintock and Baker, 2001). Moreover nutritional quality must be jointly considered, since feeding experiments have proved that some repellents are more or only effective along with low quality foods; high food quality may mask the stimuli that elicit rejection when nutrients bind to deterrent molecules or compete with these for enzymes (Duffy and Paul, 1992). Hence, highly nutritive potential preys likely require larger amounts of, or more potent, defenses to prevent consumption. Alternatively, the selection for lower nutrient content along with poor defensive chemistry could be favored (Paul et al., 2007). Thus, the palatability condition of a prey item mostly results from the combination of (1) its chemical defense, (2) its nutritional value, and/or (3) its morphological characteristics (toughness, spines…; see Fig. 3) (Cruz-Rivera and Hay, 2003). This general concept is, to some extent, reflected in some comparative assays conducted in the present PhD Thesis (Chapter 3.2).

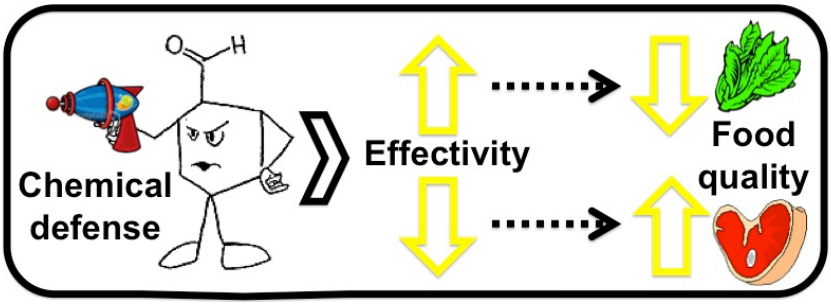


Fig. 3 Relationship between nutritional value and chemical defense effectivity

As we mentioned above, another challenge marine benthic organisms must face is prejudicial microbial invasion, and further macrobiotic epibiosis. Fouling processes, often more deleterious than beneficial to hosts, consists in a successional sequence that starts with macromolecular adsortion and bacterial colonization. Therefore, regulating initial bacterial films is a useful strategy to prevent subsequent biofouling and/or infections (Zobell and Allen, 1935). That is why our approaches to study antifouling were based on antibacterial tests. Certainly, the ability of many species to stay markedly free from epibionts is attributed to the occurrence of chemical inhibitors (Fusetani, 2004). Antifouling and antibiotic activities against co-occurring marine microbes have been reported in algae (Denys et al., 1995), sponges (*i.e.* Tsoukatou et al., 2002; Dobretsov et al., 2005; Haber et al., 2011), corals (*i.e.* Standing et al., 1984; Coll et al., 1987; Slattery et al., 1995; Kelman et al., 1998), ascidians (*i.e.* Davis and Wright, 1990; Wahl et al., 1994; Teo and Ryland, 1995; Davis and Bremner, 1999; Bryan et al., 2003), bryozoans (Konya et al., 1994), echinoderms (Selvin and Lipton, 2004; Guenther et al., 2009) etc. Concerning competitive epibiosis in Antarctic systems, here diatom invasions apparently surpass that of bacteria (Cervino et al., 2006), a pattern opposed to that in warmer regions, and some studies reflect this fact (Slattery et al., 1995; Amsler et al., 2000b; Peters et al., 2010;

Koplovitz et al., 2011). Both types of microorganisms may as well be consumed by filter feeders, yet diatoms represent a significant fouling threat to internal and external tissues during their impressive Austral seasonal summer blooms (Amsler et al., 2000b; Bavestrello et al., 2000; Cerrano et al., 2000). Finally, defenses to prevent epibiosis may as well include physical properties related to the host surface, like tissue- or mucus-sloughing or adhesiveness (Ducklow and Mitchell, 1979; Rublee et al., 1980; Barthel and Wolfrath, 1989; Vrolijk et al., 1990).

In relation to the methodologies to assess chemical defenses in marine ecology, when performing experiments on targeted species we must carefully consider the parameter used to normalize natural concentrations of crude extracts, sub-fractions, or compounds obtained from the organisms. Wet weight, dry weight, volume, and surface area are each most appropriate under differing circumstances. With respect to fouling, surface area may be most appropriate, but in sponges, for instance, surfaces within pores and large morphological variations across specimens make this approach extremely difficult. Volume-based normalization has been used in studies of chemical defenses against biting predators (*i.e*. Pawlik et al., 1995; Pisut and Pawlik, 2002), permitting to calculate the "defense per unit bite". Concentrations based on biomass use wet weight or dry weight, and have been employed in palatability assays with biting and no-biting consumers (Avila et al., 2000; Núñez-Pons et al., 2010). However, defining the "natural" concentration as the concentration calculated per unit weight approximating that in the organism is hampered by a variety of limitations, which, indeed, make impossible to mimic in the laboratory what it really happens in nature. For example, defensive compounds may be sequestered in certain regions of an organism (Furrow et al., 2003) and, therefore, be present at those areas at levels many folds higher than estimates based on the weight of the entire organism. Such limitations are common to almost all bioassays in which extracts, sub-fractions or compounds are presented in an artificial matrix or solution. We considered dry weight the most appropriate parameter to calculate natural concentrations in our samples, because it eliminates the water content, which may entail great deviations in porous, soft-bodied marine organisms, like sponges, and to a less extent, also soft corals and ascidians. Moreover, in many laboratory palatability assays the predators used, such as mosquitofish and killifish, as well as cosmopolitan strains of microbes for antifouling tests (*Staphylococcus aureus*, *Micrococcus sp.*, *Serratia sp.* and *Escherichia coli*), are not encountered in the same habitat as the organisms that contain the defensive compounds. Therefore, these types of experiments can “only suggest” a bioactivity (Paul et al., 2007). In order to prove ecologically relevant activities, realistic assays must be conducted with sympatric natural predators, competitors and fouling organisms (Munro et al., 1987; Scheuer, 1990; Hay and Fennical, 1996). This issue has been particularly taken into account in all our experiments with polar organisms

1.3. Marine natural products and chemical defense in the Antarctic realm

There are a number of classes of natural products, recognized on the basis of their biosynthetic origin. Among these are the polyketides, built primarily of acetate (C_2), occasional propionate (C_3) or, rarely, larger building blocks; terpenes, characterized by the number of C_5 isoprene units; and amino acid derived products, such as hydroquinones, depsipeptides and the major class of natural products, the alkaloids. Derivatives of other primary metabolites, including nucleosides, carbohydrates, steroids and fatty acids can also be found as secondary metabolites, though they are less common (Blunt et al., 2012 and previous reviews of the series). Secondary metabolites may derive from the diet, and be sequestered/stored by an organism, be biotransformed from a precursor, or be *de novo* biosynthesized (Paul, 1992; Avila, 1995; McClintock and Baker, 2001). A provocative hypothesis, originating primarily among marine natural products chemists, is that microbial associates are the true source of most biologically active compounds isolated from some species of chemically rich invertebrates, primarily among sponges, bryozoans, and colonial tunicates. Many of these invertebrates harbor microsymbionts and possess secondary metabolites with structural similarities to known microbial products. Yet few studies have convincingly demonstrated symbiont production of these metabolites (reviewed in Kobayashi and Ishibashi, 1993; Hildebrand et al., 2004; Piel, 2009), due to the complexity of naturally occurring microorganism assemblages in most marine invertebrates.

Although most certainly biased by the research interests of individual investigators and the isolation techniques used each phylum affords a characteristic distribution of compound structural types. For example, the great majority of metabolites isolated from cnidarians have been terpenoids; sponges, the most chemically studied marine animals, have yielded mostly terpenoids and nitrogenous metabolites; and ascidians appear to be specialized to biosynthesize amino acid derivatives (Davidson, 1993; Blunt et al., 2012). In fact, some products, especially lipids, have been often used for chemotaxonomical studies (Bergquist et al., 1991; Thiel et al., 2002; Berge and Barnathan, 2005; Imbs and Dautova, 2008), and modest contributions are also presented in this PhD Thesis for hexactinellid sponges (Chapter 3.3). Moreover, variation of secondary metabolites occurs at several scales, including intra-specimen, intraspecific, and spatial scales (Harvell and Fenical, 1989; Harvell et al., 1993; Becerro et al., 1998; Becerro et al., 2003; López-Legentil et al., 2005). This variability could respond to different reasons, among which: an intraspecific genetic variability (Harvell et al., 1993), chemical defense induction (Cronin and Hay, 1996; Slattery et al., 2001; Lindquist, 2002; Thoms et al., 2006), or symbiotic origin of certain metabolites (Sarà et al., 1998; Hildebrand et al., 2004).

Many natural products that display ecological roles but have no known primary metabolic function are considered secondary metabolites, which are costly but essential for fitness. While

a plethora of marine secondary metabolites have been identified, little is known about their functional significance (Paul, 1992; McClintock and Baker, 2001; Avila et al., 2008; Blunt et al., 2012). Some have been proposed to serve as toxins and noxins, deterrents of predation (which has been the most thoroughly studied), inhibition of fouling and/or infection and mediation of spatial competition. Secondary metabolites are produced under selective evolutionary pressure and must then serve a purpose, so the energy expenditure for biosynthesizing them compensates the decrease from that available to basic activities (Herms and Mattson, 1992; Paul, 1992; Berenbaum, 1995; McClintock and Baker, 2001). In many instances, biological activity has been measured as the ability of a compound to cause cell lysis or inhibit growth of a non-marine microbe. While such information may have utility in pharmaceutical studies (Munro et al., 1987; Scheuer, 1990; Hay and Fennical, 1996; Taboada et al., 2010), much remains to be learned about the ecological significance of bioactive compounds. This is particularly true in polar waters, where chemical ecological studies of marine invertebrates have only recently begun. The vast majority of chemical ecology studies has been conducted with shallow-water organisms (accessible via scuba diving), mainly from McMurdo Sound (Ross Sea) and the Western Antarctic Peninsula. A few studies also investigated some sub-Antarctic Islands and deep-water species from the eastern Weddell Sea (McClintock and Baker, 1997a; Lebar et al., 2007; Avila et al., 2008; McClintock et al., 2010; Taboada et al., 2012). In contrast, the coverage of East Antarctica is far from complete, and we know nothing about the chemical defense of the benthos of the almost inaccessible Amundsen and Bellingshausen Seas. The knowledge acquired until now though, can be expected to have a wide applicability, as Antarctic macrofauna is generally circumpolar and eurybathic in its distribution (Dell, 1972; Arnaud, 1977; White, 1984). Ecological studies in Antarctica have dealed mainly on how defensive chemistry may inhibit feeding by predators, similar to those in temperate and tropical marine environments (Paul, 1992; Pawlik, 1993; Hay, 1996). Nonetheless, in very few cases have the responsible molecules of the activity been identified (Núñez-Pons et al., 2010; Núñez-Pons et al., 2012a; and previously revised in Avila et al., 2008).

Examples of Antarctic natural products with ecologically relevant defensive activities are mostly reported from poriferans, red algae, cnidarian corals, molluscs, and colonial ascidians (Table 1). Interestingly, for sponges, nearly all of the compounds are responsible for their bright colorations. Colored pigments may have evolved under aposematic (warning coloration) selection, or photoprotection, in ancient, warmer Antarctic seas when visual predators, including fish and turtles were abundant. In the present, however, the main consumers are sea stars (Dayton et al., 1974; Dearborn, 1977; McClintock, 1994), which orientate to prey chemically (Sloan, 1980). Thus, brightly colored Antarctic sponges, as well as other organisms, likely

retained 'relict pigments' because of their inherent anti-foulant or anti-feedant defensive properties (McClintock and Baker, 1998; McClintock et al., 2000; and reviewed in Bandaranayake, 2006; McClintock et al., 2010). This issue is partly discussed for colonial ascidians in chapter 3.6. Many other molecules have been described from Antarctic marine sources (see Fig. 4), but, as mentioned above, only a few studies really demonstrate the activity of isolated natural compounds against sympatric species (reviewed in Avila et al., 2008; McClintock et al., 2010). In this thesis, some contributions are given on this meaningful topic of the Antarctic chemical ecology (Chapters 3.3, 3.4, 3.5 and 3.6).

Table 1: Natural products with ecological bioactivities obtained from Antarctic organisms

PHYLUM	**SPECIES**	**METABOLITE**	**REFERENCE**
ALGAE	*Plocamium cartilagineum*	Averene	Ankisetty et al., 2004
PORIFERA	*Dendrilla membranosa*	Isoquinoline	Baker et al., 1995; Amsler et al., 2001
	Isodictya erinacea	p-Hydroxybenzaldehyde; Erebusinone	Baker and Yoshida, 1994; Moon et al., 2000; Amsler et al., 2001
	Kirkpatrickia variolosa	Variolins; uncharacterized purple pigment	Perry et al., 1994; Trimurtulu et al., 1994
	Latrunculia apicalis	Discorhabdins	Furrow et al., 2003
	Suberites sp.	Suberitones	Baker et al., 1997
CNIDARIA	*Ainigmaptilon antarcticus*	Ainigmaptilones	Iken and Baker, 2003
	Alcyonium paessleri	Several steroids	Slattery et al., 1997a
	Clavularia frankliniana	Chimyl alcohol	McClintock et al., 1994c
MOLLUSCA	*Austrodoris kerguelenensis*	Several diterpenes	Iken et al., 2002
	Bathydoris hodgsoni	Hodgsonal	Avila et al., 2000
	Clione antarctica	Pteroenone	Yoshida et al., 1995
	Marseniopsis mollis	Homarine	McClintock et al., 1994a
	Tritoniella belli	Chimyl alcohol	McClintock et al., 1994c
CHORDATA	*Aplidium falklandicum* and *A. meridianum*	Meridianins	Núñez-Pons et al., 2010

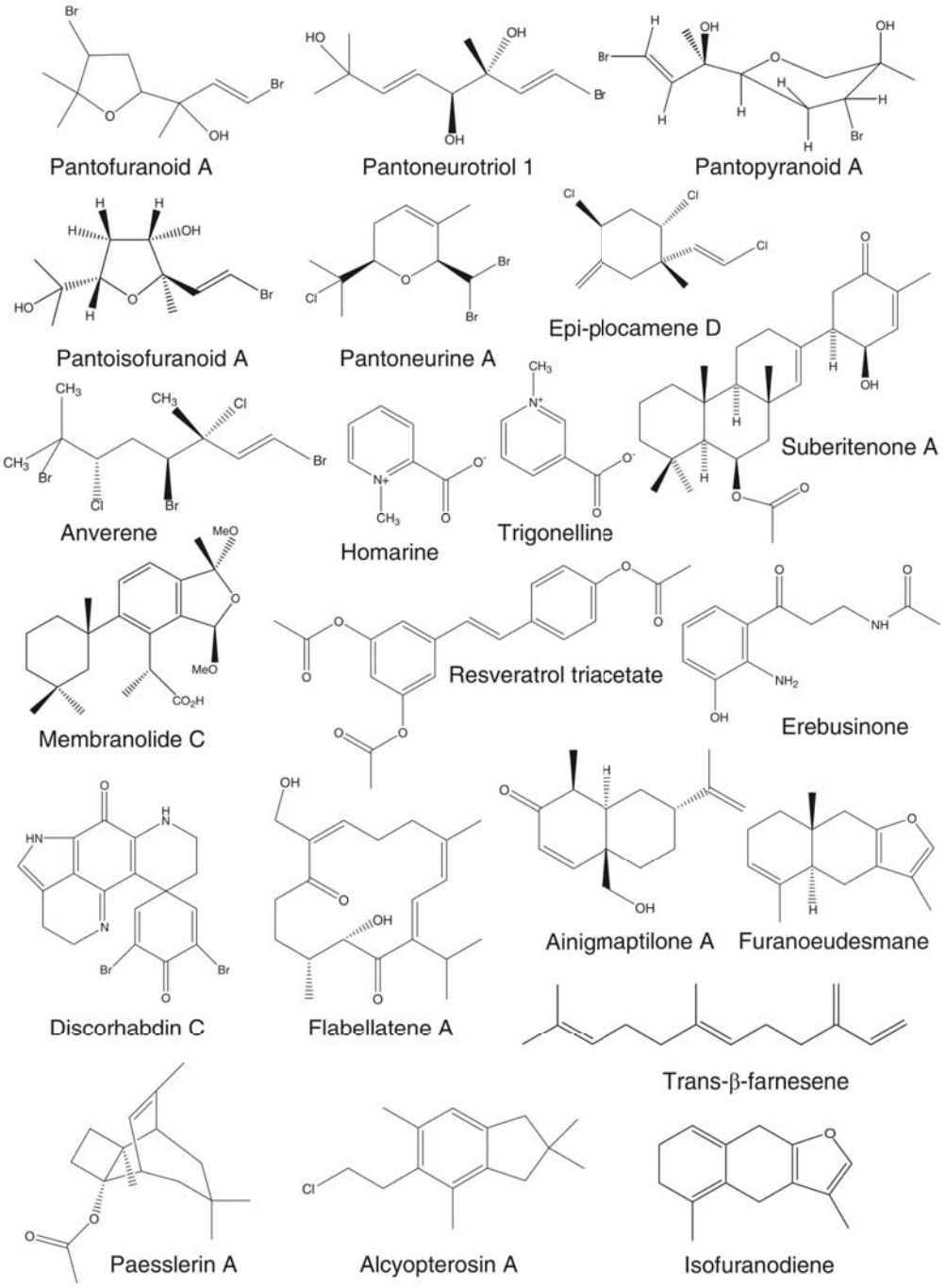


Fig. 4 Chemical structures of some natural products obtained from Antarctic organisms

In general, the predictions of the ODT vary with the type of predator and prey, and in co-ordination with other defensive traits. Localization of defensive chemistry primarily into the outer body-regions in Antarctic benthic invertebrates could be highly adaptive, protecting from a number of enemies that first encounter the prey's surface. This circumstance, coupled with the extraoral mode of feeding of ubiquitous sea star predators (Sloan, 1980), apparently follows the assumptions of the ODT (Rhoades and Gates, 1976). However, in prey with a very porous body

(with conspicuous holes), other effective consumers like small grazers able to reach inner tissues may affect the distribution of chemical defenses. This could be particularly relevant in large hexactinellids with volcano shape and large oscula, like our sponge samples (Núñez-Pons et al., 2012a). But, furthermore, the life history of some organisms may promote the storage of chemical defenses in internal tissues (gonads) for the production of defended larval stages. This strategy is actually typical of colonial ascidians (Lindquist et al., 1992). Chemical defense allocation has been described in the mantle of some Antarctic nudibranchs (Avila et al., 2000; Iken et al., 2002). But it has mainly been studied in Antarctic sponges, in which some species allocate deterrent agents mostly to their peripheral body zones (*i.e.,* (Furrow et al., 2003; Peters et al., 2009), whereas others do not (Peters et al., 2009). One of our recent Antarctic studies particularly addressed the issue of chemical defense allocation in several zoological taxons (Taboada et al., 2012). Whenever it was possible, and depending on the taxonomical group, our samples were analyzed for location of defenses, and taking in consideration the postulates of the ODT (Rhoades and Gates, 1976).

The sequestration of bioactive compounds into the outermost layers may serve additional roles beyond predation avoidance, including inhibition of fouling and mediation of allelochemical interactions (Rhoades, 1979; Paul, 1992; Slattery and McClintock, 1997; McClintock and Baker, 2001; Avila et al., 2008; McClintock et al., 2010). For instance, contact-mediated induction of tissue necrosis to the colonizer sponge *Mycale acerata* has been observed in a co-occurring *Alcyonium* soft coral, along with potent antifouling agents, likely released to the surrounding water. This suggests that important ecological properties must be allocated in the superficial mucus of these corals (Slattery and McClintock, 1997). In effect, many colonial Antarctic invertebrates, like compound ascidians and soft corals, are conspicuously devoid of fouling organisms (for review see McClintock et al., 2010; pers. obs.). Nonetheless, there is limited information about antifoulants in Antarctic marine invertebrates. Surveys of sponges and ascidians indicate a general lack of antibacterial chemistry. However, soft corals do exhibit antifouling inhibition towards surrounding marine bacteria (Slattery et al., 1995; Slattery et al., 1997a). Contrastingly, potent chemicals with broad-spectrum activity against benthic diatoms are common in all these groups (Slattery and McClintock, 1997; Peters et al., 2010; Koplovitz et al., 2011). We have tested antibacterial fouling in ascidians and soft corals (chapters 3.4, 3.5 and 3.6), attempting to contribute to a better understanding of these issues.

The state-of-the-art in marine chemical ecology draws a map in which most of the current knowledge comes from shallow tropical and temperate ecosystems, which are more accessible and where ecological interactions may be easily established (Paul, 1992, McClintock and Baker, 2001; Avila et al., 2008; McClintock et al., 2010). Polar areas instead, due to their geographical isolation and harsh conditions, have received much less attention (Lippert, 2003; Avila et al.,

2008; McClintock et al., 2012). In Antarctica, the bulk of chemical ecological research has focused on the presence of antipredatory properties, with the most studied groups being macroalgae and sponges. Within the Porifera, however, most of the investigated species are demosponges, and in spite of hexactinellids being one of the major components on the Antarctic seafloors, almost nothing is known about them in this field (Avila et al., 2008; McClintock et al., 2010). Actually, besides our work in this PhD Thesis (Chapter 3.3; Núñez-Pons et al., 2012a), only an additional reference existed addressing aspects of the nutritional and spicule content, and on the chemical defense in a few hexactinellid sponges (McClintock, 1987).

After poriferans, the more studied groups in Antarctic chemical ecology are molluscs and recently also ascidians (Avila et al., 2008; Koplovitz et al., 2010; 2011; McClintock et al., 2010). These recent studies reveal a poor presence of chemical defenses to avoid predation and bacterial invasions in Antarctic solitary and clonal ascidians (Koplovitz et al., 2010; 2011). The next group receiving more attention in terms of ecological chemistry is the cnidarians (Avila et al., 2008; McClintock et al., 2010). Nonetheless, most research with cnidarians has focused on the chemistry and isolation of new metabolites (Slattery et al., 1994; Slattery et al., 1997b; Palermo et al., 2000; Rodríguez-Brasco et al., 2001; Gavagnin et al., 2003; Iken and Baker, 2003; Carbone et al., 2009; Manzo et al., 2009; and reviewed in Avila et al., 2008). Moreover, only three soft coral species, *Alcyonium paessleri*, *Clavularia frankliniana* and *Gersemia antarctica*, were analyzed for the presence of chemical defenses, demonstrating a rich and multipurpose arsenal (Slattery and McClintock, 1997). Recently, our research group has conducted an extensive study of feeding deterrent activities in Antarctic invertebrates, where the issue of chemical defense allocation was particularly addressed. We believe this study represents a great contribution to the Antarctic chemical ecology, because the examined species came from deep-sea areas of the Weddell Sea and Bouvet Island, and most of them were studied here for the first time (Taboada et al., 2012). Indeed, the overall scenario in Antarctic chemical ecology reveals that, although the incidence of chemical defenses is quite extended in many of the organisms studied, much needs to be learned in some relevant groups. Moreover, the knowledge on the identity of the implicated defensive metabolites, as well as specific features including distribution, mode of functioning and interaction with other molecules, and origin is still in its very young infancy (Avila et al., 2008; McClintock et al., 2010). Hence, in order to fill some of the gaps in Antarctic ecology, this PhD Thesis focuses on enhancing our understanding of mechanisms of defense mediated by organic chemicals. For this purpose, we selected conspicuous Antarctic benthic organisms, like hexactinellid sponges, soft corals and colonial ascidians. Additionally, this Thesis presents the challenge of including the study benthic organisms collected at deep waters.

1.4. Antarctic keystone model predators

The selection of the experimental model predator is crucial if we seek to obtain ecologically relevant information. Experiments to demonstrate the existence of chemical defensive mechanisms in Antarctic marine invertebrates have included several methods using various putative predators. Direct feeding bioassays have been performed with Antarctic fish (*Notothenia coriiceps, Pagothenia borchgrevinki, Dissostichus mawsoni, Trematomus bernacchii* and *Pseudotrematomus bernacchii*), cnidarians (sea anemone *Isotealia antarctica*) and amphipods (*Gondogeneia antarctica* and *Paramoera walkeri*) with fresh tissues, or pellets made of agar including organic extracts from potential prey organisms (*i.e*. McClintock et al., 1991; McClintock et al., 1992; McClintock et al., 1993; Slattery and McClintock, 1995; McClintock and Baker, 1997b; Koplovitz et al., 2009).

Asteroids, one of the most relevant predator groups in Antarctic benthic communities, feed by extruding their cardiac stomachs over their prey (Sloan, 1980). Because of this unique stomach extension feeding mechanism, the approach typically used in assays with other predators may not be appropriate. On the other hand, tube-feet are a primary site for chemical reception in echinoderms (Sloan, 1980; McClintock, 1994). Their retraction is considered to be a chemoreception defensive response that occurs when sea stars detect strong sensory changes in their environment, or the presence of compounds that are irritants or repellents (Sloan, 1980). To date, most of the experiments using Antarctic echinoderms relied on an indirect measure of feeding deterrence, exploiting the chemotactile reactions of sensory tube-feet after food presentation. Thus, they did not evaluate either actual ingestion or latter reactions of the predator. These assays consisted on testing tube-foot retractions or rightening response in sea stars (*Odontaster validus, Odontaster meridionalis, Diplasterias brucei, Acodontaster conspicuus and Pesknaster fuscus*) (*i.e*. McClintock, 1987; McClintock et al., 1990; McClintock et al., 1992; McClintock et al., 1993; McClintock et al., 1994b; Slattery and McClintock, 1995; Slattery et al., 1997a; Slattery and McClintock, 1997; McClintock et al., 2000). Other tests placed treated shrimp paper disks in the mouth of sea urchins (*Sterechinus neumayeri*), or treated pellets or tissues in the ambulacral grooves of sea stars' arms, or tentacles of sea anemones*,* or, more recently, coated mucous secretions onto krill pieces, finally monitoring movement of these items towards or outwards the mouth (McClintock et al., 1994a; McClintock et al., 1994c; McClintock and Baker, 1997; Amsler et al., 1999; Koplovitz et al., 2009; Peters et al., 2009). Until now, our research group has reported most of the existing examples of repellency experiments using direct feeding bioassays with Antarctic sea stars (*i.e.* Bryan et al., 1998; Avila et al., 2000; Iken et al., 2002; Núñez-Pons et al., 2010, 2012a; Taboada et al., 2012). These tests went on long enough as to unequivocally determine ingestion or rejection. Artificial food cubes, very useful in tropical systems (*i.e.* Van Alstyne et al., 1992), were found

not suitable for feeding assays with polar asteroids (Iken et al., 2002). The use of different methodologies makes it difficult to compare results. In our previous experience, since Antarctic echinoderms may either reject or eat the offered food after several hours because of their slow feeding habits, direct feeding bioassays are much more reliable to test repellency.

Odontaster validus has a circumpolar distribution and can be extremely common across a bathymetric range from the intertidal to 940 m (Fig. 5), occuring on almost every type of substrate (Dearborn, 1977; McClintock et al., 1988; Dearborn et al., 1983). It is an omnivore opportunistic species, which displays a variety of feeding behaviors as a detritus feeder, scavenger or effective predator, depending on available prey and circumstances (Dearborn, 1977; McClintock, 1994). Dayton et al. (1974) found the diet of *O. validus* to consist of detritus, diatoms, sponges, hydroids, bivalves, gastropods nauplii, ostracods, shrimp and different crustacea, and sea stars. *O. validus* is a model starfish predator in the sense that it has been repeatedly used in feeding acceptability studies, yielding quite widespread deterrent properties in Antarctic organisms, wich makes it useful for comparative studies. Additionally, it is readily available and its feeding response facilitates laboratory bioassays. Hence, we selected this species to evaluate post-ingestion repulsive reactions (chapter 3.2), which have been scarcely tested in Antarctic echinoderms (for reviews see Avila et al., 2008; McClintock et al., 2010).

Fig. 5 The Antarctic sea star *Odontaster validus*

In Antarctic benthic communities, peracarid crustaceans, and especially the Amphipoda, are by far the most species-rich group and probably the most diversified with respect to lifestyles, trophic types (including necrophagy, carnivory, herbivory, suspension feeding, detritivory, and omnivory), habitats and size spectra (Rauschert, 1988; De Broyer and Jazdzewski, 1996; De Broyer et al., 2004, 2005, 2007). They appear in very high densities (up to 300,000 individuals m^{-2}; Huang et al., 2007) compared to reports from other latitudes (Nelson, 1980; Brawley, 1992). They are commonly associated with living substrata (frequently macroalgae and

sponges), which are often their potential (direct or incidental) prey. As benthic small consumers, Antarctic amphipods are strong influencing predators that feed on a wide array of prey items including macroalgae, sponges, cnidarians, holoturians, bryozoans, diatoms, etc. (Coleman, 1989a; Coleman, 1989b; Coleman, 1990; Graeve et al., 2001; Nyssen et al., 2005; Huang et al., 2006; Huang et al., 2007; Amsler et al., 2009; McClintock et al., 2009). Thus, they represent a very interesting group for studying the incidence of chemical defenses in benthic sessile organisms. Yet, the Antarctic amphipods that have been normally used as experimental model predators, such as *Gondogeneia antarctica* and *Paramoera walkeri,* exhibit limitations for testing unpalatable activities in algae and animals, either for being herbivorous, or because they show preference to artificial foods containing extracts (Amsler et al., 2005). The lyssianasid amphipod *Cheirimedon femoratus*, with a circumpolar distribution and eurybathic occurrence down to several hundred meters depth (De Broyer et al., 2007), is described as a voracious omnivorous-scavenger (Fig. 6). However, ovigerous females and newly hatched young ingest photosynthetic diets (algae) during summer (Bregazzi, 1972; Richardson and Whitaker, 1979). It has opportunistic habits, being a first-arrival feeder to carrion inputs (Smale et al., 2007). As a very abundant generalist consumer (algal, animal material and detritus) in the Antarctic sea bottoms, *C. femoratus* could be considered a grazer towards which most potential prey would address defensive chemistry. For all these, *C. femoratus*, never previously tested, was chosen to perform new palatability bioassays in this PhD Thesis (chapter 3.1).

Fig. 6 Antarctic lyssianasid amphipod *Cheirimedon femoratus* (photo by M. Rauschert)

Few studies of sensory ecology have been conducted in Antarctica to understand how predators find their prey items, and how prey detect potential consumers (McClintock et al., 2010). Examinations on aspects of the chemosensory biology of the asteroid *O. validus* (Kidawa, 2005b; Kidawa, 2005a; Kidawa, 2009), and field behavioral observations of the amphipod *C. femoratus* (Smale et al., 2007) suggest that both species have a notable ability for tracking food cues. This fact may favor the detection of deterrent agents in nature, rendering both species with advantageous characteristics for laboratory bioassays as well (Chapters 3.1

and 3.2.). Moreover, both organisms are very abundant and easily collectable around the BAE Gabriel de Castilla, at Deception Island, where the experiments were performed.

1.5. Antarctic invertebrate targets in the research project

1.5.A. Rossellid hexactinellid sponges

Sponges (phylum Porifera) are mostly filter-feeding, sessile metazoans with a body organization consisting of a cell-poor mesenchyme (mesohyl) sandwiched between two epithelial layers, in most cases with cells showing enormous potential for transdifferentation and migration across body regions. So far approximately 9,000 poriferan species have been described, of which around 400 are hexactinellids, about 500 are calcareous, a few belong to the recently recognized Homosclerophorida, being the rest (about 90%) demosponges (Brusca and Brusca, 2003). From a chemical point of view, the Porifera have been the focus of much interest due to their associations with a variety of microorganisms, and for their outstanding repertoire of bioactive metabolites (Taylor et al., 2007; Blunt et al., 2012).

Hexactinellid sponges, often referred to as glass sponges because of the importance of the silica skeleton relative to the soft parts of the body, possess a unique histology. The larger part of their scarce soft tissue (75%) is built by giant multinucleated (syncytial cells), with only occasional uninucleated cells. The syncytium ramifies among the skeletal framework, characteristically formed by “hexactinal” siliceous spicules and derived forms. The mesohyl is minimal, some times virtually absent. This trabecular syncytium serves as a stream way transport of nuclei, organelles and substances, similar to plants (Leys, 2003). It is a pathway for propagation of action potentials that trigger halting of flagella motion after external stimuli (disturbance, sediment in the water) and consequent arrest of feeding currents, representing a rapid electric protection response (Leys et al., 1999; Leys et al., 2007; Tompkins-MacDonald and Leys, 2008). Some glass sponges experience ‘‘gigantism’’ along with long life spans (arguably up to 15.000 years!; Leys et al., 2007). They occur mostly in the bathyal and hadal depth ranges of all oceans, where predators are rare, and collection and investigation are difficult (). However, in polar seas, the fact that silicate concentrations are notably higher than in the remainings oceans at similar depths favors occurrence of hexactinellid sponges at relatively shallow depths (Maldonado et al., 1999). In the study area the hexactinellid fauna comprise 35 reported species and can live fairly shallow (up to 20 m), dominating the megabenthos in the upper shelf, between 100 and 600 m. They form spectacular associations including 8 prevailing species from 2 genera of the family Rossellidae, *Rossella* (restricted to the Southern Ocean except for one species) and *Anoxycalyx* (Fig. 7). However, other rossellids,

such as *Caulophacus* and *Bathydorus*, are only well represented in the deep-sea (Barthel, 1992; Barthel and Gutt, 1992; Gutt, 2007; Janussen and Tendal, 2007).

Fig. 7 Assemblage of Antarctic hexactinellid sponges (photo A. Starmans)

Antarctic sponges are recognized to show high incidence of defensive chemicals, however the available results to date include mostly demosponge species (McClintock et al., 2000; McClintock et al., 2005; Avila et al., 2008; Peters et al., 2009). The general view is that hexactinellids produce no bioactive secondary metabolites and that are unattractive to predators, in part because their silican skeleton accounts on average for about 80-90% of the animal dry weight, only 10% being organic material, which suggests not much of a meal (Barthel, 1995). However, these features do not seem to deter some Antarctic spongivores, such as *Odontaster* spp. and *Acodontaster* spp. asteroids, and the nudibranch *Austrodoris kerguelenensis*, which readily feed on hexactinellids, such as *Rossella racovitzae* and *R. nuda* (Dayton et al., 1974; Dayton, 1979). Although hexactinellids represent a tridimensional shelter, and probably a source of nutrition for a diverse macro and microfauna, rich aggregations of amphipods included (Kunzmann, 1996), their internal body regions are quite pristine in terms of bacteria (Leys et al., 2007). Nonetheless, populations of diatoms are found living within their peripheral tissues, which could represent a food supply, as well as a source of other chemical compounds (Gaino et al., 1994; Cattaneo-Vietti et al., 1996; Cerrano et al., 2004a; Cerrano et al., 2004b).

Glass sponges appear to have diverged earlier than the other sponge classes (Demospongia, Calcarea, and Homosclerophorida), and are often regarded as the earliest living metazoans. However, within the phylum Porifera the relationships among the extant classes and their connection with eumetazoans are still debated (Reiswig and Mackie, 1983; Worheide et al.,

2012), and especially the class Hexactinellida is currently quite controversial (Barthel, 1992; Göcken and Janussen, 2011). Moreover, there are scarce contributions on the chemistry and ecology of hexactinellid species, in which the taxonomical information is also deficient (Guella et al., 1988; McClintock et al., 2000; Blumenberg et al., 2002; Thiel et al., 2002). Considering the relevance of glass sponges in Antarctic communities, we dedicated chapter 3.3 to their study.

1.5.B. *Alcyonium* soft corals

Corals comprise about 5100 recognized species, and live over enormous latitudinal and bathymetric ranges, some reaching amazing longevities (Hughes et al., 1992). Soft corals (order Alcyonacea) are a group of octocorals including the families Alcyoniidae, Nephtheidae, Nidaliidae and Xeniidae. They are made up of a large number of polyps connected by a fleshy tissue (coenenchyme), lacking calcium carbonate massive skeletons. Instead they have an assortment of internal, minute spiky sclerites that provide physical support to body shape and structure, (Brusca and Brusca, 2003) and are useful for taxonomy (Bayer et al., 1983). Shallow species live in association with photosynthetic zooxanthellae (Muscatine and Porter, 1977; Muscatine et al., 1981), while deep ones, for living outside photic zones, lack algal symbionts. In general soft corals represent food, host substrata and refuge for many symbiotic organisms, including animals, bacteria, fungi and algae, sharing food inputs and allelochemicals (Humes, 1990; Kerr and Paul, 1995; Slattery et al., 1998; Avila et al., 1999; Barneah et al., 2004; Barneah et al., 2007).

Fig. 8 The Antarctic soft coral *Alcyonium antarcticum* (photo by D. Schories)

Despite their flabby aspect, missing safeguarding rigid skeletons, and their nutritious nature (La Barre et al., 1986b), no predators are known to cause a notable deleterious impact on soft

coral populations. Only specialist consumers (pycnogonids and opistobranchs) readily feed on them (Sammarco and Coll, 1992; Slattery et al., 1998; Avila et al., 1999). Defensive strategies to prevent heavy generalist consumption may include nematocyst based (Stachowicz and Lindquist, 2000; Bullard and Hay, 2002; Hines and Pawlik, 2012), physical-mechanical (Harvell and Fenical, 1989; Van Alstyne et al., 1992; Van Alstyne et al., 1994), or chemical protection (La Barre et al., 1986b; Wylie and Paul, 1989; Sammarco and Coll, 1992; Hines and Pawlik, 2012). Contrasting to pelagic siphonohores, hydrozoans and scleractinian corals with potent penetrating nematocysts (Sammarco and Coll, 1992; Stachowicz and Lindquist, 2000; Bullard and Hay, 2002; Hines and Pawlik, 2012), Octocorallia are characterized by a less aggressive nematocyst system lacking stinging devices (*i.e.* mastigophores). Octocorals have low diversity (basically a single type, *i.e.* rhabdoidic heteronemes) and density of cnidos (Schmidt, 1974; Brusca and Brusca, 2003), being incompetent for active prey capture and for defensive aggressions (Mariscal and Bigger, 1977; Lasker, 1981; Sammarco and Coll, 1992). Occurrence of structural defenses, putatively mediated through the polypary armament of sclerites and coenenchyme mineralization, are still a matter of debate (Harvell and Fenical, 1989; Sammarco and Coll, 1992; Van Alstyne et al., 1992; Slattery and McClintock, 1995; Kelman et al., 1999; O'Neal and Pawlik, 2002). In fact, sclerites are primarily necessary for structural support rather than for defense, since this latter function can also be accomplished by repellent metabolites (Van Alstyne et al., 1992; West, 1998; Kelman et al., 1999; Blunt et al., 2012 and previous reviews).

Alcyonacea are indeed rich in secondary compounds which serve several ecological roles related to predator defense, competition for space, antifouling and reproduction enhancement (La Barre et al., 1986a; Coll et al., 1987; Mackie, 1987; Pass et al., 1989; Wylie and Paul, 1989; Sammarco and Coll, 1992; Kelman et al., 1999; Wang et al., 2008). Deterrent as well as antifouling properties from soft corals are often a result of the presence of several different repellent metabolites, mostly terpenes and sterols, which may act in additive or synergistic mode (Wylie and Paul, 1989; Van Alstyne et al., 1994; Kelman et al., 1998). All corals secrete a surface mucus layer, which is essential for vital processes, such as ciliary feeding, reproduction, and as a defense against a plethora of environmental stressors, providing a medium into which allelochemicals are exuded. The mucus consists of a muco-polysaccharide protein lipid complex containing wax esters (main lipidic energy reserves in corals), sterols, terpenic toxins, and also UV-absorbing compounds. Nevertheless, the exact composition may vary in response to external disturbances, such as physical damage (Sargent et al., 1977; Brown and Bythell, 2005). A rich microbial community lives in the mucus supplying corals with nutrient molecules which may provide the holobiont defensive substances (Brown and Bythell, 2005; Ritchie, 2006; Shnit-Orland and Kushmaro, 2009).

Anthozoans are the third dominant taxon in the benthic communities of the Weddell Sea, contributing much of the tridimensional community structure (Arnaud, 1977; Galerón et al., 1992; Arntz et al., 1994, 1997; Sirenko et al., 1997; Orejas, 2001). The soft-coral genus *Alcyonium* is particularly common (Fig. 8), represented in the Southern Ocean by 8 reported species, some of them with very high abundances. This genus is also extended through all oceans of the World, with approximately a total of 59 described species. In shallow Antarctic communities, only one pygnogonid species is reported to feed on *Alcyonium* spp., and in fact, soft corals are consistently avoided by dominant predators (Slattery and McClintock, 1995; author's personal observations). Up to date only the investigations of Slattery and co-workers (reviewed in Slattery and McClintock, 1997) have contributed to the knowledge on the chemical ecology of Antarctic soft corals, demonstrating extended ocurrence of mechanisms for chemical defense. The rich defensive potential displayed in the past by these organisms through substances of diverse origins, drove us to examine several *Alcyonium* spp. with the object to determine the relevance of primary and secondary metabolites as means of protection (Chapter 3.4).

1.5.C. Colonial ascidians of the genera *Aplidium* and *Synoicum*

Ascidians occur in all oceans from the Arctic to the Antarctic and from the surface to abyssal zones, with over 2800 described species (Lambert, 2005). They may be solitarian, or constitute social groups of individuals vascularly connected by the base, or be compound (truly colonial), with many minute clonal zooids embedded in a gelatinous matrix sharing the external tunic (Brusca and Brusca, 2003). This outer integumentary tissue harbors diverse cell types, including symbionts in some cases, and is multifunctional with very variable consistency, from gelatinous to leathery (Hirose, 2009). Species dispersal abilites take place through gametes and larval stages. Dispersal is usually limited to not more than a few meters, especially in colonial species producing only few, very large, yolky eggs that are brooded in the atrial cavity until released as lecithotrophic tadpole larvae (Brusca and Brusca, 2003; Lambert, 2005).

Many mechanisms have been developed to prevent predation in ascidians, most related to physical or chemical properties of the tunic (Tarjuelo et al., 2002; Lambert, 2005). Tough outer tunics composed of the proteinaceous polysaccharide tunicin occur in some colonial, but mainly solitarian ascidians (Koplovitz and McClintock, 2011). Besides, minute calcium carbonate spicules embedded within the tunics of certain species may serve to discourage predation (Lambert, 1979; Lambert and Lambert, 1997; López-Legentil et al., 2006). Some species have also developed tunics with low nutritional value (Tarjuelo et al., 2002). However, defensive chemistry is likely the fisrt line of protection evolved by most ascidians. This may include the

accumulation of heavy metals like vanadium, sulfuric and/or hydrochloric acid in tunic bladder cells (Stoecker, 1980b; Stoecker, 1980a; Pisut and Pawlik, 2002; McClintock et al., 2004). But the production of deterrent secondary metabolites is a widespread strategy too (McClintock et al., 2004; López-Legentil et al., 2006; Núñez-Pons et al., 2010). These compounds can also be incorporated into eggs, embryos and larvae to confer them protection. This is often the case in compound ascidians where the energy investment by the adult to produce the reproductive elements is substantial (Young and Bingham, 1987; Lindquist et al., 1992; Pisut and Pawlik, 2002). Redundancy of defensive strategies can operate either against diverse enemies, or also at different life stages (Wahl and Banaigs, 1991; Pisut and Pawlik, 2002; Tarjuelo et al., 2002; McClintock et al., 2004; López-Legentil et al., 2006). Colonial species tend to maintain a clean, unfouled surface, a putative indication of antifouling properties. Instead a number of solitary ascidians present their surfaces heavily fouled to become cryptic, which is assumed to be part of a defensive strategy (Stoecker, 1980b; Bryan et al., 2003; and reviewed in Lambert, 2005).

Fig. 9 Antarctic ascidian *Synoicum adareanum* (photo from Antarctic Underwater Field Guide)

Ascidians mostly possess nitrogen-bearing metabolites, particularly aromatic heterocycles (peptides, alkaloids, and amino acid derived products), but also in lesser amount non-nitrogenous compounds, such as lactones, terpenoids or quinones (Blunt et al., 2012 and previous reviews). While the vast majority of ascidian metabolites have been isolated from whole-body extractions, several compounds were obtained from specific tissues, physiological fluids, or cells (Davidson, 1993; Rottmayr et al., 2001; López-Legentil et al., 2005; Seleghim et al., 2007). Ascidians posses a more complex organized body-plan and circulatory system respect to other sessile invertebrates, which could favor the encapsulation of bioactive compounds within particular cells or other compartments to fulfill ecological roles avoiding autotoxocity (Goodbody, 1974).

Ascidiacea, and the family Polyclinidae in particular, is one of the most abundant taxa on the shelf of Antarctica (Galerón et al., 1992; Arntz et al., 1994, 1997; Sirenko et al., 1997; Ramos-Esplá et al., 2005). The ascidiofauna is relatively homogeneous across this entire geographical region. It is characterized by a high level of endemisms (25-51%) with only 0-7% of cosmopolitan species (Primo and Vazquez, 2009). Within the Class Ascidiacea and Family Polyclinidae, one of the most prolific genera is *Aplidium,* with 40 species described from the Southern Ocean. The genus *Synoicum* instead is represented by 8 Antarctic-subantarctic species. *Synoicum adareanum* produces pedunculated colonies of variable colorations (Fig. 9), whereas those of *Aplidium* species are usually globulous, varying in pigmentation inter- and intraspecifically (Varela, 2007). Bioaccumulation of acids or heavy metals have not been reported to date in none of the species analyzed here nor in their congenerics (Lebar et al., 2011). Thus, secondary metabolites are expected to be the main weapons to avoid predation. However, low prevalence of chemical defense attributable to secondary metabolites against amphipod, sea star, or fish grazing, was recorded in previous studies with Antarctic solitary and colonial ascidians (Koplovitz et al., 2009). Several bioactive natural products have been obtained from Antarctic colonial ascidians of the genera *Synoicum* and *Aplidium*, such as the palmerolide A, a group of ecdysteroids, meridianins, aplicyanins and rossinones (Hernández Franco et al., 1998; Diyabalanage et al., 2006; Miyata et al., 2007; Seldes et al., 2007; Appleton et al., 2009), but rarely have their ecological properties been investigated (*i.e.* Núñez-Pons et al., 2010). Some of these products have been found as a direct result of this PhD, thus we selected some of species of these two genera of Antarctic ascidians to elucidate characteristic features of their defensive strategies. In chapters 3.5 and 3.6 we undertake the study of tissue allocation and origin of responsible deterrent agents in colonies of *Aplidium* and *Synoicum* ascidians.

1.6. General structure of this PhD Thesis

This PhD Thesis focuses on several of the understudied aspects mentioned above, in order to improve our understanding of the Chemical Ecology of Antarctic Benthos. Our aims were: 1) the identification of defensive natural products, 2) the study of the primary ecological mechanisms through which they operate, and 3) the allocation and origin of these compounds within selected benthic organisms.

This Ph.D. Thesis has been structured in different chapters, including 6 publications (Chapters 3.1 to 3.6) in the results section. Each publication addresses a different aspect of the chemical ecology of the Antarctic marine benthos: The first two **publications** (**I** & **II**) are interconnected approaches to investigate general aspects of the unpalatability of some benthic organisms towards two different types of predators. The other four **publications** (**III** to **VI**) are

specific studies focusing on particular invertebrate groups and the elucidation of some of their ecologically bioactive natural products.

CHAPTER 2.

OBJECTIVES

CHAPTER 2. OBJECTIVES

The overall goal of this research is to better understand the mechanisms of chemical defense in Antarctic benthic communities. For this aim, ecologically realistic experiments were designed and conducted using relevant sympatric potential predators and fouling bacteria. Whenever it was possible, the potential site of storage of the defensive agents was estimated. Moreover exhaustive chemical analyses were performed to identify some of the actively responsible defensive metabolites in three conspicuous groups of Antarctic benthic animals (see Fig. 10).

More specifically, the objectives were:

1) To select a predator model for evaluating the incidence of chemical defenses against Antarctic sympatric predators. We screened the natural communities searching for a suitable Antarctic predator to develop a new protocol for feeding preference bioassays with choice. Upon selection, *Cheirimedon femoratus* was used to determine the presence of unpalatable defenses in marine invertebrates and algae (Chapter 3.1, **publication I**). This influencing omnivorous amphipod had never been previously assessed. The experiments intended to evaluate the impact of generalist small consumers, topic that has received scarce attention in Antarctic waters so far.

2) To compare the defensive mechanisms developed by different Antarctic benthic invertebrates and macrophytes towards two different sympatric consumers. By using the sea star *Odontaster validus* and the amphipod *C. femoratus* as putative predators, we comparatively performed diverse direct feeding experiments (Chapter 3.2, **publication II**), to determine similar or divergent responses in the tested prey organisms. We also tried to estimate potential interactions between chemical defense and nutritional value of the assessed prey items.

3) To analyze chemo-ecology in glass sponges. In Chapter 3.3, **publication III**, we conducted one of the very few studies on the chemical ecology of hexactinellid sponges available to date. Our aim was to determine the existence of repellent properties in species belonging to the family Rossellidae, as well as possible within-body allocation of defenses. The approach was replicated in some Antarctic demosponges for comparison. Additionally, this objective involved the isolation of particular metabolites to test for potential bioactivity and usefulness as chemical markers for chemotaxonomical purposes.

4) To identify ecologically functional natural products from Antarctic soft corals. The purpose here was to evaluate the effectiveness of defensive chemistry against predation and bacterial fouling in Antarctic soft corals pertaining to the genus *Alcyonium* (Chapter 3.4,

publication IV). The study was also addressed to the identification of their chemicals and to the study of possible interactive mechanisms occurring among them. With this case example, it was also intended to evaluate the importance of metabolites derived from both, primary and secondary metabolism in the defensive arsenal of this group of animals.

5) To characterize singular features of the chemical protective strategies in Antarctic colonial ascidians. Chapters 3.5 and 3.6 (**publications V & VI**) aimed at the localization of defenses and their storage at colony level, as well as to the interpretation of their possible origin. The ascidian species analyzed here belonged to the common Antarctic genera *Aplidium* and *Synoicum*. Moreover, we attempted to elucidate and quantify the mixtures of the responsible metabolites participating in the antipredatory and antibacterial mechanisms.

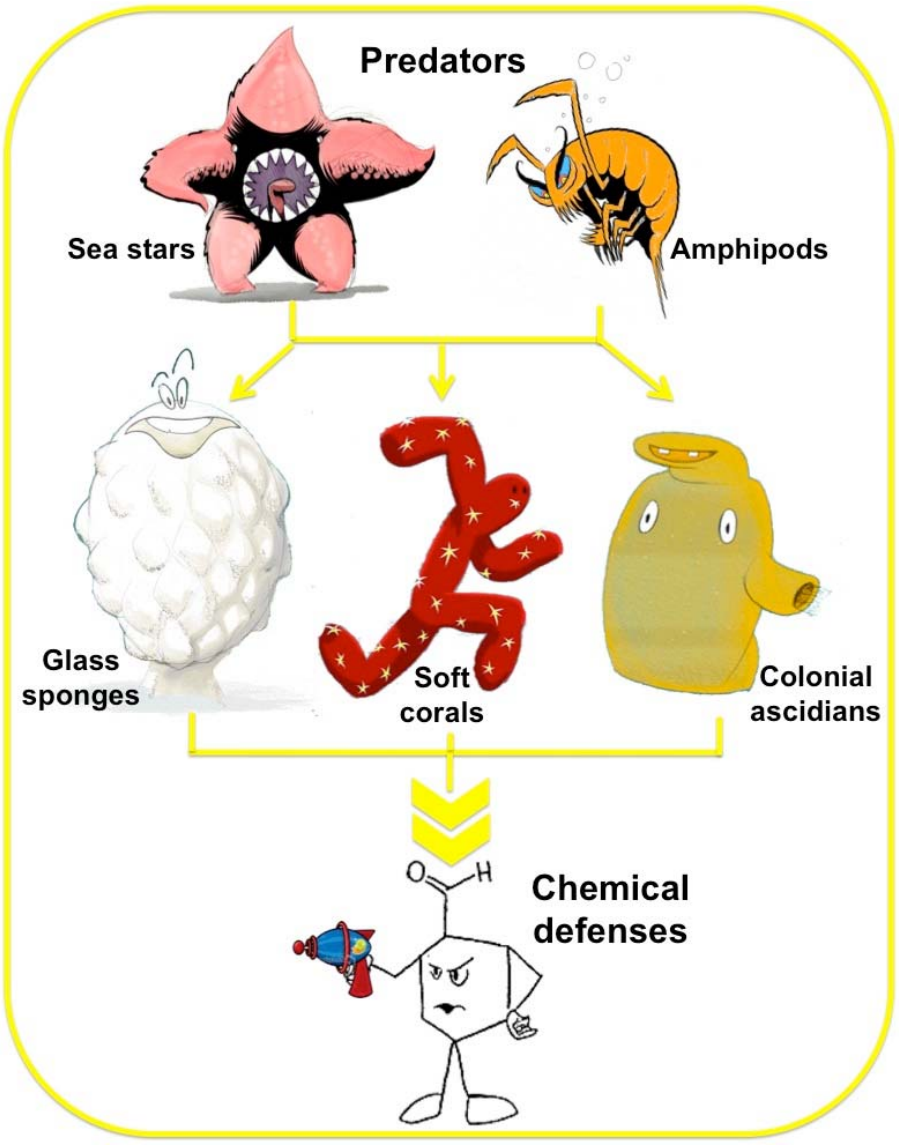
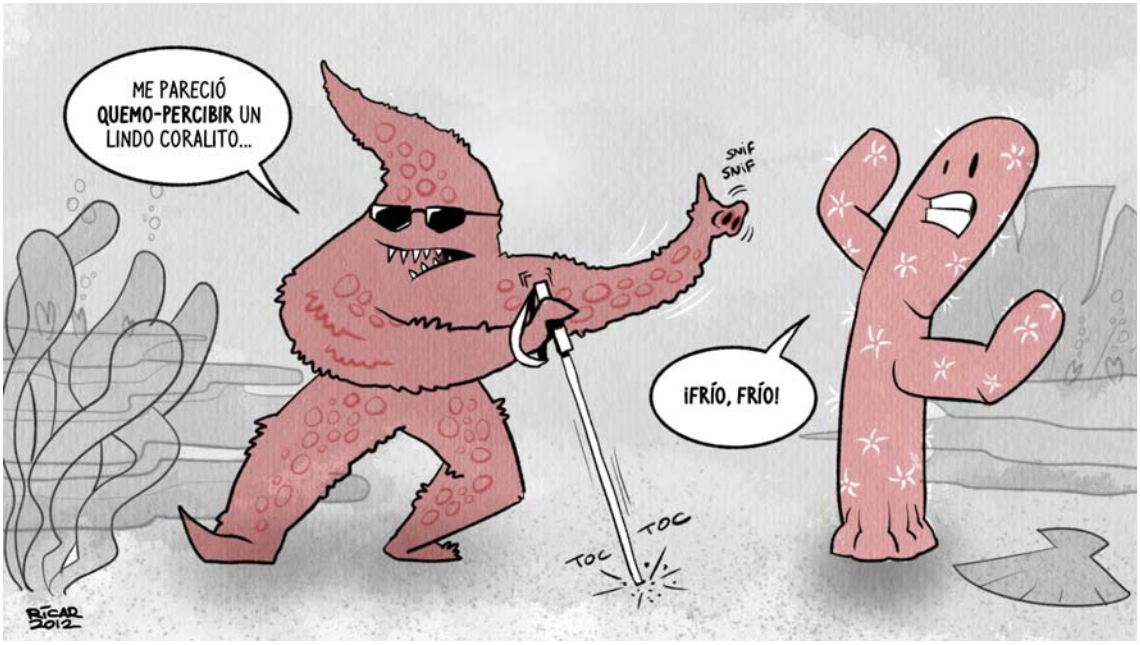


Fig. 10 General diagram of the PhD Thesis

CHAPTER 3.

RESULTS: PUBLICATIONS



CHAPTER 3.1. PUBLICATION I

NÚÑEZ-PONS L, RODRÍGUEZ-ARIAS M, GÓMEZ-GARRETA A, RIBERA-SIGUÁN A and AVILA C. 2012. Feeding deterrency in Antarctic marine organisms: bioassays with an omnivorous lyssianasid amphipod. ***Marine Ecology Progress Series***. *in press*. DOI: 10.3354/meps09840.

Feeding deterrency in Antarctic marine organisms: bioassays with an omnivorous lyssianasid amphipod

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ABSTRACT: Predation in the Antarctic benthos is intense and mainly provoked by macroinvertebrates and dense amphipod populations. Moreover, marked seasonalities of food availability drive consumers to develop opportunistic behaviors. This favors the evolution of defensive chemistry in potential prey. The circumpolar omnivorous amphipod *Cheirimedon femoratus* was selected to examine the incidence of lipophilic deterrents in Antarctic benthic organisms. A new feeding preference assay using alginate caviar-textured food pearls was performed. The protocol revealed methodological advantages and a remarkable discriminatory potential for distasteful metabolites. Thirty-one species, comprehending 40 samples from the Weddell Sea and South Shetland Islands including sponges (8), cnidarians (13), ascidians (8), bryozoans (1), echinoderms (1), hemichordates (1) and algae (8) yielded 52 fractions. Feeding unpalatability was found in 42 extracts from 26 species. The remaining 10 extracts from seven samples did not exhibit deterrency, and therefore either deterrents are contained in other fractions not tested here, or alternative defensive traits might protect these organisms. Within the four major taxonomical groups the ascidians showed the highest repellencies, followed by sponges, cnidarians, and algae. These organisms from distant Antarctic locations may represent both host biosubstrata and potential prey for this amphipod species, which could be a meaningful agent inducing chemical protection. Defense sequestration in specific body-structures, as predicted by the Optimal Defense Theory (ODT), was detected in an octocoral sample. Other organisms could display a combination of strategies to prevent predation. Our results indicate that chemical ecology is a key aspect to better understand the role of amphipods in Antarctic ecosystems.

KEY WORDS: *Cheirimedon femoratus* · Antarctic invertebrates · Antarctic algae · chemical ecology · omnivorous amphipod · unpalatability · feeding preference assays

INTRODUCTION:

Antarctic marine ecosystems are characterized by low temperatures and pronounced seasonality, with broad periodic limitations of food resources. Despite some coastal shallow regions (less than aprox. 30 m) exposed to ice scour and anchor ice, the benthos appears to be a stable, 'biologically accommodated' environment (Gutt 2000). Hence, these communities are supposed to be largely regulated in distribution and abundance by predatory and competitive interactions (Dayton et al. 1974). The continental shelf of Antarctica houses a rich suspension-feeding macroinvertebrate assemblage comprised of dominating sponges, soft corals, bryozoans, hydroids, and ascidians, as well as abundant macroalgae in the photic zone (Gutt et al. 2000, Wiencke et al. 2007). Higher trophic levels include mostly high densities of

opportunistic crustacean amphipods (De Broyer & Jazdzewski 1996, Huang et al. 2007), as well as generalist macroinvertebrate predators like nemerteans and asteroid echinoderms, and fish (Richardson 1975, Dearborn 1977, Gutt et al. 2000, Obermuller et al. 2010). These keystone predators, especially sea stars and populations of amphipods, cause intense ecological pressures, and are commonly circumpolar in distribution. Thus, sessile and sluggish organisms from distant Antarctic regions are likely affected in a similar way by their foraging activities (Dayton et al. 1974; De Broyer et al. 2007). These biological factors, along with the ancient (22 million years) and endemic nature of the Antarctic biota (Gutt 2000), suggest many opportunities for the evolution of predator-prey defensive mechanisms. One extended tactic is chemical defense, characterized by the biosynthesis or dietary storage of toxic, noxious or distasteful metabolites (Paul 1992). Presumably, the organisms must balance the energetic costs of defense against those of growth and reproduction. According to the Optimality Theory (OT), common defensive traits should be addressed for a variety of enemies to save energy (Herms & Mattson 1992). Moreover, the Optimal Defense Theory (ODT) also predicts that chemical defenses should be sequestered in the most vulnerable tissues in terms of fitness, attending to predators' habits, and in co-ordination with other defensive mechanisms (Rhoades & Gates 1976). Thus, defenses should be primarily allocated into the outermost zones, where they would be most effective against a number of predators that firstly encounter the victim's surface. But in perforated prey in which small grazers may reach to inner tissues, defense should be also found in the internal parts.

In Antarctic benthic communities, peracarid crustaceans, and especially Amphipoda, are by far the most species-rich group and probably the most diversified with respect to lifestyles, trophic types (including necrophagy, carnivory, herbivory, suspension feeding, detritivory, and omnivory), habitats and size spectra (De Broyer & Jazdzewski 1996). They commonly associate in a non-specifical way with living substrata (frequently macroalgae and sponges but also others), which are often their potential (direct or incidental) prey (De Broyer et al. 2001). Amphipods appear in very high densities (up to 300,000 individuals m^{-2} were reported in the Western Antarctic Peninsula; Huang et al. 2007), even if compared to other latitudes (Nelson 1980). As benthic grazers, Antarctic amphipods are highly influencing consumers feeding on a wide array of prey items: macroalgae, sponges, cnidarians, holoturians, bryozoans, diatoms… (Coleman 1989a, b, 1990, Graeve et al. 2001, Nyssen et al. 2005, Huang et al. 2006, 2007, Amsler et al. 2009, McClintock et al. 2009), and are relevant in terms of energy flux in the shelf ecosystem being an important food source for demersal fishes (Richardson 1975).

Feeding deterrents are quite widespread in Antarctic communities, but the impact of generalist amphipods has received scarce attention (for reviews see Avila et al. 2008, McClintock et al. 2010), even if these consumers are considered important inducers of defensive chemistry

(Cronin & Hay 1996, Toth et al. 2007). The lyssianasid amphipod *Cheirimedon femoratus*, with a circumpolar distribution and eurybathic occurrence down to 1500 meters depth (De Broyer et al. 2007, Krapp et al. 2008), is described as a voracious omnivorous-scavenger. It has opportunistic habits. In fact, it is a first-arrival feeder to carrion inputs (Smale et al. 2007). As a very abundant generalist feeder (animal, micro- and macroalgal material and detritus) (Bregazzi 1972b), *C. femoratus* could be considered a consumer towards which most potential prey inhabiting the Antarctic shelf would address defensive chemistry. Our samples came from variable depths of the scarcely studied Weddell Sea and the South Shetland Archipelago. Hence, here for the first time, this ubiquitous amphipod was chosen as experimental predator to perform palatability bioassays. Considering that most of the known marine repellents are lipid-soluble (Sotka et al. 2009), lipophilic extracts from Antarctic benthic invertebrates and algae were selected for experimentation. The aim of this study is to evaluate the presence of unpalatable defenses in target organisms and to determine the hypothetical within-body allocation of defenses when possible. Furthermore, a new protocol for feeding preference assays in Antarctic waters is proposed.

MATERIALS AND METHODS:

Collection of samples and taxonomical identification:

Marine benthic invertebrate and algal samples of 31 different species were collected in the Southern Ocean in four Antarctic campaigns: two of them took place in the Eastern Weddell Sea (Antarctica) on board the R/V Polarstern, from the Alfred Wegener Institute for Polar and Marine Research (AWI Bremenhaven, Germany) during the ANT XV/3 (January -March 1998) and ANT XXI/2 cruises (November 2003-January 2004). A third one was done on board the R/V BIO Hespérides during the ECOQUIM-2 cruise (January 2006) around the South Shetland Islands. And finally, the ACTIQUIM-1 cruise took place at Deception Island (December 2008 - January 2009; Fig. 1). Sampling was done in a total of 24 stations between 0 m and 1524 m depth by using bottom and Agassiz trawls, epibenthic sledge, and also by scuba diving (Table 1). Organisms were sorted on deck, photographed, and a voucher portion or specimen of each sample was fixed in 10% formalin or 70% ethanol and stored at Dept. of Animal Biology (Invertebrates) for taxonomical identification. Further identification studies were carried out at the Faculties of Biology and Pharmacy (University of Barcelona). All individuals or colonies of each species from a collection site were grouped as a single sample in order to represent mean values of each particular population, and were conserved at -20 °C for further examination.

Hundreds of individuals of the amphipod *Cheirimedon femoratus* were captured in the shoreline of the Spanish Antarctic Base (BAE) “Gabriel de Castilla” in Deception Island (62º 59.369' S, 60º 33.424' W) between 2 to 7 m depth during the Antarctic cruise ACTIQUIM-2 in January

2010 (Fig. 1) for feeding experiments. Collection was done by scuba diving employing fishing nets to capture aggregations of individuals attached to the piroclast sediment and algae, and also by displaying baited traps with canned sardines for 48h, which congregated dense swarms of amphipods. This further illustrates the omnivory and ability of this species to find food cues. After testing, a few specimens were fixed in 70% ethanol for taxonomy and the rest were returned to the sea.

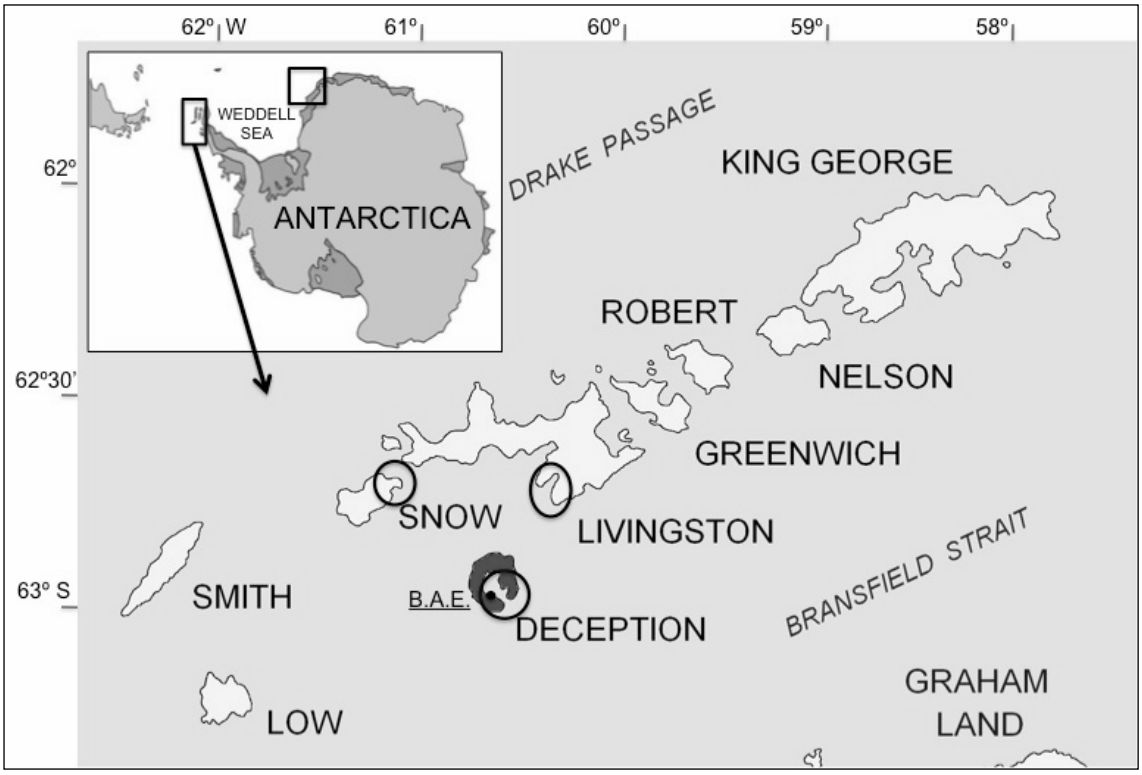

Fig. 1. Map of the Antarctic continent with the main sampling areas in the squares for the four campaigns, and the South Shetland Archipelago in detail, showing in circles the sampling points of that particular zone. In dark, Deception Island housing the Spanish Antarctic Base "Gabriel de Castilla" (B.A.E.) where the experiments took place.

Chemical extractions:

Some invertebrate samples were dissected for within-body allocation of defensive chemicals when possible. The sections were done attending to the predictions of the ODT for Antarctic prey, which should likely accumulate defenses in the outer layers for predation avoidance (Rhoades & Gates 1976). Depending on the organism these were sectioned into: internal (visceral) and external (tunic) tissues, in ascidians; external/internal or apical/basal parts, in sponges; and polyparium and axial regions in octocoral cnidarians. The resulting samples from

invertebrates and algae, each consisting on several individuals or colonies, were exhaustely extracted with acetone, and sequentially partitioned into diethyl ether and butanol fractions at the Faculty of Biology (University of Barcelona). All steps were repeated three times, except for the butanol, which was done once. Organic solvents were evaporated using a rotary evaporator. Chemical profiles of the obtained fractions were screened by thin layer chromatography (TLC). Only diethyl ether extracts (comprising most apolar lipophilic metabolites) were further used in the bioassays (Table S1 in the Supplement at: www.int-res.com). Butanolic fractions and water residues were not tested here, but were kept for future investigations.

Table 1: Antarctic benthic invertebrate and algal samples collected in the Southern Ocean. AGT: Agassiz Trawl, BT: Bottom Trawl, ES: Epibenthic Sledge, SD: Scuba Diving. BAS: basal, API: apical, EXT: external, INT: internal, POL: polyparium, AX: axis body-parts; B&W: Black & White, Br: Browm, O: orange morphotypes.

Taxonomic group and species name	Location	Latitude	Longitude	Gear	Depth (m)
PORIFERA					
Demospongiae					
Isodictya toxophila Burton, 1932	Weddell Sea	70° 57.00' S	10° 33.02' W	BT	332.8
Hexactinellida					
Anoxycalyx (Scolymastra) joubini Topsent, 1916 (1)	Weddell Sea	71° 06.30' S	11° 32.04' W	AGT	175.2
Anoxycalyx (Scolymastra) joubini Topsent, 1916 (2)	Weddell Sea	70° 52.16' S	10° 43.69' W	BT	290.8
Rossella fibulata Schulze & Kirkpatrick, 1910	Weddell Sea	70° 57.00' S	10° 33.02' W	BT	332.8
Rossella nuda Topsent, 1901	Weddell Sea	71° 4' S	11° 32' W	BT	308.8
Rossella vanhoffeni (Schulze & Kirkpatrick, 1910)	Weddell Sea	72° 28' S	17° 51’ W	ES	882
Rossella villosa Burton, 1929	Weddell Sea	70° 55.92' S	10° 32.37' W	AGT	288.0
Rossella sp.1 (Orange) Carter, 1872	Weddell Sea	70° 55.92' S	10° 32.37' W	AGT	288.0
CNIDARIA					
Anthozoa					
Alcyonium antarcticum Wright & Studer, 1889	Weddell Sea	70° 56' S	10° 31’ W	BT	337.2
Alcyonium haddoni Wright & Studer, 1889	Deception Island	62º 59.55' S	60º 33.68' W	SD	9
Alcyonium roseum van Ofwegen, Häussermann & Försterra, 2007	Weddell Sea	71º 17.1’ S	12º 36’ W	AGT	416
Primnoisis antarctica (Studer, 1878) (1)	Weddell Sea	70° 52.75' S	10° 51.24' W	BT	294.8
Primnoisis antarctica (Studer, 1878) (2)	Weddell Sea	70° 52.75' S	10° 51.24' W	BT	294.8
Thouarella laxa Versluys, 1906 (1)	Weddell Sea	71° 4' S	11° 32' W	BT	308.8
Thouarella laxa Versluys, 1906 (2)	Weddell Sea	70° 52.16' S	10° 43.69' W	BT	290.8

Thouarella laxa Versluys, 1906 (3)	Weddell Sea	70° 52.75' S	10° 51.24' W	BT	294.8
Thouarella laxa Versluys, 1906 (4)	Deception Island	63º 02.29' S	60º 36.36' W	AGT	100.4
Thouarella minuta Zapata-Guardiola & López-González 2009	Weddell Sea	70° 56' S	10° 32' W	BT	338
Umbellula antarctica Kükenthal and Broch, 1911	Weddell Sea	70° 56' S	10° 32' W	BT	338
Hydrozoa					
Staurotheca antarctica Hartlaub, 1904	Weddell Sea	72° 51.43' S	19° 38.62' W	BT	597.6
Symplectoscyphus glacialis (Haderholm 1904)	Weddell Sea	71° 06.30' S	11° 32.04' W	AGT	175.2
CHORDATA (ASCIDIACEA)					
Aplidium falklandicum Millar, 1960	Weddell Sea	70° 57.00' S	10° 33.02' W	BT	332.8
Aplidium fuegiense Cunningham, 1871	Weddell Sea	71° 7' S	11° 26' W	AGT	228.4
Aplidium meridianum (Sluiter, 1906)	Weddell Sea	70° 56.42' S	10° 31.61' W	BT	284.4
Synoicum adareanum (Black & white) (Herdman, 1902) (1)	Weddell Sea	70° 56' S	10° 32' W	BT	337.2
Synoicum adareanum (Black & White) (Herdman, 1902) (2)	Weddell Sea	70° 55.92' S	10° 32.37' W	AGT	288.0
Synoicum adareanum (Brown) (Herdman, 1902)	Weddell Sea	71° 06.44' S	11° 27.76' W	AGT	277.2
Synoicum adareanum (Orange) (Herdman, 1902) (1)	Weddell Sea	70° 55.92' S	10° 32.37' W	AGT	288.0
Synoicum adareanum (Orange) (Herdman, 1902) (2)	Weddell Sea	70° 56' S	10° 32' W	BT	337.2
BRYOZOA					
Isoschizoporella secunda Hayward and Taylor, 1984	Weddell Sea	71° 06.44' S	11° 27.76' W	AGT	277.2
ECHINODERMATA (HOLOTUROIDEA)					
Peniagone vignioni Herouard, 1901	Weddell Sea	70° 47.88' S	11° 24.13' W	AGT	1524.8
HEMICHORDATA (PTEROBRANCHIA)					
Cephalodiscus nigrescens Lankester, 1905	Weddell Sea	70° 56.42' S	10° 31.61' W	BT	284.4
ALGAE					
Ochrophyta					

Adenocystis utricularis (Bory de Saint-Vincent) Skottsberg 1907	Snow Island	62º 44.01' S	61º 12.2' W	SD	1.5
Ascoseira mirabilis Skottsberg 1907	Livingston Island	62º 45' S	60º 20' W	SD	0.7
Desmarestia anceps Montagne 1842	Deception Island	62º 59.37' S	60º 33.42' W	SD	7.5
Desmarestia antarctica Moe & Silva 1989 with *Geminocarpus austrogeorgiae* Skottsberg, 1907	Livingston Island	62º 45' S	60º 20' W	SD	0.7
Desmarestia menziesii J.Agardh 1848	Deception Island	62º 59.02' S	60º35.85' W	AGT	109.7
Rodophyta					
Georgiella confluens (Reisch) Kylin 1956	Livingston Island	62º 45' S	60º 20' W	SD	0.7
Gigartina skottsbergii Setchell & Gardner, 1936	Deception Island	62º 59.37' S	60º 33.42' W	SD	12
Palmaria decipiens (Reinsch) Ricker 1987	Deception Island	62º 58.59' S	60º 40.58' W	SD	1.3

Selection of the putative predator:

In our search for an appropriate experimental consumer, three of the most abundant species around the Antarctic Spanish Base were assessed. Selective trials were performed with the limpet *Nacella concina*, the sea urchin *Sterechinus neumayeri*, and the amphipod *Cheirimedon femoratus*. Our attempts revealed that the limpet and the urchin would consistently eat barely anything in the lab and did not fulfill the minimum grazing rates, according to previous reports (*i.e.* Amsler et al. 2005b). Moreover the predator should be ubiquitous and omnivorous, since extracts from animals and algae coming from distant locations were being evaluated. Thus *Cheirimedon femoratus* was finally chosen. This species is eurybathic, circumantarctic and a voracious omnivorous-scavenger (De Broyer et al. 2007, Krapp et al. 2008), providing ecologically relevant information. Ovigerous females and newly hatched young though, ingest more photosynthetic material (algae) during summer (Bregazzi 1972b).

Artificial food preparation:

Artificial foods were prepared in two formats: the first one consisted on the traditional agar strips on a base of window screen derived from the modified method of Hay et al. (1994). The agar proportion though was reduced from 20 mg ml^{-1} to 10 mg ml^{-1} and the quantity of food stimulant was increased from 55.6 mg ml^{-1} to 80 mg ml^{-1}. The second format was new, and it used alginate caviar pearls made with the "Kit Sferificacion®" (comprising various ingredients) of the famous cook Ferran Adrià. Agar-strips showed disadvantages compared to alginate spheres: 1) lower consumption rates, resulting in longer experimental times, 2) required larger amounts of extract, and moreover, 3) the agar had to be heated up to 70 ºC, which could entail degradation of some compounds in the extracts. Hence, alginate food pearls were selected for our assays after several trials. A concentrated blend of powdered dried aquarium food called Phytoplan® (17-19 KJ g^{-1} dry wt) was chosen as feeding stimulant for providing the highest consumption rates. Phytoplan® was selected among diverse diets, including vegetarian sources: *Chlorella*, Nori, liofilized Antarctic seaweeds (*Desmarestia anceps, D. menziesii, Gigartina skottsbergii*), *Spirulina*-based feed and phytoplancton, as well as carnivorous sources: krill, Cyclop-eeze mixed zooplancton, fish feed and anchovy paste. The final artificial food recipe consisted of 10mg/mL alginate (Algin® of "Kit Sferificacion®") aqueous solution containing 66.7 mg ml^{-1} of feeding stimulant (Phytoplan®) and a drop of green or red food coloring (see below). The mixture was introduced into a syringe without needle, and added dropwise into a 0.09 M (1%) $CaCl_2$ (Calcic® of "Kit Sferificacion®") water solution, where it gelatinized/polymerized to form spheroid pearls approximately 2.5 mm in diameter. These yielded a protein content of 3.3%, 1.36% carbohydrates and 1.3% of lipids (based on nutrition facts from Phytoplan®). Extracts, applied at natural tissue concentrations, were dissolved in

solvent carrier (diethyl ether) to totally wet the food stimulant and the solvent was then evaporated, resulting in a uniform coating of the extract on the powdered food concentrate prior to being added into the alginate aqueous mix. The factor employed to normalize tissue concentrations of each lipophilic fraction (hereafter referred to as the 'natural concentration') was calculated on a dry-weight basis attending to the total dry weight (DW_T = DW dry weight of the extracted sample + EE weight of the ethereal fraction + BE weight of the butanolic fraction). Volume-based normalization is usually applied when dealing with biting predators to calculate the "defense per unit bite" (*i.e.* Pisut & Pawlik 2002). Concentrations based on biomass employing wet or dry weight are used with no-biting and biting predators (*i.e.* Amsler et al. 2005b, Núñez-Pons et al. 2010). When using food pearls "defense per pearl" can be measured. We chose dry weight, because it is the most constant parameter for eliminating the water content, which entails notable deviations when manipulating aquatic samples, especially sponges (Table S1 in the Supplement). The relative quantity of each extract at natural concentration was then calculated referred to the total dry weight of artificial food mix required to elaborate a whole set of extract-treated pearls for a single experiment: 0.23 g (0.03 g alginate + 0.2 g Phytoplan®). This guarantees the formation of 150 pearls (15 replicates x 10 pearls per replicate), and a few extra pearls, useful to check for autogenic modifications (see below). Control food pearls were prepared identically but without extract, adding an equal volume of solvent alone. Control extract-free pearls and treatment pearls (containing extract) were visually distinguished in paired assays by adding different liquid tasteless food colorings (red and green) to the alginate mix before spherification in $CaCl_2$ solution. Several previous trials confirmed the null effect of food colorings in feeding preferences respect to not colored pearls ($p = 0.47$, n.s.), and also between red and green colored pearls ($p = 0.47$, n.s.). Nevertheless, control and treatment food pearls were randomly swapped to green or red colorations throughout the experiments.

Feeding-preference bioassays with amphipods:

Alive organisms of *Cheirimedon femoratus* were maintained with fresh seawater in 8L aquariums at the BAE and were starved for 3-5 days. Every assay consisted on 15 replicates, which were run in 15 500-ml containers, each filled with sea water with 15-20 randomly selected amphipods and a simultaneous choice of 10 pearls of each food type (20 in total: 10 control extract-free and 10 extract-treated pearls, with different colorations: green or red). Amphipods attached to the pearls and ate individually or in groups of up to 5 specimens per pearl, according to their gregarious habits (Bregazzi 1972b). They were extremely voracious and so they were periodically monitored and video filmed previously to determine the time course of the experiments. We concluded that on average a group of 15 to 20 individuals ingested 10 pearls in about 4 to 5 hours. Thus, the assays were considered to be over when

approximately one-half or more of either food types (control and/or treatment) had been consumed, or 4 hours after food presentation, and amphipods were not re-used. The number of consumed and not consumed pearls of each color (control or treatment) was recorded. In previous trials food pearls proved to be resistant to degradation after 72-hr exposure in seawater. Since our feeding assays were short in time (4 hrs), we eliminated the need to run other "controls" in the absence of consumers for autogenic changes unrelated to consumption (Peterson & Renaud 1989). Finally, statistics were calculated to determine feeding preference of extract-treated foods versus the paired simultaneous controls to consequently establish repellent activities. For each of the 15 replicate containers and each food type the quantity of ingested food was counted, and the differences for each experimental unit (replicate) were calculated. The changes in the two foods held in the same container are not independent and possess correlated errors. Each replicate is thus represented by a paired result yielding two sets of data (treatments and controls). Both sets of data can be compared, since assumption of normality and homogeneity of variances are not met, by non-parametric procedures, that is by applying the Exact Wilcoxon test, calculated using R-command software (Fig. 2). Uneaten pearls, or extra pearls conserved in seawater, were preserved for further extraction and analysis by TLC, to check for possible alterations in the extracts after testing. No major changes were observed in any case, plus, theoretically the compounds solved in diethyl ether extracts are not hydrophilic, hence there should be little, if any, loss to the water column.

RESULTS:

The incidence of unpalatable defenses in Antarctic benthic organisms against the omnivorous lysianassid amphipod *Cheirimedon femoratus* was very high. A total of 31 species were tested including 40 samples: sponges (8), cnidarians (13), tunicates (8), bryozoans (1), echinoderms (1), hemichordates (1) and algae (8). The majority of our samples came from the Weddell Sea area, while two anthozoan samples and the eight algal samples came from shallower bottoms of the South Shetland Islands (Table 1). No apparent pattern related the incidence of unpalatable chemicals with location or depth. *C. femoratus* was very voracious towards Phytoplan®-alginate control pearls, which were ingested at extraordinary high rates. And in spite of its gregarious behavior, feeding preferences were fairly evident. In fact, most of the extracts that yielded unpalatability were barely consumed when included in food pearls, while the paired controls were mostly completely eaten.

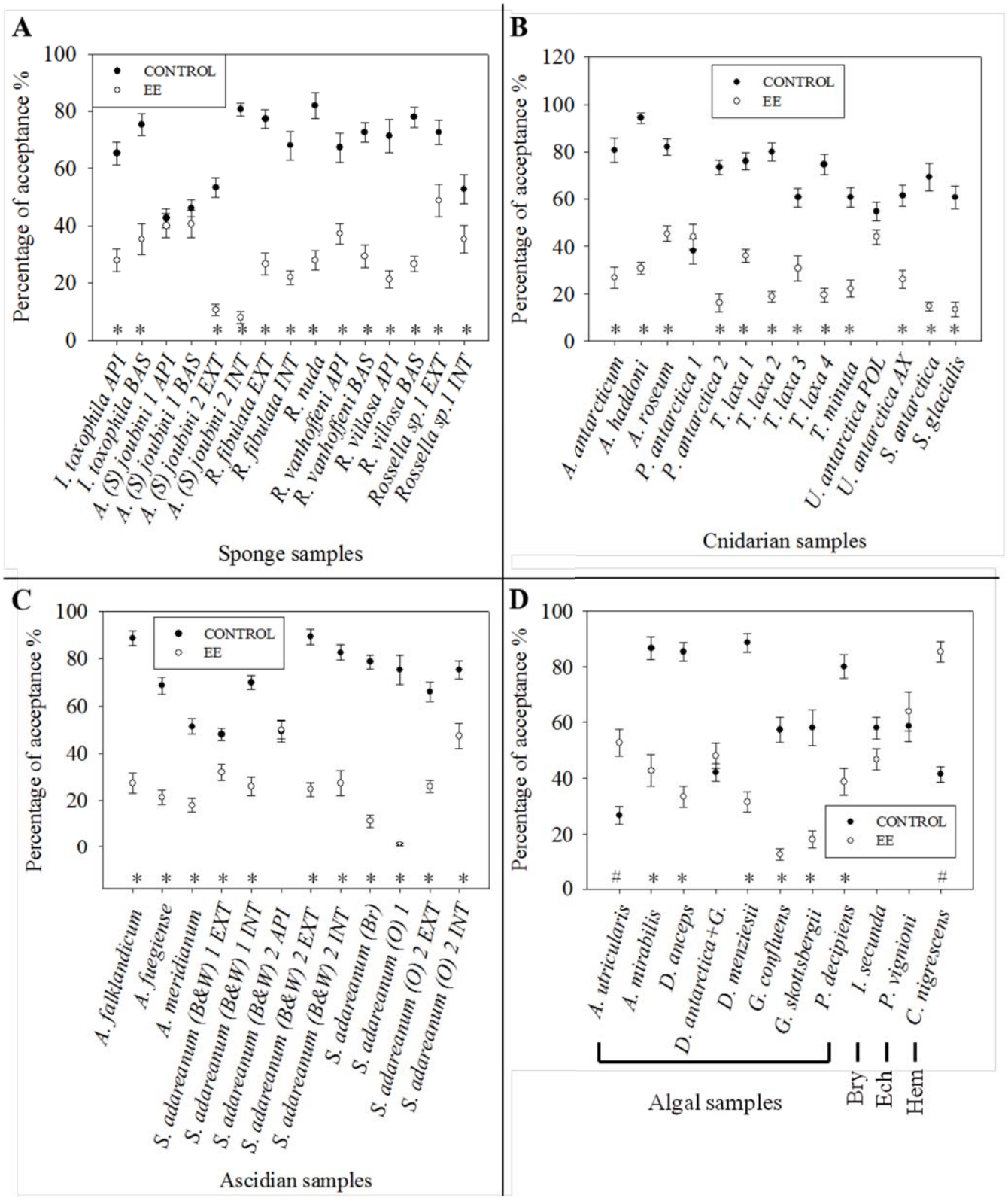

Fig. 2. *Cheirimedon femoratus*. Scatter plot diagrams of the feeding preference bioassays with the amphipod for the four major groups assessed: (A) sponges, (B) cnidarians, (C) ascidians (in *Synoicum adareanum*: B&W: Black & White, Br: Browm, O: orange morphotypes) and (D) algae and other minor groups (Bry: Bryozoa, Ech: Echinodermata, Hem: Hemichordata). API/BAS: apical/basal, EXT/INT:

external/internal, POL/AX: polyparium/axial body-regions. The mean percentage of acceptance and standard error bars of the paired results with control and extract treated food pearls are represented for each test. *: significant differences ($p<0.05$) with control as preferred food; #: significant differences ($p<0.05$) with extract preferred to controls (Exact Wilcoxon test).

From a total of 52 extracts assayed, of 40 samples pertaining to 31 species, 33 samples showed feeding unpalatability (26 of the species) towards *Cheirimedon femoratus*, revealing 42 repellent lipophilic extracts (80.8%). The remaining 10 extracts, obtained from 7 samples belonging to 5 different species, did not exhibit any activity (Fig. 2). The four major taxonomical groups tested yielded high percentages of significant feeding repellence against the amphipod, with the ascidians in the lead displaying 91.7% of unpalatable extracts, followed by the sponges 86.7%, cnidarians 85.7%, and algae 75%. One of the two samples of the gorgonian *Primnoisis antarctica*, and one *Anoxycalyx (Scolymastra) joubini* hexactinellid poriferan caused no rejection when testing their extracts, as well as the bryozoan, the holothurian and the hemichordate. Actually, food pearls containing extracts from the pterobranch (hemichordate) *Cephalodiscus nigrescens* ($p = 0.002$*), as well as from the algae *Adenocystis utricularis* ($p = 0.002$*) were preferred from control pearls. Regarding dissected samples, the pennatulacean *Umbellula antarctica* exhibited a deterrent axis, but the diethyl ether fraction from the polyparium was readily ingested. Moreover the apical ethereal fraction (API) from the ascidian *Synoicum adareanum* (B&W: black and white morphotype) 2 was palatable contrasting with the barely consumed basal-external and visceral (EXT and INT) extracts (Table S1 in the Supplement; Fig. 2).

DISCUSSION:

Grazing describes a type of feeding on part of a prey without killing it outright. This promotes strategies of resistance to further consumption. Indeed, amphipods are meaningful inducers of chemical defense, since they can congregate in high densities on their biosubstratum, using it as potential diet too, exerting an intense localized pressure (Cronin & Hay 1996, Toth et al. 2007). Nonetheless, and according to the OT (Herms & Mattson 1992), hosts tend to be protected concurrently from a wide range of predators, also representing chemical refuges for small "commensal" grazers from larger mobile enemies. Moreover, defenses are mostly addressed towards generalists (Sotka et al. 2009, Paul et al. 2011). This is certainly more useful in places like Antarctica, where unpredictable food availabilities drive consumers to develop flexible foraging strategies. In Antarctic sea bottoms putative biosubstrata include seaweeds, sponges, cnidarians, ascidians… (*i.e*. Kunzmann 1996, De Broyer et al. 2001, Loerz & Coleman 2003, Amsler et al. 2009, McClintock et al. 2009, Zamzow et al. 2010). Marine non-specific amphipods usually select for chemically protected hosts (Poore et al. 2008). Thus, *Cheirimedon*

femoratus, a bottom-dweller with limited swimming capacities and opportunistic habits for feeding and for host relationships (Bregazzi 1972a; field and baited traps authors' pers. obs.) is expected to influence in the defensive potential of many benthic organisms. The widespread incidence of unpalabilities found towards *C. femoratus* in our samples coming from a broad depth range of the Weddell Sea and South Shetland Archipelago could actually be due to its great ubiquity. This may moreover reflect the importance of this amphipod as generalist consumer provoking the evolution of defenses in Antarctic communities.

The most active unpalatable group, the tunicates, presented repellents in all the samples tested, indicating a predominant reliance on organic chemical protection. This contrasted with other Antarctic surveys (Koplovitz et al. 2009). Our samples pertained to common Antarctic colonial species (Varela 2007), some reported to harbor bioactive products (see Avila et al. 2008, Blunt et al. 2011, and previous reviews). In fact, two species, *A. falklandicum* and *A. meridianum*, are known to posses the meridianins, defensive alkaloids that cause rejection to the asteroid *Odontaster validus*, and which are found in inner as well as in outer tissues (Núñez-Pons et al. 2010). Similarly, no allocation of defenses was detected in our tunicate samples. Apparently, repellents are often sequestered in the gonads of tunicates providing protected larval stages (Pisut & Pawlik 2002). Other antipredation strategies not measured here describe tunics containing poor nutritional value, or accumulating acid or heavy metals (McClintock et al. 1991, Pisut & Pawlik 2002, McClintock et al. 2004; Koplovitz et al. 2009, Lebar et al. 2011). Colonial ascidians frequently exhibit intraspecific variability (Varela 2007), and we found 3 morphotypes for *Synoicum adareanum*: black and white (B&W), brown (Br) and orange (O). All were significantly unpalatable except for the apical diethyl ether fraction from a *S. adareanum* B&W. This region concentrated siphon mouths and common cloaca, and is where waste matter from digestion accumulates (Table S1 in the Supplement, Fig. 2). Fresh colonies of *S. adareanum* were previously reported to provoke rejection to the fish *Notothenia coriiceps* and to *O. validus*, while crude extracts were positively preferred for the amphipod *Gondogeneia antarctica* (Koplovitz et al. 2009). Chemically defended ascidians may also represent refuges for inquiline crustaceans from prospective fish predators, as described in *Distaplia cylindrica* with the amphipod *Polycheria antarctica* (McClintock et al. 2009).

Glass sponges, even if representing not much of a meal due to a low energetic value, are subject to intense predation by Antarctic invertebrates (McClintock 1987, Barthel 1995). They are considered to be poor in secondary metabolites (see Blunt et al. 2011 and previous reports) and their extracts have usually displayed little bioactivity (McClintock 1987). However, our results greatly contradict these postulates, and all but one of the hexactinellid samples assayed yielded significant unpalatable activity. The palatable extracts from *Anoxycalyx (Scolymastra) joubini* 1, had lower natural concentrations respect to the unpalatable extracts from a conspecific sample, *A (S.). joubini* 2 (Table S1 in the Supplement). Hydrophilic and lipophilic extracts from *A (S.)*

joubini caused strong tube-foot retractions to the spongivorous *Perknaster fuscus*, although this could be due to the highly specialized diet of this asteroid on *Mycale acerata* (McClintock et al. 2000). Our sponge samples displayed unpalatable activities in outer and inner tissues, indicating no defense allocation. This differs with the clear distribution found by Furrow et al. (2003), but it is in accordance with what Peters et al. (2009) reported for some of their poriferans. The expected localization of defensive chemistry primarily to the outermost layers could be highly adaptive against asteroids that feed by extrusion of the cardiac stomach according to the ODT (Rhoades & Gates 1976). But this might be ineffective towards smaller biting grazers approaching to inner body parts through perforations, like large hexactinellid oscula. Amphipods, which occur in high abundance and diversity in association with Antarctic sponges with no obligate host relationships, are clear examples (Kunzmann 1996, Loerz & De Broyer 2004, De Broyer et al. 2007). However, Amsler and co-workers have ruled out amphipods as a source of significant spongivory and as responsible for the evolution of defenses in Antarctic sponges, after observing that sponge extracts stimulated rather than inhibited feeding. However, the amphipod used in those tests (*Gondogeneia antarctica*) frequently exhibits skewed increased preferences to foods containing extracts (Amsler et al. 2005b, Koplovitz et al. 2009). Contrarily, in our case, the amphipod *C. femoratus* might certainly affect sponges' chemical protection.

Cnidarians are rich sources of bioactive metabolites. In fact, our octocoral samples included species known to possess characteristic natural products (see Avila et al. 2008, Blunt et al. 2011 and previous reviews). Soft corals and gorgonians do not usually have stinging nematocysts, hence chemistry is likely an important resort of protection in these groups (Paul 1992, Sammarco & Coll 1992). Our results agree with this assumption, and most of the extracts displayed unpalatability. Only the anthozoan *Primnoisis antarctica* displayed acceptance with one sample (1), but the highly concentrated extract from another conspecific sample (2) was rejected by the amphipod (Table S1 in the Supplement; Fig. 2). This is probably due to natural variability in compounds concentrations.

Umbellula antarctica is a pennatulacean devoid of sclerites with a flower shape (Pasternak 1962). In the dissection, the sample was separated into the more exposed and nutritiously rich crowns with few (15-30) voluminous polyps, and the axis. The polyparium yielded a rich but palatable extract, reflecting a lack of lipophilic deterrents in this region. Thus, either defensive agents are present in more polar fractions, or the prominent autozooids (3-4 cm long) are protected by their effective penetrating nematocysts. Actually, pennatulid species in oligotrophic regions tend to have fewer but larger autozooids, allowing them to practice active macrophagy or carnivory (Dolan 2008). Here, the axial stalk, deprived of nematocysts, did significantly repel *C. femoratus.* Also, different chemical profiles were found in the extracts from both regions in the TLC plates. This could partly be due to the unpalatable metabolites

present in the axis but absent in the polyparium. In this case, chemical and nematocyst-based defenses would not be redundant since they protect different body-regions. Instead, in hydroids, where lipophilic defenses seem to be as common as defensive nematocysts, both strategies are not supposed to co-exist according to the OT (Herms & Mattson 1992, Stachowicz & Lindquist 2000, Lindquist 2002). But both hydrozoan extracts tested here revealed deterrency. In the case of *Symplectoscyphus glacialis*, from the family Sertulariidae, they rarely possess penetrating nematocysts. However, species of the Syntheciidae family, like *Staurotheca antarctica*, do possess them (Shostak 1995). Such co-existence of both defenses would seem redundant.

The ether extracts of the bryozoan and the holothurian tested were not repellent against *C. femoratus*. Indeed, certain large sized acanthonotozomatid and stilipedid Antarctic amphipods include prey from these taxa in their diet (Coleman 1989a, 1990). The sea cucumber in particular, *Peniagone vignioni*, might not be so vulnerable to amphipods, thanks to its locomotive benthopelagic activities and ability to swim (Galley et al. 2008). As to the bryozoan, *Isoschizoporella secunda*, the apparent lack of chemical defenses might be offset by the possession of avicularia that can act as traps for small crustaceans (Carter et al. 2010) and by its calcified structure (Winston & Bernheimer 1986). The extract from *Cephalodiscus nigrescens* was inactive, even phagostimulatory (Fig. 2). This pterobranch lives sheltered within a secreted reinforced encasement (Ridewood 1911), which could already provide with enough physical protection. The reliance on more polar defensive metabolites however, cannot be ruled out.

Some results of our assays with macroalgal samples agree with previous studies. For instance, the lipophilic extract from *Desmarestia menziesii* elicited unpalatability as was recorded for other grazers (Amsler et al. 2005b). Also the diethyl ether extract of *Desmarestia antarctica* epiphyted by *Geminocarpus austrogeorgiae* was not repellent in our assay. This corroborated the assumptions of this alga combining acid sequestration along with low organic defenses (Amsler et al. 2005b), but deterrents might occur in other fractions not tested here. *Adenocystis utricularis* yielded a palatable extract that elicited preference respect to controls. This was reported with *Gondogeneia antarctica* as well, but rejection to sea stars has been also described in this alga (Amsler et al. 2005b). Some of our results do as well disagree with previous findings. For instance, *Palmaria decipiens* was rejected in our test, but it has been described as palatable to other amphipods. Fresh thallus from this alga and from *Gigartina skottsbergii* were found suitable to various consumers (Amsler et al. 2005b, Huang et al. 2006, Aumack et al. 2010, Bucolo et al. 2011). The remaining seaweed extracts tested here reflected deterrence towards *C. femoratus* (Table S1 in the Supplement; Fig 2).

In Antarctic macroalgae, an inverse correlation between amphipod density in the field and feeding preference for that same alga in the laboratory, as well as a correlation with the level of defense in the algae seem to exist. This could explain why chemically defended algae often harbor high amphipod densities (Amsler et al. 2005a, b, Huang et al. 2006, 2007, Zamzow et al.

2010). Contrary to coastal zones where detailed investigations of amphipod habitats may be done directly by scuba diving (*e.g.* Bregazzi 1972a), in the deeper shelf, determination of temporal host associations relies on indirect approaches (De Broyer et al. 2001). Our deep samples reflect this lack of information. In marine ecosystems, host organisms offer structural and/or chemical asylum from predation. They also represent sources of nutrition, either by direct profit of host tissues, or often by indirect (casual) ingestion when grazing on detritus or associated microbiota, such as diatoms readily fouling host surfaces (Kunzmann 1996, Amsler et al. 2000, De Broyer et al. 2001, Graeve et al. 2001, Amsler et al. 2009, Zamzow et al. 2010). Hence, amphipods associate with defended invertebrates, in particular as they do with algae in photic zones, because they provide chemical refuges from prospective fish (Richardson 1975, Huang et al. 2007, McClintock et al. 2009, Zamzow et al. 2010). Most of the invertebrate samples tested here from both, Weddell Sea and South Shetland Islands, do contain chemical repellents, and could replace macroalgae as hosts. Moreover, generalist amphipods usually associate with chemically defended biosubstrata (Poore et al. 2008), maybe because their assorted diets reduce the consumption of recurrent host repellents (Sotka et al. 2009, Paul et al. 2011). Even if in nature defended organisms maybe foraged fortuitously while profiting other resources (Graeve et al. 2001), in the lab, repellence for these tissues can be notable. Some of our results with the opportunistic *C. femoratus* could be related to this phenomenon.

When conspecific samples displayed different palatabilities in our assays, the active one was that with the richer extract (like in *A. (S.) joubini* and *P. antarctica*), suggesting possible higher quantities of repellents. This may be due to different composition in samples collected in distant stations (Table 1), subjected to diverse environmental conditions and/or genetic variability (Cutignano et al. 2011). Furthermore, variabilty could be related to chemical defense induction (Cronin & Hay 1996, Lindquist 2002, Paul et al. 2011), even though in Antarctica events of inductive defenses have not yet been proved (McClintock et al. 2010).

Effectiveness of deterrents reflects biochemical interactions between a defensive metabolite and a particular consumer. We examined diethyl ether extracts because most of the reported repellents from sea organisms are lipid-soluble, and because amphipods seem more affected by defensive lipophilic metabolites (Koplovitz et al. 2009, Sotka et al. 2009, Aumack et al. 2010). Nevertheless, less lipophilic fractions are kept for future studies. Scavenging lysianassid amphipods have well-developed gustatory gnathopods able to typify items chemically and physically while eating (Kaufmann 1994). Actually necrophagous Antarctic amphipods, including *C. femoratus,* are highly sensitive to food cues (Smale et al. 2007), which may explain the amazing ability of this species in detecting repellence.

The new experimental protocol proposed here evaluated the incidence of chemical defenses against a significant Antarctic opportunistic consumer, the amphipod *Cheirimedon femoratus* that resulted in a very suitable experimental model. On balance, the test provided many

methodological benefits: (1) requirement of small quantities of extract; (2) short experimental timings (4 h); (3) omnivorous amphipods allowed to assess algal and invertebrate samples; (4) easiness of reading the results; (5) simple statistical analysis; (6) lack of heating, thus avoiding chemical damage; (7) high discriminatory potential of *C. femoratus* for unpalatable metabolites; and (8) the great ubiquity of this amphipod makes it suitable for assessing chemical defense in organisms from many Antarctic locations. Amphipods often provide excellent models for studying feeding behavior since they can be easily manipulated and fed on artificial diets.

More studies are needed to determine the importance of amphipods and their role inducing the production of defensive metabolites in Antarctic communities. These should include field experiments to better mimic natural ecological interactions.

Ackowledgements: We thank J. Vázquez, B. Figuerola, F.J. Cristobo and S. Taboada for their precious support in the Antarctic cruises. Thanks are due to W. Arntz and the crew of R/V Polarstern for their help on board. UTM (CSIC), "BIO-Hespérides", "BIO-Las Palmas" and BAE "Gabriel de Castilla" crews provided logistic support. We are thankful to M. Mota for statistical advice and to P. Ríos, M. Varela and A. Bosch for taxonomical contributions. Funding was provided by the Ministry of Science and Innovation of Spain (CGL2004-03356/ANT, CGL2007-65453/ANT and CGL2010-17415/ANT). Thanks also to F. Adrià for inventing his "Kit Sferificacion"®.

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On-line Supplementary material:

Table S1: Data of the diethyl ether extracts of the studied samples. WW: wet weight of the sample, DW: dry weight of the sample, EE: dry weight of the diethyl ether extract, [N_{EE}] respect DW (%): percentage of the natural tissular concentration of EE in the sample calculated by dividing EE by DW; n.a.: not available; BAS: basal, API: apical, EXT: external, INT: internal, POL: polyparium, AX: axis body-parts; B&W: Black & White, Br: Browm, O: orange morphotypes.

Taxonomic group, species and bodypart	WW (g)	DW (g)	EE (mg)	% [N_{EE}] respect DW
PORIFERA				
Demospongiae				
Isodictya toxophila API	88.00	17.30	913.00	5.28
Isodictya toxophila BAS	61.00	12.30	835.00	6.79
Hexactinellidae				
Anoxycalyx (S) joubini 1 API	137.30	18.11	233.20	1.29
Anoxycalyx (S) joubini 1 BAS	53.40	12.51	91.39	0.73
Anoxycalyx (S) joubini 2 EXT	266.30	32.90	855.50	2.60
Anoxycalyx (S) joubini 2 INT	80.40	16.10	294.90	1.83
Rossella fibulata EXT	254.70	120.75	1432.75	1.19
Rossella fibulata INT	518.70	104.73	1833.61	1.75
Rossella nuda	422.20	118.37	1384.30	1.17
Rossella vanhoffeni API	226.20	16.12	381.22	2.36
Rossella vanhoffeni BAS	253.00	45.61	279.67	0.61
Rossella villosa API	336.70	81.15	851.96	1.05
Rossella villosa BAS	452.40	98.71	980.16	0.99
Rossella sp,1 EXT	424.40	88.73	1062.30	1.20
Rossella sp,1 INT	572.90	83.33	898.71	1.08
CNIDARIA				
Anthozoa				
Alcyonium antarcticum	1.01	0.52	10.15	1.97
Alcyonium haddoni	118.90	17.65	813.41	4.61
Alcyonium roseum	9.32	1.66	53.21	3.21
Primnoisis antarctica 1	38.80	19.69	88.74	0.45
Primnoisis antarctica 2	32.30	13.99	81.11	0.58
Thouarella laxa 1	12.50	5.56	80.14	1.44
Thouarella laxa 2	122.60	44.43	562.09	1.27

Thouarella laxa 3	79.40	27.38	240.02	0.88
Thouarella laxa 4	62.51	22.94	330.24	1.44
Thouarella minuta	8.60	4.87	97.65	2.00
Umbellula antarctica POL	25.68	4.17	482.93	11.59
Umbellula antarctica AX	3.84	2.46	26.47	1.08
Hydrozoa				
Staurotheca antarctica	28.40	5.13	201.77	3.93
Symplectoscyphus glacialis	140.60	22.50	229.30	1.02
CHORDATA (ASCIDIACEA)				
Aplidium falklandicum	199.10	19.93	837.16	4.20
Aplidium fuegiense	n.a.	14.60	1125.50	7.71
Aplidium meridianum	30.29	1.30	167.27	12.85
Synoicum adareanum (B&W) 1 EXT	36.60	7.20	144.30	2.00
Synoicum adareanum (B&W) 1 INT	89.90	6.90	228.30	3.31
Synoicum adareanum (B&W) 2 API	41.60	4.00	222.71	5.57
Synoicum adareanum (B&W) 2 EXT	66.00	14.29	258.90	1.81
Synoicum adareanum (B&W) 2 INT	50.20	5.46	149.03	2.73
Synoicum adareanum (Br)	43.00	4.43	163.34	3.68
Synoicum adareanum (O) 1	820.00	49.1	1002.23	2.04
Synoicum adareanum (O) 2 EXT	122.30	13.66	382.91	2.80
Synoicum adareanum (O) 2 INT	426.50	23.65	625.07	2.64
BRYOZOA				
Isoschizoporella secunda	19.20	7.60	57.00	0.75
ECHINODERMATA (HOLOTUROIDEA)				
Peniagone vignioni	361.90	8.70	190.91	2.19
HEMICHORDATA (PTEROBRANCHIA)				
Cephalodiscus nigrescens	92.64	2.26	167.66	7.43
ALGAE				
Ochrophyta				
Adenocystis utricularis	278.10	10.45	613.64	5.87
Ascoseira mirabilis	147.45	31.67	882.55	2.79
Desmarestia anceps	122.60	8.04	613.75	7.63
D.antarctica + *Geminocarpus austrogeorgiae*	139.00	17.43	782.97	4.49
Desmarestia menziesii	153.90	28.88	389.75	1.35
Rodophyta				
Georgiella confluens	168.00	20.38	197.92	0.97

Gigartina skottsbergii	173.40	53.73	136.97	0.25
Palmaria decipiens	292.60	17.32	114.05	0.66

Capítulo 3.1. Resumen en castellano de la Publicación I

Repelencia alimentaria en organismos antárticos marinos: experimentos contra un anfípodo lyssianásido omnívoro

LAURA NÚÑEZ-PONS, MARIANO RODRÍGUEZ-ARIAS, AMELIA GÓMEZ-GARRETA ANTONIA RIBERA-SIGUÁN y CONXITA AVILA C. 2012. *Marine Ecology Progress Series* Accepted, in press. DOI: 10.3354/meps09840.

Resumen

La depredación en el bentos antártico es intensa y principalmente provocada por macroinvertebrados y densas poblaciones de anfípodos. Además, la marcada estacionalidad en la disponibilidad de alimento lleva a los consumidores a desarrollar hábitos oportunistas. Todo ello favorece la evolución de defensas químicas en las posibles presas. El anfípodo circumpolar y omnívoro *Cheirimedon femoratus* fue elegido para determinar la incidencia de repelentes alimentarios de tipo lipofílico en organismos bentónicos antárticos. Se diseñó un nuevo experimento de preferencia alimentaria usando perlas de caviar de alginato. El nuevo protocolo demostró una serie de ventajas metodológicas y un gran potencial discriminatorio para detectar metabolitos repelentes. Treintaiuna especies, que incluían 40 muestras del Mar de Weddell y de la zona de las Islas Shetland del Sur, comprendiendo esponjas (8), cnidarios (13), ascidias (8), briozoos (1), equinodermos (1), hemicordados (1) y algas (8) proporcionaron 52 fracciones orgánicas para probar. Se encontró actividad repelente en 42 extractos, pertenecientes a 26 especies. Los 10 extractos restantes de siete muestras distintas no exhibieron repelencia alguna, con lo que, o bien existen repelentes alimentarios en otras fracciones no probadas aquí, o bien estos organismos podrían explotar otras estrategias defensivas alternativas. Entre los cuatro grupos taxonómicos mayoritarios del estudio, las ascidias demostraron mayor actividad, seguidas por las esponjas, los cnidarios, y las algas. Estos organismos, de áreas antárticas distantes, podrían representar a la vez un sustrato biológico o huésped, y también una fuente potencial de nutrición para esta especie de anfípodo, que podría convertirlo en un agente inductor de protección química. La concentración de defensas químicas en estructuras corporales específicas, como predice la Teoría de Defensa Optimizada (ODT), se ha demostrado en una muestra de octocorales. Otros organismos, en cambio, podrían combinar varios mecanismos para prevenir la depredación. Nuestros resultados indican que la ecología química es un aspecto clave para comprender el papel de los anfípodos en los ecosistemas antárticos.

Capítol 3.1. Resum en català de la Publicació I

Repel·lència alimentària en organismes antàrtics marins: experiments contra un amfípode lyssianàsid omnívor

LAURA NÚÑEZ-PONS, MARIANO RODRÍGUEZ-ARIAS, AMELIA GÓMEZ-GARRETA ANTONIA RIBERA-SIGUÁN i CONXITA AVILA C. 2012. *Marine Ecology Progess Series* Accepted, in press. DOI: 10.3354/meps09840.

Resum

La depredació al bentos antàrtic és intensa i principalment provocada per macroinvertebrats i denses poblacions d'amfípodes. A més, la marcada estacionalitat en la disponibilitat d'aliment porta els consumidors a desenvolupar hàbits oportunistes. Tot plegat afavoreix l'evolució de defenses químiques en les possibles preses. L'amfípode circumpolar i omnívor *Cheirimedon femoratus* fou escollit per determinar la incidència de repel·lents alimentaris de tipus lipofílic en organismes bentònics antàrtics. Es va dissenyar un nou experiment de preferència alimentària utilitzant perles de caviar d'alginat. El nou protocol va demostrar una sèrie d'avantatges metodològiques i un gran potencial discriminatori per detectar metabòlits repel·lents. Trenta-una espècies, que incloïen 40 mostres del Mar de Weddell i de la zona de les Illes Shetland del Sud, comprenent esponges (8), cnidaris (13), ascidis (8), briozous (1), equinoderms (1), hemicordats (1) i algues (8) varen proporcionar 52 fraccions orgàniques per experimentar. Es va trobar activitat repel·lent en 42 extractes, pertanyents a 26 espècies. Els 10 extractes restants de set mostres distintes no varen exhibir cap repel·lència, amb el què, o bé existeixen repel·lents alimentaris en altres fraccions no provades ací, o bé aquests organismes podrien explotar altres estratègies defensives alternatives. Entre els quatre grups taxonòmics majoritaris de l'estudi, les ascidies demostraren major activitat, seguides per les esponges, els cnidaris, i les algues. Aquests organismes, d'àrees antàrtiques distants, podrien representar alhora un substrat biològic o un hoste, i també una font potencial de nutrició per aquesta espècie d'amfípode, que el podria convertir en un agent inductor de protecció química. La concentració de defenses químiques en estructures corporals específiques, como prediu la Teoria de Defensa Optimizada (ODT), s'ha demostrat en una mostra d'octocorals. Altres organismes, en canvi, podrien combinar diferents mecanismes per previndre la predació. Els nostres resultats indiquen que l'ecologia química és un aspecte clau per comprendre el paper dels amfípodes als ecosistemes antàrtics.

CHAPTER 3.2. PUBLICATION II

NÚÑEZ-PONS L and AVILA C. 2012. Comparative study of unpalatability in Antarctic benthic organisms towards two relevant sympatric consumers: does it taste matter? ***Polar Biology*** Submitted.

Comparative study of unpalatability in Antarctic benthic organisms towards two relevant sympatric consumers: does it taste matter?

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Abstract Many ecosystems are structured by generalist predation, and this constitutes a driving force for the evolution of defensive strategies, such as chemical defense. This, in conjunction with low nutritional quality, helps prey to avoid being consumed. Producing protective metabolites is expensive, and the Optimal Defense Theory (ODT) postulates their administration and distribution to guarantee survival. Antarctic benthos is influenced by opportunistic feeders, mainly asteroids and also abundant mesograzers. Hence, feeding-deterrence experiments were performed with the circumpolar asteroid macropredator *Odontaster validus*, to determine the presence of apolar unpalatable defenses in extracts from Antarctic benthic invertebrates and macroalgae. Moreover, feeding acceptabilities towards the circum-Antarctic omnivorous amphipod *Cheirimedon femoratus*, were assessed using the same lipophilic fractions. In this study, we aim to contrast the results obtained in both types of bioassays against two relevant sympatric consumers. A 44.9% of the extracts were unpalatable for both consumers, versus a 10.2% resulting suitable. Furthermore, 38.8% were repellent to the amphipod but edible for the asteroid, and 6.1% of the fractions were rejected only by sea stars. Overall more deterrent activities were reported towards amphipods than against asteroids, principally in fractions coming from algae and sponges, in which amphipods may especially have an effect in defenses distribution. Generalist mesograzers through casual host-prey associations may be significant promoters of defensive chemistry on their living substrata, because of the localized pressure they exert. Only a few of the samples tested did allocate repellents in specific body-regions following the ODT, and several species seem to combine different defensive traits.

Key words Antarctic invertebrates · Antarctic algae · chemical ecology · sea star *Odontaster validus* · amphipod *Cheirimedon femoratus* · chemical defense

Introduction

Predation constitutes an interaction in which an organism attacks another one driving to its eventual digestion, while grazing describes a type of predation occurring only on a part of the victim, rarely provoking its death (Heck and Valentine 1995; Sánchez et al. 2004). To combat these struggles, many prey exhibit plastic antipredatory behavior, morphology and/or chemistry, thus stabilizing the community (Harvell 1984; Vermeij 1994). But protected organisms pay costs, diverting part of the energy income that otherwise would be assigned to growth or reproduction, for defensive functions. This, at the same time compensates for the investment by providing greater survival potential (Harvell 1990). According to this, the Optimality Theory

(OT) promotes the use of all-purpose defensive tactics against a variety of enemies to save energy (Herms and Mattson 1992).

One efficient weapon for facing predation is chemical defense. Actually many natural products display ecological roles but have not known primary metabolic function, and are thus considered secondary metabolites, which are costly but essential for fitness. The mechanisms by which defensive metabolites promote predation avoidance are still unknown, since many are not highly poisonous (Paul 1992; McClintock and Baker 2001). Moreover, nutritional quality must be jointly considered too, since some repellents are more effective (or only effective) along with low quality foods. High food quality may mask the stimuli that elicit rejection when nutrients bind to deterrent molecules or compete with these for enzymes (Duffy and Paul 1992). Hence, highly nutritive potential preys likely require larger amounts of, or more potent, defenses to avoid consumption. Alternatively, the selection for lower nutrient content could be favored. For these reasons there are also costs for consumers ingesting defended items: on the one hand they may need detoxification mechanisms energetically expensive, but also they resign on eating poor quality foods. In fact, specialist predators usually feeding on one or a few chemically protected species, obtain protection through dietary sequestration of defense and/or host refuge from enemies, but in exchange, get less profitable diets (Hay et al. 1987; Lindquist and Hay 1995; Cruz-Rivera and Hay 2003). Indeed, defenses tend to be developed against generalists, which are more common than specialists (Paul et al. 2007). These may dilute possible negative effects derived from ingesting defensive metabolites by consuming discreet amounts from a variety of foods, also obtaining more benefits in nutrient supply from a mixed diet (Bernays et al. 1994; Stachowicz et al. 2007).

Inducible chemical defense has been described in marine organisms provoking increased levels of repellents, such as phlorotannins or terpene alcohols in algae, alkaloids in poriferans, or dithiocarbamates in hydrozoans, where grazing acts as the selecting force for mechanisms that decrease the vulnerability to future attacks (Cronin and Hay 1996; Lindquist 2002; Thoms et al. 2007; Toth et al. 2007). Mesograzers (0.2 - 20 mm length consumers) have not been generally considered very significant, but can be highly congregated on their living substratum (often also their prey, and chemical retreat), constituting a potential threat, sometimes worse than larger wandering echinoderms or fish (Hay et al. 1987; McClintock and Baker 2001; Toth et al. 2007).

Antarctic sea floors are characterized by unpredictable food availability driving most consumers to develop flexible opportunistic foraging strategies. Moreover, there is a predominant circumpolar distribution of many of the dominant benthic organisms, coupled with that of the keystone macroinvertebrate predators, majorly nemertean worms and asteroids (Dayton et al. 1974; McClintock 1994). But also important populations of amphipod mesograzers with diversified diets are found in extremely high densities in association with

biosubstrata (Coleman 1989a; b; 1990; Kunzmann 1996; Graeve et al. 2001; Nyssen et al. 2005; De Broyer et al. 2007; Huang et al. 2007; Amsler et al. 2009; McClintock et al. 2009; Zamzow et al. 2010).

Sea stars feed by extruding the cardiac stomach against their food items (Sloan 1980; Brusca and Brusca 2003). In Antarctic waters, where sea stars are keystone predators (McClintock 1994), defenses are expected to concentrate primarily in the outermost body parts of the prey. This circumstance apparently follows the assumption of the Optimal Defense Theory (ODT). This is because the ODT postulates that deterrent metabolites, since they are expensive, should be managed efficiently attending to the type of predator and compensating with other co-existing defensive traits. Thus, defensive chemicals should be allocated in the most valuable tissues (Rhoades and Gates 1976). However, it should be considered that other effective consumers, like small grazers, may promote different distributions.

The asteroid *Odontaster validus* and the amphipod *Cheirimedon femoratus* are abundant devouring opportunistic feeders in the Antarctic benthos. Perhaps they could constitute also potential inducing agents for the production of defensive chemicals in sessile prey organisms to avoid being consumed. In Antarctic marine organisms the occurrence of repellent activities has been well established; however, post-ingestion repulsive reactions in sea stars have been scarcely tested, and deterrency towards omnivorous mesograzers is understudied (for reviews see Avila et al. 2008; McClintock et al. 2010). Hence, we decided to evaluate feeding palatability in Antarctic benthic invertebrates and algae towards these two relevant sympatric predators. Considering that the known repellents from the sea are majorly lipid-soluble (Sotka et al. 2009), we used the lipophilic fractions to compare and determine: (1) the presence of apolar unpalatable defenses in the selected organisms; (2) the hypothetical within-body allocation of deterrents in some of the samples; and (3) the importance of comparing different kinds of experiments using several types of consumers. Here we present the results obtained in feeding deterrency tests using the asteroid *O. validus* and the amphipod *C. femoratus*, and we discuss the obtained results in both assays attending to lipid-soluble agents.

Materials and methods

Field collection of experimental asteroids and amphipods

Individuals of the Antarctic asteroid *Odontaster validus*, between 6 and 10.5 cm diameter, were collected at different sites within Port Foster Bay, Deception Island, South Shetland I. (Antarctica) (62º 59.369' S, 60º 33.424' W) for feeding-repellence assays during the ACTIQUIM-1 (December-February 2008-2009) and ACTIQUIM-2 (January 2010) cruises by scuba diving from 3 to 15 m depth. Once testing was over, the sea stars were brought back to the sea.

As part of our research, the circumpolar eurybathic amphipod, *Cheirimedon femoratus*, (Bregazzi 1972; De Broyer et al. 2007), was used as putative generalist mesograzer in previous feeding preference experiments (Núñez-Pons et al. in press). Hundreds of individuals were captured by scuba diving with fishing nets between 2 to 7 m depth, and also by displaying baited traps with canned sardines along the coastline of the Antarctic Spanish Base (BAE) Gabriel de Castilla (Deception Island) during the campaign ACTIQUIM-2. After experimentation was concluded, amphipods were returned to the sea.

Feeding-deterrence bioassays with macro-predators

The asteroid *Odontaster validus* is a predator model used in feeding acceptability studies (for review see Avila et al. 2008), is readily available, and its feeding response lends itself quite well to laboratory bioassays. The collected sea stars were kept in large tanks with seawater at the Spanish Antarctic Base (BAE) "Gabriel de Castilla" (Deception Island, Antarctica), and were left to starve for five days. The crude extracts assessed in the present feeding experiments came from marine samples from the Southern Ocean (Table 1). Benthic invertebrates and algae were collected during four Antarctic cruises: two in the Eastern Weddell Sea (Antarctica) on board the R/V Polarstern (ANT XV/3 and ANT XXI/2 cruises); a third one on board the R/V BIO Hespérides around the South Shetland Islands (ECOQUIM-2 cruise), and the fourth at Deception Island (ACTIQUIM-1 campaign). Sampling took place in a total of 24 stations between 0 m and 1524 m depth by using bottom and Agassiz trawls, epibenthic sledge, and by scuba diving in shallow sites. The procedures of how samples were collected, identified, dissected and extracted are described in a recent study in Núñez-Pons et al. (in press). The methodology followed in the experiments is detailed elsewhere (Avila et al. 2000; Iken et al. 2002). In brief, the assays consisted on 10 replicates, each with a 2.5 L container filled with seawater and one sea star, which was presented to a shrimp cube sufficiently small (5 x 5 x 5 mm and 13.09 ± 3.43 mg of dry mass) to be fully gobbled by the asteroid. These tiny shrimp food items contained 12.4% protein, 9.1% carbohydrates and 1.5% lipids, and 17.8 KJ g^{-1} in dry wt and 4.1 KJ g^{-1} wet wt, according to nutrition facts and the Atwater factor system (Atwater and Benedict 1902). They were loaded either with lipophilic extracts from Antarctic invertebrates and algae applied at their respective natural concentration in the treatment tests, or with solvent carrier alone (diethyl ether) in the control tests. In both cases the solvent was left to totally evaporate under flow hood. The factor to normalize tissue concentrations of each fraction (hereafter 'natural concentration') was calculated on a dry-weight basis employing the total dry weight of each sample. Dry weight was chosen, rather than volume or wet weight, because it eliminates the water content, which may entail notable deviations in aquatic porous samples (Table 1). Thus, considering sea star extraoral feeding habits (Sloan 1980), everting the cardiac stomach and bolting down whole shrimp chunks, we could measure the "defense per shrimp

cube". After 24 hours the food items eaten were counted, and feeding repellence was evaluated by applying Fisher's Exact tests for each assay referred to the control run simultaneously (Sokal and Rohlf 1995; Fig. 1). Extract-treated shrimp pieces that were left uneaten, were further preserved frozen for further extraction and analysis by TLC to check for alterations in the extracts. No major changes were observed, plus, theoretically, ether extracts are not hydrophilic and the water temperature was fairly cold (~ -1ºC), hence there should be little, if any, loss to the water column.

Table 1 Data from the benthic organisms analyzed. % [N_{EE}] respect DW: natural concentration in percentual values of diethyl ether extracts (EE) in each sample obtained by dividing EE weight by the total dry weigth (DW). Dissected body parts: API: apical; BAS: basal; EXT: external; INT: internal; POL: poliparium; AX: axis. B&W: Black & White, Br: Browm, O: orange morphotypes. Gear: BT: bottom trawl, AGT: Agassiz trawl, ES: epibenthic sledge, SD: scuba diving in shallow sites. Values of % [N_{EE}] respect DW, from Núñez-Pons et al. in press

Taxonomic group and species name	Body parts; % [N_{EE}] in DW	Location	Gear	Depth (m)
ALGAE				
Ochrophyta				
Adenocystis utricularis (Bory de Saint-Vincent) Skottsberg 1907	5.87	Snow Is.	SD	1.5
Ascoseira mirabilis Skottsberg 1907	2.79	Livingston Is.	SD	0.7
Desmarestia anceps Montagne 1842	7.63	Deception Is.	SD	7.5
Desmarestia antarctica Moe & Silva 1989 epiphyited by *Geminocarpus austrogeorgiae* Skottsberg, 1907	4.49	Livingston Is.	SD	0.7
Desmarestia menziesii J.Agardh 1848	1.35	Deception Is.	AGT	109.7
Rhodophyta				
Georgiella confluens (Reisch) Kylin 1956	0.97	Livingston Is.	SD	0.7
Gigartina skottsbergii Setchell & Gardner, 1936	0.25	Deception Is.	SD	12
Palmaria decipiens (Reinsch) Ricker 1987	0.66	Deception Is.	SD	1.3
PORIFERA				
Desmospongiae				
Isodictya toxophila Burton, 1932	API: 5.28; BAS: 6.79	Weddell Sea	BT	332.8
Hexactinellida				
Anoxycalyx (Scolymastra) joubini Topsent, 1916 (1)	API: 1.29; BAS: 0.73	Weddell Sea	AGT	175.2
Anoxycalyx (Scolymastra) joubini Topsent, 1916 (2)	EXT: 2.60; INT: 1.83	Weddell Sea	BT	290.8

Rossella fibulata Schulze & Kirkpatrick, 1910	EXT: 1.19; INT: 1.75	Weddell Sea	BT	332.8
Rossella nuda Topsent, 1901	1.17	Weddell Sea	BT	308.8
Rossella vanhoffeni (Schulze & Kirkpatrick, 1910)	API: 2.36; BAS: 0.61	Weddell Sea	ES	882
Rossella villosa Burton, 1929	API: 1.05; BAS: 0.99	Weddell Sea	AGT	288.0
Rossella sp.1 (Orange) Carter, 1872	EXT: 1.20; INT: 1.08	Weddell Sea	AGT	288.0
CNIDARIA				
Anthozoa				
Alcyonium antarcticum Wright & Studer, 1889	1.97	Weddell Sea	BT	337.2
Alcyonium haddoni Wright & Studer, 1889	4.61	Deception Is.	SD	9
Alcyonium roseum van Ofwegen, Häussermann & Försterra, 2007	3.21	Weddell Sea	AGT	416
Primnoisis antarctica (Studer, 1878) (1)	0.45	Weddell Sea	BT	294.8
Primnoisis antarctica (Studer, 1878) (2)	0.58	Weddell Sea	BT	294.8
Thouarella laxa Versluys, 1906 (1)	1.44	Weddell Sea	BT	308.8
Thouarella laxa Versluys, 1906 (2)	1.27	Weddell Sea	BT	290.8
Thouarella laxa Versluys, 1906 (3)	0.88	Weddell Sea	BT	294.8
Thouarella laxa Versluys, 1906 (4)	1.44	Deception Is.	AGT	100.4
Thouarella minuta Zapata-Guardiola & López-González 2009	2.00	Weddell Sea	BT	338
Umbellula antarctica Kükenthal and Broch, 1911	POL: 11.59; AX: 1.08	Weddell Sea	BT	338
Hydrozoa				
Staurotheca antarctica Hartlaub, 1904	3.93	Weddell Sea	BT	597.6
Symplectoscyphus glacialis (Haderholm 1904)	1.02	Weddell Sea	AGT	175.2
CHORDATA (ASCIDIACEA)				
Aplidium falklandicum Millar, 1960	4.20	Weddell Sea	BT	332.8
Aplidium fuegiense Cunningham, 1871	7.71	Weddell Sea	AGT	228.4
Aplidium meridianum (Sluiter, 1906)	12.85	Weddell Sea	BT	284.4
Synoicum adareanum (B&W (Herdman, 1902) (1)	EXT: 2.00; INT: 3.31	Weddell Sea	BT	337.2
Synoicum adareanum (B&W) (Herdman, 1902) (2)	API: 5.57; EXT: 1.81; INT: 2.73	Weddell Sea	AGT	288.0
Synoicum adareanum (Br) (Herdman, 1902)	3.68	Weddell Sea	AGT	277.2
Synoicum adareanum (O) (Herdman, 1902) (1)	2.04	Weddell Sea	AGT	288.0
Synoicum adareanum (O) (Herdman, 1902) (2)	EXT: 2.80;	Weddell Sea	BT	337.2

	INT: 2.64			
BRYOZOA				
Isoschizoporella secunda Hayward and Taylor, 1984	0.75	Weddell Sea	AGT	277.2
ECHINODERMATA (HOLOTUROIDEA)				
Peniagone vignioni Herouard, 1901	2.19	Weddell Sea	AGT	1524.8
HEMICHORDATA (PTEROBRANCHIA)				
Cephalodiscus nigrescens Lankester, 1905	7.43	Weddell Sea	BT	284.4

Feeding-preference bioassays with mesograzers

The omnivore-scavenger amphipod, *C. femoratus*, (De Broyer et al. 2007), was used in a recently designed feeding preference assays as putative mesograzer (Núñez-Pons et al. in press). Artificial caviar-textured foods were prepared with 10mg/mL alginate aqueous solution containing 66.7 mg/mL of a concentrated dried feeding stimulant (Phytoplan®), and a drop of green or red food coloring. The mixture was introduced into a syringe without needle and added drop-wise into an aqueous 0.09 M (1%) $CaCl_2$ solution, where it polymerized into spheroid pellets (~2.5 mm diameter). The energetic value of these food pellets was 19 KJ g^{-1} dry wt, and 1.5 KJ g^{-1} wet wt (by nutrition facts and Atwater factors; Atwater and Benedict 1902). For treatment pearls, extracts were added dissolved in diethyl ether at their natural concentration in a dry weight basis, attending to that exposed above. Afterwards, they were left to evaporate onto the feeding stimulant. Control pellets were prepared similarly but with solvent alone. Amphipods were maintained in large 8L aquariums and were starved for 3-5 days. Every assay consisted on 15 replicate 500-mL containers filled with sea water and 15 amphipods each, which were offered a simultaneous choice of 10 treatment (extract-treated) and 10 control extract-free pellets (20 food pearls in total) of different colorations, green or red. The assays ended when one-half or more of either food types had been consumed, or 4 hours after food presentation. The number of consumed and not consumed pearls of each color (control or treatment) was recorded for each replicate container. Finally, statistics were calculated by applying the Exact Wilcoxon to determine feeding preferences to consequently establish unpalatable activities. The detailed procedure is described in Núñez-Pons et al. (in press)

Results

A total of 31 species comprising 40 Antarctic samples from sponges (8), cnidarians (13), ascidians (8), bryozoans (1), echinoderms (1), hemichordate pterobranchs (1) and macroalgae (8) yielded 52 lipophilic extracts, which were tested in feeding acceptability assays using two types of consumers. Further data on the invertebrate and algal species, samples, and amounts of extracts used are available in Núñez-Pons et al. (in press). For the Antarctic macropredator sea

star *Odontaster validus*, the control assays yielded a minimum consumption of seven pieces of shrimp out of ten in all tests. Out of the 31 species from which 49 fractions were assessed towards *O. validus*, repellent activities were detected in 17 species, which yielded 25 unpalatable fractions (51%;). The remaining 14 species provided 24 extracts (49%) that resulted suitable for sea star consumption. In terms of groups, tunicates and cnidarians exhibited the highest activity with 83.3% and 61.5% of repellent extracts respectively, followed by algae (25%) and sponges (23.1%; Fig. 2). The only bryozoan and the holoturian samples were also significantly unpalatable, differently to the hemichordate pterobranch, which yielded an inactive ether fraction. In the group of the tunicates, both *Synoicum adareanum* (B&W: black and white morphotype) 1 and 2 samples caused rejection with their internal fraction to *O. validus* but not with their external lipophilic extracts (with no significant activity). Most seaweed and poriferan fractions were suitable for sea star consumption, except for two algal extracts from *Ascoseira mirabilis* and *Georgiella confluens*, and two sponge fractions from *Isodictya toxophyla* and *Rossella nuda*, which elicited deterrency. Finally, five cnidarian extracts including those from the polyparium and axis of *Umbellula antarctica*, two from samples of the gorgonians *Primnoisis antarctica* and *Thouarella laxa,* and one from the hydrozoan *Staurotheca antarctica* were accepted by the star (Table 1; Fig. 1).

In the feeding preference assays with *Cheirimedon femoratus* 33 samples displayed feeding unpalatability (26 of the species). Hence 42 extracts were unpalatable (80.8%) out of the 52 tested. The remaining 10 extracts, obtained from 7 samples belonging to 5 different species, were not active (Fig. 1). The ascidians leaded in incidence of unpalatable extracts (91.7%), followed by the sponges (86.7%), cnidarians (85.7%), and algae (75%; Fig. 2). One extract coming from a *Primnoisis antarctica* gorgonian, and one *Anoxycalyx (Scolymastra) joubini* poriferan caused acceptance, as well as the bryozoan, the holothurian and the hemichordate extracts. In fact, lipophilic extracts from *Cephalodiscus nigrescens,* as well as from the algae *Adenocystis utricularis* caused positive preference to the amphipod. Finally, the sea pen *Umbellula antarctica* exhibited a deterrent axial fraction, but the polyparium was instead consumed. Also the apical ethereal fraction (API) from the ascidian *Synoicum adareanum* (B&W) 2 was palatable contrasting with the basal-external and visceral (EXT and INT) extracts (Fig. 1).

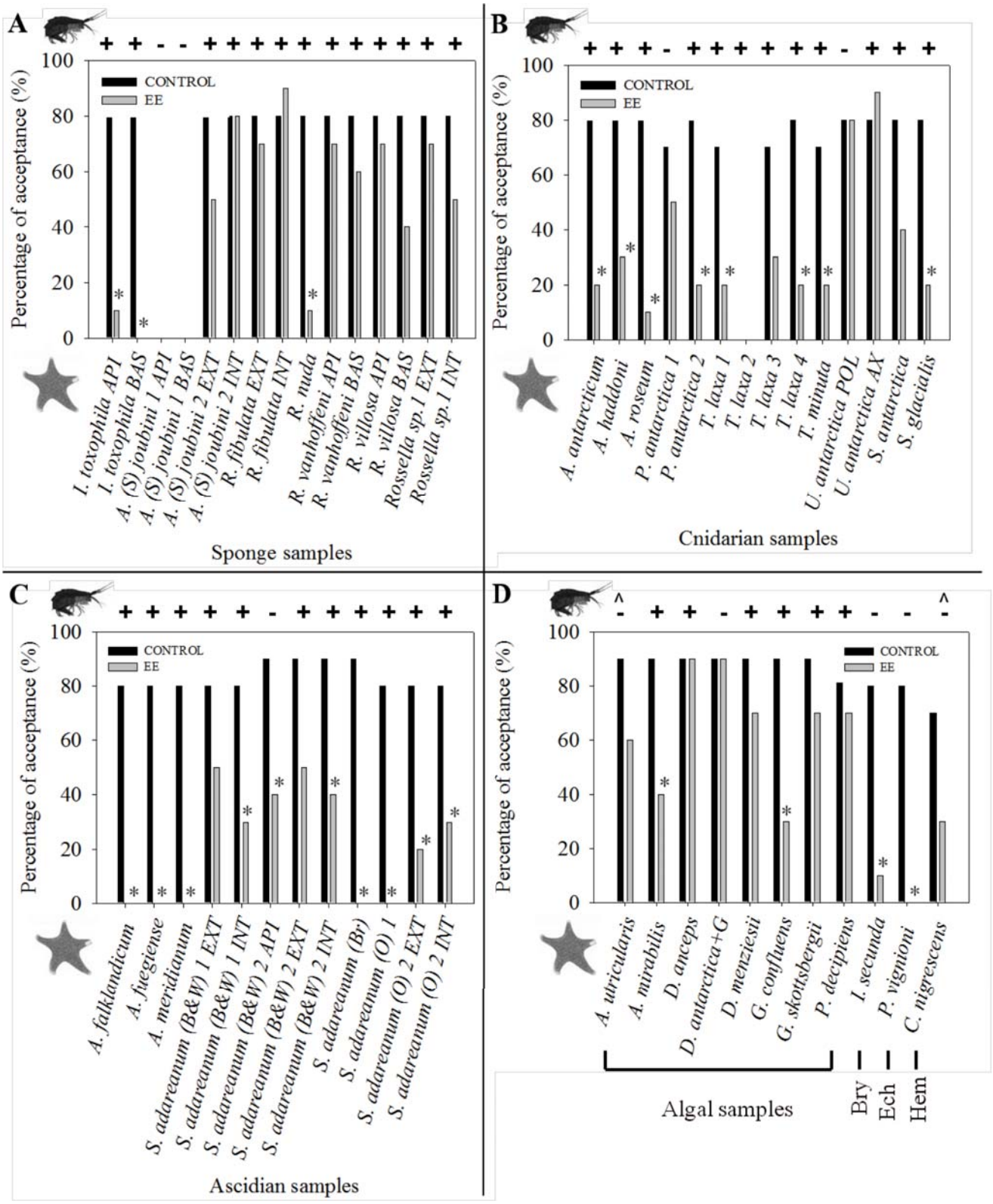

Fig. 1 Bar diagrams of the feeding repellence bioassays with the sea star *Odontaster validus* for the four major groups assessed: (A) sponges, (B) cnidarians, (C) ascidians, (D) algae + minor groups (Bry: Bryozoa, Ech: Echinodermata, Hem: Hemichordata), showing the results of each paired test with control and extract-treated diets represented by the percentage of acceptance. The hexactinellid *S. (A.) joubini* 1 and the octocoral *Thouarella laxa* 2 samples were not assayed in this experiment. *: significant differences ($p<0.05$) with control as preferred food (Fisher's exact test). On top of each graph the results

are contrasted with data from feeding preference bioassays ran in parallel with the amphipod *Cheirimedon femoratus* (Núñez-Pons et al. in press), where -: not significant preferences ($p>0.05$); +: significant differences ($p<0.05$) with control as preferred food; ^: significant differences with extract-treated food preferred from control diet (Exact Wilcoxon test)

Discussion

Deterrence against sea star predators and amphipod mesograzers

The sea star *Odontaster validus* rejected most of the fractions from cnidarian and ascidian samples, demonstrating a widespread presence of lipophilic deterrents within these groups. In lieu, for sponge and algal extracts, the predominant palatability reflected a scarce incidence of effective lipid-soluble repellents to avoid asteroid predation. Comparing these data with those obtained in feeding preference experiments with the amphipod *Cheirimedon femoratus*, we can observe that out of the 49 lipophilic extracts tested in both bioassays, 22 (44.9%) were unpalatable and 5 (10.2%) were accepted for both predators. This yielded a 55.1% (27 fractions) of coincident activities in the two assays. Instead 22 fractions showed discrepancies, being 19 (38.8%) negatively preferred to the amphipod but palatable for the asteroid *O. validus*, and the remaining 3 fractions were repellent just for the sea star. Overall we observed a 32.7% higher incidence of deterrency towards amphipod feeding than against sea star ingestion, especially in the groups of sponges and algae (Fig. 2).

Even if the asteroid *Odontaster validus* and the amphipod *Cheirimedon femoratus* have both circumpolar-eurybathic distributions, and extensive generalist diets (scavenger, detritivore, planktivore; Bregazzi 1972; McClintock 1994; De Broyer et al. 2007), the distinct habits of both predadors in nature may promote variable defensive responses in potential prey. In general, sea stars are mobile macropredators that start extraoral digestion from the surface of the victims (Sloan 1980). Amphipods feed with minute peripheral bites, and similarly rarely arrive to internal tissues unless feeding is prolonged in time. However, when preys have openings, their small size may allow them to reach inner regions. We compared the data obtained in our experiments using apolar fractions and different predators, and estimated divergent prey responses.

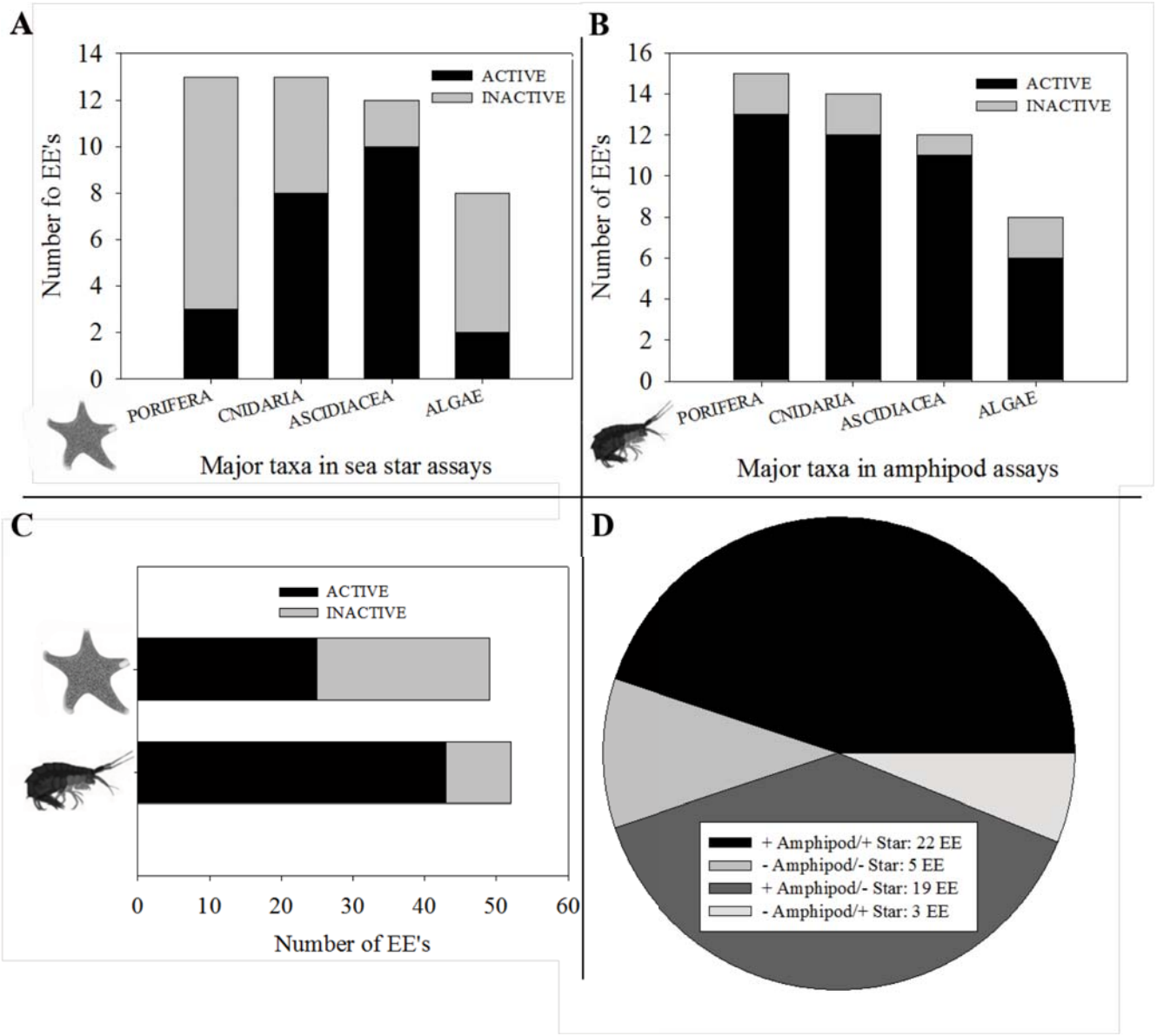

Fig. 2 Comparison of data from bioassays against the sea star *Odontaster validus* and the amphipod *Cheirimedon femoratus*, referring to the incidence of unpalatable activities in diethyl ether extracts (EE's) from the four major groups assessed: sponges, cnidarians, ascidians and algae. In the top vertical bar two diagrams are shown: (A) feeding repellence assays against the sea star, and (B) feeding preference assays towards the amphipod. In the horizontal bar diagram (C), the total activity of all extracts assessed is contrasted in both tests, with asteroids and amphipods. The pie diagram (D) represents coincident and different deterrent activities of all the fractions tested comparing the two experiments

Methodological considerations

In the sea star deterrence tests, there was no other option than rejection or acceptance of feeding items. Instead, preference assays with-choice and savoury control foods (like those conducted with *Cheirimedon femoratus*), may favor discrimination of unpalatable agents since there is a suitable alternative. But these also eliminate skewed repellencies of organisms not ingesting

anything at all. Likewise, the two base diets should be pondered: alginate food pearls used with the amphipod *C. femoratus* even if containing equal energetic value in dry mass (19 KJ g^{-1} dry wt), when prepared as food pellets, they had less energetic content (1.5 KJ g^{-1} wet wt) than that of the shimp cubes offered to the asteroid *Odontaster validus* (17.8 KJ g^{-1} dry wt and 4.1 KJ g^{-1} wet wt; by Atwater factors; Atwater and Benedict 1902). This could influence in palatability, but allows comparative approximations related to deterrence potential. Nutritious food may interact with defensive metabolites constraining the types or concentrations that could be efficacious as deterrents within the original organisms (Duffy and Paul 1992; Cruz-Rivera and Hay 2003). Thus, repellent activities in some extracts might have been less evident in the sea star tests because of a higher nutritional quality of shrimp cubes respect to the alginate pearls, and maybe respect to the original samples extracted too. Our study allowed us to detect repellent activities by changing the predator and the diet. Actually, most of the fractions unpalatable to amphipods but accepted by sea stars came from samples with apparently less nutritional content: hexactinellid sponges; and from less attractive body parts: the tunics of *S. adareanum* (B&W) and the stalk from *U. antarctica*. In these cases, moderate-to-poor chemical defense along with low nutritional value might co-operate. It would be optimal to test foods that are energetically equivalent as the studied prey, even if some species are problematic to assess with certain artificial diets, and *O. validus* is an example (authors' pers. obs.). Furthermore, we should keep in mind that other possible defenses could be present in other fractions not tested here.

Macroalgae and sponges as potential prey or as potential host-and-prey

In the case of algae, the larger deterrencies found towards the amphipod could be partly explained by a supposedly higher pressure produced by ovigerous females and juveniles of *C. femoratus*, which apparently need more algal material during summer (Bregazzi 1972). *O. validus*, even if consuming seaweeds does not have such a requirement (McClintock 1994). However, polar chemicals (like phlorotanins), not tested here, could participate in asteroid repellence. Furthermore, Antarctic benthic amphipods associate with living substrata with generally no obligate relationships (De Broyer et al. 2001), obtaining tridimensional habitat and food. Moreover, defended hosts represent chemical refuges from prospective enemies, like fish (McClintock et al. 2009, Zamzow et al. 2010). The preferred biosubstrata for *C. femoratus* are macrophytes, as well as poriferans in aphotic areas with low (or none) algal cover (authors' pers. obs.). Northern cold-water *Cheirimedon* species reside on sponges too (Vader 1984). Amphipod habits can be directly studied in nearshore areas (e.g. Huang et al. 2007) but become more approximative in deeper waters (De Broyer et al. 2001), and *C. femoratus* occurs down to 1500 m depth (Krapp et al. 2008). As opposed to algae, our deep sponges could not reflect

spongicolous fauna, since non-strict inquilines that establish lax associations are hardly recovered by trawling (Table 1).

Dense host-associations derive in diverse interactions depending on the chemical potential of the host and the feeding adaptations of the grazers. *C. femoratus* as a generalist amphipod with reduced swimming activity, associates opportunistically with substrata (authors' pers. obs.), and as part of its varied diet it may graze on host tissues directly, or while profiting adhered detritus, diatoms… (Bregazzi 1972; Graeve et al. 2001). This leads to a more constant pressure on host-and-prey organisms than more wandering macropredators (Hay et al. 1987; Toth et al. 2007), like *O. validus*, that focuses on ubiquitous prey with less recurrent encounters (McClintock 1987; McClintock 1994).

Our Antarctic algal samples came from common brown and red seaweed, which are energetically rich food sources (11-13 KJ g^{-1} dry wt; Montgomery and Gerking 1980, Gomez and Westermeier 1995), and producers of peculiar metabolites. The sponges were mostly hexactinellids, which are believed to have a poor secondary metabolism (Blunt et al. 2011 and previous reports), and have high spicule content with low energetic value (5-6 KJ g^{-1} dry wt; McClintock 1987, Barthel 1995), which in conjunction could serve to diminish predation. The average ether-lipophilic fraction yields was in fact quite low in hexactinellids (≈ 1.3%), was ≈ 4.4% in brown algae and ≈ 0.6% in Rodophyta (from data of Table 1). The small lipidic percentual characterizing red algae may explain these low values, even if being very nutritive (Montgomery and Gerking 1980). Antarctic poriferans and macroalgae hold high diversities of amphipods (Kunzmann 1996; Huang et al. 2007; Amsler et al. 2009) representing potential prey. Moreover, some algae are known to harbor deterrents for amphipods and to act as chemical refuges repelling fish, like *Desmarestia menziesii* and *D. anceps*. Others instead, like *Palmaria decipiens,* are more preferred as food (not for our amphipod though) but unpreferred as host (Amsler et al. 2005a; Aumack et al. 2010). Analogous chemical refuges have not been described in Antarctic sponges, yet some defended species host dense amphipod populations (Amsler et al. 2009). Most of the seaweed and sponge fractions reflected no activity against *O. validus* (respect to other studies; for review see Avila et al. 2008) but were rejected by *C. femoratus* that may use them as substrata and casual prey. The few lipophilic fractions that caused repellency to the asteroid were actually rejected in both assays, such as those from the macroalgae *Ascoseira mirabilis* and *Georgiella confluens*, and also the desmosponge *Isodictya toxophila* and the hexactinellid *Rossella nuda*. This last sponge is among the most abundant and one of the primarily foraged by spongivorous generalists including *O. validus*, justifying the selection for chemical defense. *I. toxophila* seems more nutritious than glass sponges from analysis carried out with congeneric species (McClintock 1987; Barthel 1995), and had much richer fractions, which could suggest greater amounts of or stronger repellent compounds. Similarly, the few extracts palatable for the amphipod were also accepted by sea stars, such as

those from the algae *Adenocystis utricularis* and *Desmarestia antarctica* epiphyted by *Geminocarpus austrogeorgiae* (Table 1; Fig 1). In *D. antarctica* it was proposed that acid sequestration could be effective against asteroids (Amsler et al. 2005b). Many glass sponges are readily preyed upon by *Odontaster* and *Acanthaster* sea stars (McClintock 1987), however most of the fractions tested here were deterrent only for amphipods. Moreover, no defense allocation was observed to the outermost layers for asteroid avoidance (Furrow et al. 2003), which indeed may not serve against smaller grazers biting inner and outer sponge-parts (Peters et al. 2009), especially in volcano-shaped rossellids with conspicuous pores. All this suggests that opportunistic amphipods, through lax associations, may particularly influence the chemical ecology of lipidic nature in Antarctic macroalgae and sponges and on the expectations of the ODT (Rhoades and Gates 1976). Thus, in some cases amphipods could replace asteroids as main inducers of defense distribution.

Anti-predatory protection in other invertebrate groups

Antarctic cnidarians and ascidians are not usual hosts for casual mesograzers, although there are some described associations (Loerz 2003; McClintock et al. 2009). Actually, in shelf communities that are not dominated by sponges (Dayton et al. 1974), these organisms represent transitory biosubstrata for opportunistic amphipods (De Broyer et al. 2001), like *C. femoratus*. Antarctic Cnidaria and Tunicata are rich in secondary metabolites, and many species assessed produce them (Blunt et al. 2011 and previous reports). In fact, some of the species tested here have revealed in the past deterrent activities and effective defensive metabolites (Koplovitz et al. 2009; Núñez-Pons et al. 2010). In our study, the cnidarian samples comprised hydrozoans and anthozoans, whereas the tunicates included exclusively colonial ascidians. Soft corals and colonial ascidians have high energetic contents (16 KJ g^{-1}; and 15 KJ g^{-1} dry wt respectively; Slattery and McClintock 1995; McClintock et al. 2004). Hydroids and gorgonians, which contain more inorganic skeletal material, can be quite nutricious as well (Coma et al. 1998). The average natural concentrations of the ether fraction in these groups may to some extent illustrate these facts (ascidians ≈ 4.9%, soft corals ≈ 3.3%, gorgonians ≈ 1.2%; Table 1). Indeed, most extracts were unpalatable in both assays; however, other defensive tactics might co-occur in these groups.

Hydrozoans may present lipophilic as well as nematocyst-based defenses, even if the OT considers this redundant (Herms and Mattson 1992; Lindquist 2002; Stachowitcz and Lindquist 2000). *Staurotheca antarctica* yielded a rich fraction containing deterrents to amphipods, but did not cause sea star rejection. Nematocysts may be involved in protection too, since the Syntheciidae family is generally armored with penetrating cnidos (often injecting polar proteinaceous venoms; Shostak 1995, Ostman 2000). Asteroids could be particularly vulnerable to stinging, since contact happens with the sensitive ambulacral feet or the thin mucose of the

cardiac stomach. Thus, *S. antarctica* might not follow the OT and could hypothetically repel crustaceans with unpalatable lipids and echinoderms with nematocyts. Nonetheless polar fractions may contain other defenses too. The hydroid *Symplectoscyphus glacialis* (Family Sertulariidae) rarely presents penetrating nematocysts (Shostak 1995), supporting the selection for protective chemistry. Actually, it was significantly unpalatable to both predators. Regarding Anthozoa, *Umbellula antarctica* is a pennatulacean with a thin fibrous axis and a distal crown of giant polyps (Pasternak 1962; Dolan 2008). Both regions separately processed revealed distinct palatabilities and TLC profiles, indicating that lipid repellents were present only in the axis. The polyparium was not repellent in any of the assays. Indeed, such an exposed and apparently energetic structure must be defended, either by hydrophilic defenses or probably by effective nematocysts lodged in the prominent polyps (3-4cm long), utilized for its macrophagous carnivorous diet (Dolan 2008). The pennatullid sea pansy *Renilla kollekeri* for instance, keeps sea star predators away thanks to the nematocysts when autozooids are expanded (Kastendiek 1976). The stalk of *U. antarctica* is denuded of nematocysts, but possessed deterrents for amphipods, which along with a low nutritional attractiveness may repel consumers. Since both types of defenses might occur in different body regions, the OT and the ODT could be sustained for *U. antarctica*. In contraposition, soft corals and gorgonians generally lack stinging nematocysts and likely rely on chemical defense (Sammarco and Coll 1992), as demonstrated in our octocoral samples. Only one fraction from *Primnoisis antarctica* 1, was accepted in the two assays, and one extract from *Thouarella laxa* repelled *C. femoratus* but not the sea star. Both fractions were less abundant than active conspecific extracts (Table 1; Fig. 1).

Many colonial ascidians protect their tunic with alternative tactics other than organic deterrents, such as sequestration of inorganic chemistry (acid, heavy metals); *i. e. Distaplia cylindrica* and *D. colligans* combine lipidic defenses and inorganic acids (McClintock et al. 2004, Koplovitz et al. 2009). None of the species studied here reflects this, though, as reported in recent analysis (Koplovitz et al. 2009; Lebar et al. 2011). Also tunics may be nutritiously unattractive (McClintock et al. 1991; Pisut and Pawlik 2002). Among our *Synoicum adareanum* samples, as it is usual in colonial species (Varela 2007), there were 3 color morphs: black and white (B&W), brown (Br) and orange (O) morphotypes. Similar to previous findings (Núñez-Pons et al. 2010), the dissected O sample exhibited no defense allocation. Instead both B&W samples caused rejection in both feeding assays with internal extracts, but tunic fractions were only unpalatable to the amphipod. As opposed to the tunic from the O morph, B&W tunics were thick and tough and yielded poorer lipophilic fractions than their corresponding inner regions (Table 1, Fig. 1). Several strategies might co-occur in our B&W samples: lipophilic distasteful metabolites could avoid amphipod and asteroid feeding in inner tissues. Instead, smaller quantities of these deterrents along with a low nutritional value could keep asteroids from

attacking the tunic. The participation of polar products, again, cannot be discarded. Finally, the rich apical fraction from one B&W sample, which included most colonial siphon mouths and common cloaca was only rejected by the star. *C. femoratus* could be attracted to cloacal material since it is a scavenger (Smale et al. 2007). The allocation of deterrent activities within the internal regions of the B&W *S. adareanum* samples is reported here for the first time in Antarctic tunicates, and supports the ODT. An inverse pattern was expected though, since predators encounter outer parts first (Rhoades and Gates 1976; Furrow et al. 2003). However the trend towards larval brooding in colonial ascidians (Lambert 2005), suggests that they should defend internal parts of higher fitness value, rather than the tunic, or at least both. This may represent a coordinated energy-saving protection strategy.

In the less represented taxa, the abundant extract from the hemichordate pterobranch displayed no repellency in any assay. These animals may not need chemical protection, since they live hidden from major enemies inside the coenoecia, colonial encasements hardened with agglutinated foreign material (Ridewood 1911; Brusca and Brusca 2003). But the existence of polar deterrents cannot be discharged. Instead, the bryozoan and holothurian fractions reflected the presence of feeding deterrents against *O. validus*. These extracts were not effective repelling amphipods. *Isoschizoporella secunda* is a calcified bryozoan that produced poor extract yields, and harbors sessile "trap-door" avicularia, which are proposed as defensive devices. These might function as active mechanical deterrents to zooid-level predators (amphipods, nematodes, polychaetes), and/or as chemical ones by secretion of bioactive compounds (Carter et al. 2010). Hence, this branched bryozoan could avoid amphipod attacks through entrapments of appendages by the avicularia, and rely on the chemistry against asteroids, potentially serving as refuge from large predators (Bryan et al. 1998). Finally, the elpidiid holothurian *Peniagone vignioni* is known to practice swimming (Wigham et al. 2008), and may then escape easily from many bottom-dwelling consumers. Thus, repellents for sea stars would be useful while the animal feeds on the substrate surface (Table 1; Fig. 1).

Levels of deterrence can vary among extracts from conspecifics. In other latitudes organisms with clonal growth (colonial animals, sponges, algae…) may react after episodes of grazing by increasing defense production (Cronin and Hay 1996; Lindquist 2002; Thoms et al. 2007). This, however, was not measured here. When different activities were recorded in conspecifics, the unpalatable sample was always that with the richer extract (*A. (S.) joubini, T. laxa*, *P. antarctica*). This could be attributed to larger amounts of repellents. Further studies are needed to fully determine this.

Concluding remarks

Antarctic benthic ecosystems are classically considered stable, but adapted to marked seasonalities of nutrient supply, and composed of many defended sessile species with long life-

spans subjected to intense generalist predation (Dayton et al. 1974; Avila et al. 2008). Most feeding deterrents do not totally avoid attacks, but they reduce the attractiveness of the organism respect to other co-occurrent susceptible prey, which presumably would develop defenses as well, to avoid predisposition to attacks. Hence, prey organisms are frequently defended at some level, and generalist strategies allow feeders to mitigate possible toxicities of secondary metabolites and compensate poor quality diets (Bernays et al. 1994; Stachowicz et al. 2007; Sotka et al. 2009). Combinations of nutrient content with defensive chemicals are not appreciable prior ingestion, and diverse predators may process deterrent metabolites differently. Sea stars lacking eyes rely on chemoreception (Sloan 1980; Kidawa 2005) and may experience gustation with the cardiac stomach or the ambulacral system. Scavenging lysianassoid amphipods instead have well-developed gustatory gnathopods (Kaufmann 1994). For instance, amphipods often react to lipidic deterrents (Duffy and Paul 1992; Cruz-Rivera and Hay 2003; Sotka et al. 2009). Macroalgae and invertebrates have mostly been reported to possess lipid-soluble repellents actually, which appear normally sequestered and in concentrations of < 2% dry mass, ruling out a fast chemoreception (Sotka et al. 2009). Hence, we considered convenient here to assess more than one consumer and one diet, and to focus firstly on the ether extracts. We should, however, be aware of the limitations of testing only lipophilic extracts and further studies should analyze other fractions as well. Our assays, measuring the actual ingestion of consumers, allowed the evaluation of pre- and post-ingestive responses respect to other Antarctic studies (for reviews see Avila et al. 2008; McClintock et al. 2010).

Antarctic seaweed and sponges that commonly host mesograzers, with hexactinellids considered energetically scant, yielded apolar extracts that were majorly unpalatable towards *C. femoratus*. Instead, fractions from ascidians and cnidarians were fairly deterrent to both, sea stars and amphipods. Both ascidians and cnidarians are prolific bioactive metabolite producers, and they are considered rich prey items, while they are less remarkable as hosts, Besides, some samples likely display several anti-predation strategies simultaneously. The divergent results, the majority showing unpalatability only in the amphipod assay, correspond to samples possessing lower amounts of repellents, possibly correlated with poor energetic values. In fact, the great percentage of coincident activities reflected that apolar deterrents were operative for both consumers, even if amphipods appear more sensitive. Further studies should keep filling the gaps of knowledge still existing in chemical ecology of Antarctic benthic organisms.

Ackowledgements We thank M. Rodríguez-Arias, J. Vázquez, B. Figuerola, F.J. Cristobo and S. Taboada for their precious help in the Antarctic cruises. Thanks are due to the crew of R/V Polarstern. UTM (CSIC), and "Las Palmas" and BAE "Gabriel de Castilla" crews gave logistic support. We are thankful to A. Gómez-Garreta, A. Ribera-Siguán, P. Ríos, M. Varela and A. Bosch for taxonomical support. Funding was provided by the Ministry of Science and

Innovation of Spain (CGL2004-03356/ANT, CGL2007-65453/ANT and CGL2010-17415/ANT).

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Capítulo 3.2. Resumen en castellano de la Publicación II

Estudio comparativo sobre la repelencia alimentaria en organismos bentónicos antárticos frente a dos consumidores simpátricos relevantes: el gusto importa?

LAURA NÚÑEZ-PONS y CONXITA AVILA. 2012. *Polar Biology* Submitted.

Resumen

Muchos ecosistemas están estructurados por depredadores generalistas, y esto constituye una fuerza selectiva para la evolución de estrategias defensivas, como la defensa química. Esto, junto con un valor nutricional bajo, puede favorecer a las presas en su lucha para evitar ser consumidas. La producción de metabolitos defensivos es costosa, y la Teoría de la Defensa Optimizada (ODT) postula una administración y distribución eficientes de los mismos para garantizar la supervivencia. El bentos antártico está influenciado por consumidores oportunistas, mayoritariamente estrellas de mar, pero también abundantes anfípodos. Por ello, se realizaron experimentos de repelencia alimentaria utilizando el asteroideo macrodepredador circumpolar *Odontaster validus*, para determinar la presencia de defensas repelentes de naturaleza apolar en extractos obtenidos a partir de invertebrados y macrófitos antárticos. También se evaluó la aceptación alimentaria de estas mismas fracciones ante el anfípodo circumpolar y omnívoro *Cheirimedon femoratus*. En este estudio pretendemos contrastar los resultados obtenidos en ambos tipos de tests, utilizando dos consumidores simpátricos relevantes. Un 44.9% de los extractos resultaron rechazados por ambos depredadores, en contraposición con un 10.2% de aceptados. Además, un 38.8% provocó repelencia al anfípodo, pero fue aceptado por la estrella, y el otro 6.1% de las fracciones fueron rechazadas solamente por las estrellas de mar. En conjunto, hubo más actividad repelente hacia los anfípodos que hacia las estrellas, especialmente en aquellas fracciones procedentes de macroalgas y esponjas, en las que los anfípodos podrían particularmente influir en la distribución de sus defensas químicas. Los anfípodos generalistas, a través de sus asociaciones con biosustratos huésped pueden ser importantes inductores de defensas químicas, debido a la presión localizada que ejercen sobre ellos. Sólo en unas pocas muestras se demostró la localización de repelentes alimentarios en regiones anatómicas específicas, como propone la ODT, mientras que otras especies en cambio, parece que podrían combinar diferentes características defensivas.

Capítol 3.2. Resum en català de la Publicació II

Estudi comparatiu sobre la repel·lència alimentària en organismes bentónics antàrtics en dos consumidors simpàtrics relevants: el gust importa?

LAURA NÚÑEZ-PONS i CONXITA AVILA. 2012. *Polar Biology* Submitted.

Resum

Molts ecosistemes estan estructurats per predadors generalistes, i açò constitueix una força selectiva per l'evolució d'estratègies defensives, com la defensa química. Açò, conjuntament amb un valor nutricional baix, pot afavorir a les presses en la seua lluita per evitar ser consumides. La producció de metabòlits defensius és costosa, i la Teoria de la Defensa Optimitzada (ODT) postula una administració i distribució eficients dels mateixos per garantir la supervivència. El bentos antàrtic esta influenciat per consumidors oportunistes, majoritàriament estrelles de mar, però també abundants amfípodes. Per això, es varen realitzar experiments de repel·lència alimentaria utilitzant l'asteroideu macropredador circumpolar *Odontaster validus*, per determinar la presència de defenses repel·lents de naturalesa apolar en extractes obtinguts a partir d'invertebrats i macròfits antàrtics. També es va avaluar l'acceptació alimentaria d'aquestes mateixes fraccions front l'amfípode circumpolar i omnívor *Cheirimedon femoratus*. En aquest estudi pretenem contrastar els resultats obtinguts en ambdós tipus de tests, utilitzant dos consumidors simpàtrics rellevants. Un 44.9% dels extractes varen resultar rebutjats per ambdós predadors, en contraposició amb un 10.2% d'acceptats. A més, un 38.8% va provocar repel·lència a l'amfípode, però fou acceptat per la estrella, i l'altre 6.1% de les fraccions varen ser rebutjades solament per les estrelles de mar. En conjunt, va haver més activitat repel·lent cap els amfípodes que cap les estrelles, especialment en aquelles fraccions procedents de macroalgues i esponges, en les que els amfípodes podrien influir particularment en la distribució de llurs defenses químiques. Els amfípodes generalistes, a través de les seues associacions amb biosustrats hoste poden ser importants inductors de defenses químiques, degut a la pressió localitzada que exerceixen sobre ells. Només en unes poques mostres es va demostrar la localització de repel·lents alimentaris en regions anatòmiques específiques, com proposa la ODT, mentre que altres espècies en canvi, sembla que podrien combinar diferents característiques defensives.

CHAPTER 3.3. PUBLICATION III

NÚÑEZ-PONS L, CARBONE M, PARIS D, MELCK D, RÍOS P, CRISTOBO J, CASTELLUCCIO F, GAVAGNIN M and AVILA C. 2012. Chemo-ecological studies on hexactinellid sponges from the Southern Ocean. ***Naturwissenschaften*** 99(5):353-368.

Naturwissenschaften (2012) 99:353–368
DOI 10.1007/s00114-012-0907-3

ORIGINAL PAPER

Chemo-ecological studies on hexactinellid sponges from the Southern Ocean

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Received: 10 January 2012 / Revised: 1 March 2012 / Accepted: 5 March 2012 / Published online: 20 March 2012

Abstract Hexactinellids (glass sponges) are an understudied class with syncytial organization and poor procariotic associations, thought to lack defensive secondary metabolites. Poriferans, though, are outstanding sources of bioactive compounds; nonetheless, a growing suspicion suggests that many of these chemicals could be symbiont-derived. In Polar latitudes, sponges are readily invaded by diatoms, which could provide natural products. Hexactinellids are typical of deep waters; but in Antarctica, they dominate the upper shelf providing shelter and food supply to many opportunistic mesograzers and macroinvertebrates, which exert strong ecological pressures on them. Aiming to examine the incidence of defensive activities of hexactinellids against consumption, feeding experiments were conducted using their lipophilic fractions. Antarctic hexactinellid and demosponge extracts were tested against the asteroid *Odontaster validus* and the amphipod *Cheirimedon femoratus* as putative sympatric, omnivorous consumers. Hexactinellids yielded greater unpalatable activities towards the amphipod, while no apparent allocation of lipophilic defenses was noted. After chemical analyses on the lipophilic fractions from these Antarctic glass sponges, quite similar profiles were revealed, and no peculiar secondary metabolites, comparable to those characterizing other poriferans, were found. Instead, the lipidic compounds 5α(H)-cholestan-3-one and two glycoceramides were isolated for their particular outspread presence in our samples. The isolated compounds were further assessed in asteroid feeding assays, and their occurrence was evaluated for chemotaxonomical purposes in all the Antarctic samples as well as in glass sponges from other latitudes by NMR and MS. Characteristic sphingolipids are proposed as chemical markers in Hexactinellida, with possible contributions to the classification of this unsettled class.

Keywords Antarctic hexactinellid sponges · Chemical defense · Chemotaxonomy · Glycoceramide · Keto-steroid

Communicated by: Sven Thatje

Electronic supplementary material The online version of this article (doi:10.1007/s00114-012-0907-3) contains supplementary material, which is available to authorized users.

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Introduction

Sponges are mostly filter-feeding sessile metazoans with cellular level organization, consisting of an unstructured mesohyl sandwiched between two cellular layers with migrating pluripotent cells (Brusca and Brusca 2003). So far approximately 9,000 poriferan species have been described, of which around 400 are hexactinellids, about 500 are calcareous and the rest (90%) are demosponges. However there are still unexplored habitats. Poriferans have been the focus of much interest due to their associations with a variety of microorganisms and for their outstanding repertoire of bioactive metabolites (Taylor et al. 2007b; Blunt et al. 2011).

Hexactinellid sponges, often referred to as glass sponges, possess a unique histology, with 75% of their soft tissue occupied by a single multinucleated syncytium sharing the external membrane, that ramifies in the framework of "hexactine" siliceous spicules, and the rest consisting of connected uninucleated cells. The mesohyl is absent or minimal. This trabecular syncytium serves as a stream transporting nuclei, organelles and substances, similar to plants (Leys 2003). It is a pathway for propagation of action potentials that triggers halting of flagella motion, and consequent feeding arrests after external disturbance (sediment in the water). This represents a rapid electric protection response throughout the entire sponge from the entry of unsuitable materials and clogging of filtering systems, not reported in other poriferans (Leys et al. 1999, 2007; Tompkins-MacDonald and Leys 2008).

The fossil record from glass sponges goes as far as the Precambrian, making them possibly the earliest living metazoans, with some of them experiencing "gigantism" along with very long life spans (Rossellidae spp.). Their notable porous construction enables them to filter bacteria and microalgae efficiently from their typical deep habitats in all oceans, where predators are rare, and collection and investigation are difficult (Leys et al. 2007). However, in Antarctic sea floors, hexactinellids, which comprise 35 reported species, can live fairly shallow (up to 20 m), dominating the megabenthos in the upper shelf, between 100 and 600 m. They form spectacular associations including mainly eight species from two genera of the family Rossellidae, *Rossella* (restricted to the Southern Ocean except for one species) and *Anoxycalyx*. However, other rossellids, such as *Caulophacus* and *Bathydorus*, are well represented in the deep sea (Barthel 1992; Barthel and Gutt 1992; Gutt 2007; Janussen and Tendal 2007). Antarctic peculiar conditions with long oligotrophic periods pose problems for food supply to filter feeders. Hence, summer highly productive phytoplankton blooms dominated by sea ice microalgae, which flocculate and sink to the bottom, are crucial food sources for poriferans (Hayakawa et al. 1996; Cerrano et al. 2004a). In fact, a conspicuous presence of diatoms has been described, most prevalent in polar poriferans than in tropical or temperate systems (Gaino et al. 1994; Bavestrello et al. 2000; Amsler et al. 2000; Cerrano et al. 2000, 2004a, b; Taylor et al. 2007b). Furthemore, symbiotic diatoms producing extracellular metabolites are suggested to be resources in glass sponges, since long spicules act as optical fibres collecting and delivering light to internal body parts (Cattaneo-Vietti et al. 1996; Müller et al. 2006).

Although Hexactinellids represent tridimensional shelters for diverse fauna (Kunzmann 1996), their living regions are quite pristine in bacteria (Leys et al. 2007). Moreover, they are believed to lack defensive secondary metabolites, and to be unattractive to predators, in part because their skeleton accounts for almost 90% dry weight (Barthel 1995). However, this does not seem to deter Antarctic spongivore consumers, such as asteroids from the genera *Odontaster* and *Acodontaster*, and *Austrodoris* nudibranchs, which readily feed on hexactinellids (Dayton et al. 1974; Dayton 1979). Besides, dense populations of amphipods (up to 300,000 individuals/m^2 benthos) with diversified trophic habits, exert relevant influences to their associated living substrata, frequently macroalgae and sponges, which provide direct or indirect sources of nutrition and structural or chemical refuge (Kunzmann 1996; Nyssen 2005; Huang et al. 2007). All this ecological pressure must be regarded in the frame of the Optimal Defense Theory (ODT), which presumes within body allocation of protective chemicals in the most effective manner, integrating fitness benefits and metabolic costs of defenses, along with other complementing strategies (Rhoades 1979). Hence, in Antarctic sponges, defenses are expected to be stored in external regions, since keystone macropredators firstly encounter surface layers, as has been already reported in some demosponges (Furrow et al. 2003). Peters et al. (2009), however, reported contradictory results in different species. Antarctic poriferans have yielded a high incidence of chemical defense and some active metabolites. But these results again include mostly demosponges, such as the discorhabdins from *Latrunculia apicalis*, suberitenones from *Suberites sp*., or picolinic acid from *Dendrilla membranosa* (for reviews Avila et al. 2008; McClintock et al. 2005, 2010).

Within the phylum Porifera, the relationships among the three extant classes and their connection with eumetazoans are still debated (Reiswig and Mackie 1983). Especially the class Hexactinellida is currently quite controversial (Barthel 1992; Göcken and Janussen 2011). Considering the relevance of glass sponges in Antarctic communities and the poor status of knowledge on their chemistry and ecology, a multidisciplinary research has been undertaken here. As part of a wider research on Antarctic chemical ecology, we first focused on the analysis of lipidic fractions of our samples because most of the reported effective marine bioactive metabolites are lipid soluble (Sotka et al. 2009; Abbas et al. 2011). Hence, out of the most influencing Antarctic benthic consumers, the macropredator sea star *Odontaster validus* and the mesograzer amphipod *Cheirimedon femoratus* were selected to conduct feeding assays in order to evaluate the presence and body allocation of lipophilic defenses against predation in hexactinellids, as well as in a few demosponges from the Weddell Sea for comparison. Chemical studies led to the purification of two selected lipidic metabolites, and an attempt to estimate if they could derive from particular diatoms invading the sponges was made. Moreover, lipid biomarkers have proved a chemotaxonomical value in previous studies in hexactinellids (Thiel et al. 2002). The isolated products were examined

for their unpalatable activity towards asteroid predation, and finally, for their chemotaxonomical potential as well in Antarctic and non-Antarctic samples.

Material and methods

Collection and extraction of sponges

Nineteen hexactinellid sponges pertaining to the family Rossellidae and three demosponges from three different orders (Hadromerida, Haplosclerida and Poecilosclerida) were collected in the Eastern Weddell Sea and vicinities of Bouvet Island (sub-Antarctica) during the ANT XXI/2 cruise of R/V Polarstern (AWI, Bremerhaven, Germany) from November 2003 to January 2004. Sampling was performed in a total of 15 stations between 175 and 882 m depth by using epibenthic sledge, bottom trawls and Agassiz trawls. A portion of each sample was conserved and pictures of fresh animals were taken on board for further taxonomical identification. Voucher specimens are kept at the Centro Oceanográfico de Gijón (IEO, Asturias, Spain) where they were identified by the authors. The remaining material was frozen at −20 °C and transported to the Department of Animal Biology (Invertebrates) at the University of Barcelona. In addition, several non-Antarctic hexactinellid sponges where examined for chemotaxonomical purposes, including the species: *Caulophacus* (*Caulophacus*) *arcticus* (SMF 11724 and SMF 11725) from Fram Strait, Arctic Ocean, from 2,500 m depth, AWI-HAUSGARTEN (ARK XXIII/1, 2005); *Aphrocallistes vastus* from Hosie Islets, Barkley Sound British Columbia, Canada, from 160 m depth; and *Oopsacas minuta* from a cave in La Ciotat, France, from 22 m depth. These samples were kindly supplied and identified by D. Janussen, S. Leys and T. Pérez, respectively (Table 1).

When possible, sponges were dissected into external/internal and apical/basal regions. This was directed to the study of the allocation of possible chemical defenses or particular compounds, attending to the ODT predictions (Rhoades and Gates 1976). Each sample was then broken into pieces and exhaustively extracted with acetone (3×200 mL) at room temperature (~20 °C) with 10 min ultrasonic bath. The organic fraction was evaporated *in vacuo*, and the resulting aqueous suspension was partitioned into diethyl ether (3×100 mL) and butanol fractions (2×100 mL). Diethyl ether fractions were used for bioassays and chemical analysis, while butanolic fractions and water residues were kept for future research.

Nine glass sponges and two demosponges were studied under scanning electron microscopy (SEM) for the presence of diatoms. Cubes of 1×1 cm from internal and external body parts were immersed in H_2O_2 30%, sonicated for 10 min and left 12 h, and then filtered and rinsed through a kitasato with distilled water. Filters were dried under stove, coated with gold at the "Centres Científics i Tecnològics" at the University of Barcelona and examined with a Quanta 200 scanning electron microscope.

Feeding deterrence assays with macropredators

Alive individuals of the voracious eurybathic Antarctic sea star *O. validus*, with omnivorous habits and circumpolar distribution (McClintock 1994), were captured for bioassays at Port Foster Bay in Deception Island, South Shetland Islands (62º 59.369′ S, 60º 33.424′ W). Sampling took place during three campaigns: ECOQUIM-2 (January 2006), ACTIQUIM-1 (December 2008–January 2009) and ACTIQUIM-2 (January 2010). Sea stars were collected by scuba diving from 3 to 15 m depth (n>1,300) and measured between 4.5 and 10.5 cm in diameter. The asteroids were maintained alive in large tanks with fresh seawater at the Spanish Antarctic Base BAE "Gabriel de Castilla" (Deception Island) and were starved for 5 days. Lipophilic fractions and/or isolated compounds from Antarctic poriferans were diluted in diethyl ether and were included in shrimp food cubes. The solvent was left to evaporate and feeding items resulted uniformly charged in extract, following the methodology detailed previously (Avila et al. 2000). Briefly, the tests consisted of 10 replicates each with a 2.5-L container filled with seawater and one sea star. Each animal was offered one shrimp item small enough (5×5×5 mm and 13.09±3.43 mg of dry mass) to be wholly gobbled by the asteroids and treated with either extract or compound in the treatment tests, or solvent alone in the control tests. Extracts were applied at their natural tissue concentrations, respect to the total dry weight (DWT = DW dry weight of the extracted sample + EE weight of the ethereal fraction + BE weight of the butanolic fraction). Biomass-based calculations to normalize natural concentrations using wet or dry weight have been employed in palatability assays against biting and no-biting predators permitting to calculate the "defense per unit bite". In our case, considering sea star extraoral feeding, extruding the cardiac stomach and bolting down whole shrimp chunks (McClintock 1994), the "defense per shrimp feeding item" was evaluated. Wet weight and volume were ruled out because they may entail deviations derived from considering the water content, very variable when manipulating poriferans (Table 1). Isolated compounds 1 and 2 (mixture of 2a and 2b approximately 8:1) were tested as well at their natural concentrations from dry weight yields. We combined laboratory data with published data (only available for 1). The concentrations selected were those corresponding to average quantities recovered in chromatographic purifications. These were 5.7 mg g^{-1} dry sponge for the ceramide mixture (2a and 2b) and 2.5 mg g^{-1} dry sponge for the keto-steroid (1). According to the literature, the value taken for 1 actually accounted for an average concentration in which this metabolite was recorded

Table 1 Data of the lipophilic extracts from Antarctic and non-Antarctic sponge samples

Sponge species and body part	Location	N_{EE} (mg g^{-1} DW)	Comp 1/2
Antarctic and Subantarctic Hexactinellida			
Anoxycalyx (*A.*) *ijimai*	Weddell Sea (Antarctica)	13.46	1, 2
Anoxycalyx (*S.*) *joubini* 1 API	Weddell Sea (Antarctica)	12.88	2
Anoxycalyx (*S.*) *joubini* 1 BAS	Weddell Sea (Antarctica)	7.31	2
Anoxycalyx (*S.*) *joubini* 2 EXT	Weddell Sea (Antarctica)	18.32	1, 2
Anoxycalyx (*S.*) *joubini* 2 INT	Weddell Sea (Antarctica)	26.00	1, 2
Anoxycalyx (*S.*) *joubini* 3 EXT	Weddell Sea (Antarctica)	19.32	1, 2
Anoxycalyx (*S.*) *joubini* 3 INT	Weddell Sea (Antarctica)	28.16	1, 2
Anoxycalyx (*S.*) *joubini* 4 EXT	Weddell Sea (Antarctica)	27.28	2
Anoxycalyx (*S.*) *joubini* 4 INT	Weddell Sea (Antarctica)	15.31	2
Anoxycalyx (*S.*) *joubini* 5 API	Weddell Sea (Antarctica)	24.98	1, 2
Anoxycalyx (*S.*) *joubini* 5 BAS	Weddell Sea (Antarctica)	3.37	1, 2
Rossella antárctica	Weddell Sea (Antarctica)	17.74	1, 2
Rossella fibulata 1 EXT	Weddell Sea (Antarctica)	11.97	1, 2
Rossella fibulata 1 INT	Weddell Sea (Antarctica)	10.79	2
Rossella fibulata 2 API/EXT	Weddell Sea (Antarctica)	14.05	1, 2
Rossella fibulata 2 BAS/INT	Weddell Sea (Antarctica)	6.45	1, 2
Rossella fibulata 3 API/EXT	Bouvet Island (Southern Ocean)	8.67	1, 2
Rossella fibulata 3 BAS/INT	Bouvet Island (Southern Ocean)	10.74	1, 2
Rossella nuda 1	Weddell Sea (Antarctica)	12.03	1, 2
Rossella nuda 2	Weddell Sea (Antarctica)	14.26	2
Rossella nuda 3	Weddell Sea (Antarctica)	16.51	2
Rossella nuda 4 API/EXT	Weddell Sea (Antarctica)	44.86	1, 2
Rossella nuda 4 API/INT	Weddell Sea (Antarctica)	12.66	1, 2
Rossella racovitzae 1	Weddell Sea (Antarctica)	14.93	2
Rossella racovitzae 2	Weddell Sea (Antarctica)	23.11	1, 2
Rossella vanhoffeni API	Weddell Sea (Antarctica)	23.65	2
Rossella vanhoffeni BAS	Weddell Sea (Antarctica)	6.13	2
Rossella villosa 1 EXT	Weddell Sea (Antarctica)	11.87	2
Rossella villosa 1 INT	Weddell Sea (Antarctica)	17.51	n.a.
Rossella villosa 2 API	Weddell Sea (Antarctica)	10.50	1, 2
Rossella villosa 2 BAS	Weddell Sea (Antarctica)	9.93	1, 2
Antarctic Demospongiae			
Gellius sp.	Weddell Sea (Antarctica)	19.17	
Homaxinella balfourensis API	Weddell Sea (Antarctica)	77.82	
Homaxinella balfourensis BAS	Weddell Sea (Antarctica)	116.73	
Isodictya toxophila API	Weddell Sea (Antarctica)	53.17	
Isodictya toxophila BAS	Weddell Sea (Antarctica)	65.47	
Non-Antarctic Hexactinellida			
Aphrocallistes vastus	British Columbia (Pacific Ocean)	35.00	
Caulophacus (*C.*) *arcticus* 1	Fram Strait (Arctic Ocean)	18.92	1, 2
Caulophacus (*C.*) *arcticus* 2	Fram Strait (Arctic Ocean)	15.20	1, 2
Oopsacas minuta	France (Mediterranean)	86.56	GSL[a]

N_{EE} *(mg g^{-1} DW)* natural concentration of the ether extract (EE) in mg per gram of sample total dry weight (DW), *Comp 1/2* presence of compound 1 by HSQC experiments and ceramide mixture 2 by HSQC along with LC-MS analysis, *n.a.* not available, *BAS* basal, *API* apical, *EXT* external, *INT* internal body parts

[a]In *O. minuta* the presence of GSL refers to a different unidentified glycoceramide

in 20 closely related glass sponges, 0.29–4.32 mg g^{-1} dry weight (Blumenberg et al. 2002). Thus, these concentrations were chosen as representatives for samples examined here. After 24 h, the number of eaten food units was recorded for each test, and the remaining (not eaten) were frozen. Later on, these items were extracted and checked on a thin layer chromatography (TLC) screening for ensuring the presence of the extracts or compounds, which was always the case. Diethyl ether fractions are not hydrophilic, hence fast diffusion to the cold Antarctic (~1 °C) water column is theoretically

implausible. Feeding repellence was statistically evaluated by Fisher's Exact tests for each experiment referred to the simultaneous control (Sokal and Rohlf 1995). Afterwards, the stars were returned to the sea.

Feeding preference assays with mesograzers

The abundant eurybathic Antarctic amphipod, *C. femoratus*, is a devouring opportunistic omnivore scavenger with circumpolar distribution (Bregazzi 1972; De Broyer et al. 2007) and was employed for our experiments following the protocol recently described by Núñez-Pons and co-authors (unpublished results). Hundreds of individuals were captured between 2 to 7 m depth by scuba diving with fishing nets, and also by displaying baited traps with canned sardines along the coastline of the Antarctic Spanish Base (BAE) during the campaign ACTIQUIM-2 (January 2010). Artificial caviar-textured foods were prepared with 10 mg/mL alginate aqueous solution containing 66.7 mg/mL of a concentrated dried feeding stimulant (Phytoplan®). The powdered food was mixed into the cold alginate solution with a drop of green or red food coloring (see below), introduced into a syringe without needle. The mixture was then added drop-wise into an aqueous 0.09 M (1%) $CaCl_2$ solution, where it polymerized into spheroid pellets, approximately 2.5 mm in diameter. For treatment pearls, extracts were dissolved in a minimum volume of diethyl ether to totally wet the powdered food and the solvent was evaporated, resulting in a uniform coating of extract on the feeding stimulant prior to being added into the alginate aqueous mixture. The relative quantity of each poriferan lipophilic fraction was calculated according to the natural concentration in a dry weight basis attending to the explanations exposed above, and considering the small size of the food pellets and the minute bites of the amphipods, which turn volumetric calculations intricate. Control pellets were prepared similarly but with solvent alone. Alive organisms were maintained in large 8 L aquariums and were starved for 3–5 days. Every assay consisted on 15 replicate containers filled with 500 mL of sea water and 15 amphipods each, which were offered a simultaneous choice of 10 treatment and 10 control extract-free pellets of different colorations (20 food pearls in total: 10 control and 10 extract-treated), green or red easily distinguished. The colors for treatment or control pearls were randomly switched throughout the experimentation period. Previous trials confirmed the null effect of the different colorations in feeding preferences (p=0.47, n.s.). The assays ended when approximately one-half or more of either food types had been consumed, or 4 h after food presentation, and amphipods were never reused. The number of consumed and not consumed pearls of each color (control or treatment) was recorded for each replicate container, considering that a food pearl was eaten when it was ingested up to at least 1/8 its original size. Finally, statistics were calculated to determine feeding preference of extract-treated pearls respect to the paired extract-free controls to consequently establish unpalatable activities. Every replicate was represented by a paired result yielding two sets of data (treatments and controls). Since assumption of normality and homogeneity of variances were not met, our data were compared by non-parametric procedures by applying the Exact Wilcoxon test with R-command software. Uneaten treatment food beads were preserved for extraction and analysis by TLC to check for possible alterations after testing. No major changes were observed. Once testing was over, amphipods were brought back to the sea.

General chemical experimental procedures

Silica-gel chromatography was performed using pre-coated Merck F_{254} plates and Merck Kieselgel 60 powder (Darmstadt, Germany). NMR experiments were recorded at ICB-NMR Service Centre. 1D and 2D NMR spectra were acquired in $CDCl_3$ or CD_3OD (shifts are referenced to the solvent signal: $CDCl_3$ ^1H δ 7.26 and ^{13}C δ 77.0; CD_3OD ^1H δ 3.34 and ^{13}C δ 49.9) on a Bruker Avance-400 operating at 400 MHz, using an inverse probe fitted with a gradient along the *Z*-axis, and on a Bruker DRX-600 operating at 600 MHz, using an inverse TCI CryoProbe fitted with a gradient along the *Z*-axis. ^{13}C NMR were recorded on a Bruker DPX-300 operating at 300 MHz using a dual probe. Liquid chromatography–mass spectrometry analysis was carried out on a HPLC (Alliance, Waters) on line with a Q-Tof instrument (micro Q-Tof, Micromass) equipped with an ESI source in negative ion mode and a diode array UV detector (scan range 190–400 nm) for a dual monitoring of the chromatographic runs. For ESI–Q-Tof–MS/MS experiments, argon was used as collision gas at a pressure of 22 mbar (CE¼30). Gas chromatography–mass spectrometry analysis were performed by an ion-trap MS instrument in EI mode (70 eV) (Thermo, Polaris Q) connected with a GC system (Thermo, GCQ) by a 5% diphenyl/95% dimethyl polysiloxane (30 m×0.25 mm×0.25 mm) column (Thermo, Trace TR-5) using helium as gas carrier.

Isolation of 5α(H)-cholestan-3-one (1) and the ceramides 2a and 2b

The diethyl ether extracts from *Rossella antarctica*, *Rossella nuda* 1 and *Anoxycalyx* (*Scolymastra*) *joubini* 2 and 4 (100, 345.7, 200 and 291.4 mg, respectively) were separately fractioned by silica gel chromatography using a gradient of light petroleum ether/diethyl ether. The fractions eluted with 10% of diethyl ether contained pure 5α(H)-cholestan-3-one (1) (except in *A.* (*S.*) *joubini* 4), whereas the 100% diethyl ether fraction provided a glycosphingolipid (GSL) mixture (2) (Fig. 1). The isolated metabolites were identified by

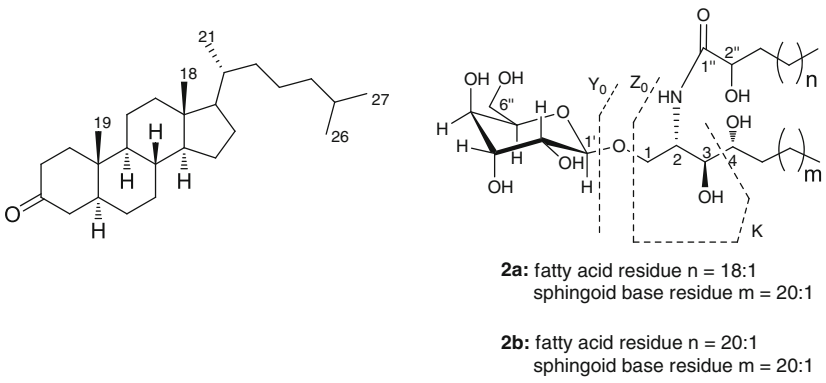

Fig. 1 Chemical structures of the compounds purified from the sponges *Rossella antarctica*, *Rosella nuda* and *Anoxycalyx* (*S.*) *joubini*: (*1*) Keto-steroid 5α-cholestan-3-one. (*2*) Glycoceramides (*2a*, *2b*)

spectroscopic analysis and comparison with literature data (Falsone et al. 1987; Breitmaier and Voelter 1989). Detailed information on the chemical methods, isolation and identification of compounds as well as analyses on the remaining extracts are reported in Online Resource 1.

Results

Chemical extraction and SEM observation of the sponges

In total, 22 Antarctic sponges (19 rossellid hexactinellids and 3 demosponges), along with 4 non-Antarctic glass sponges (2 rossellids and 2 pertaining to different families), yielded 40 diethyl ether fractions (31 from Antarctic rossellids, 5 from demosponges and 4 from Northern glass sponges) which were further analysed (Table 1).

The Antarctic hexactinellids and demosponges examined by SEM shared an identical qualitative composition of species of phytoplanktonic diatoms, dominated by large centric and elongated pennate species, in solitary forms or forming chains, along with silicoflagellates (Fig. 2).

Feeding deterrence assays with macropredators

A total of 24 lipophilic extracts (19 from glass sponges and 5 from demosponges) were tested at their natural concentration and 6 possessed deterrent agents against the asteroid *O. validus*. These active fractions derived from 3 hexactinellids, *Anoxycalyx* (*Anoxycalyx*) *ijimai*, *R. antarctica* and *R. nuda*, and from 2

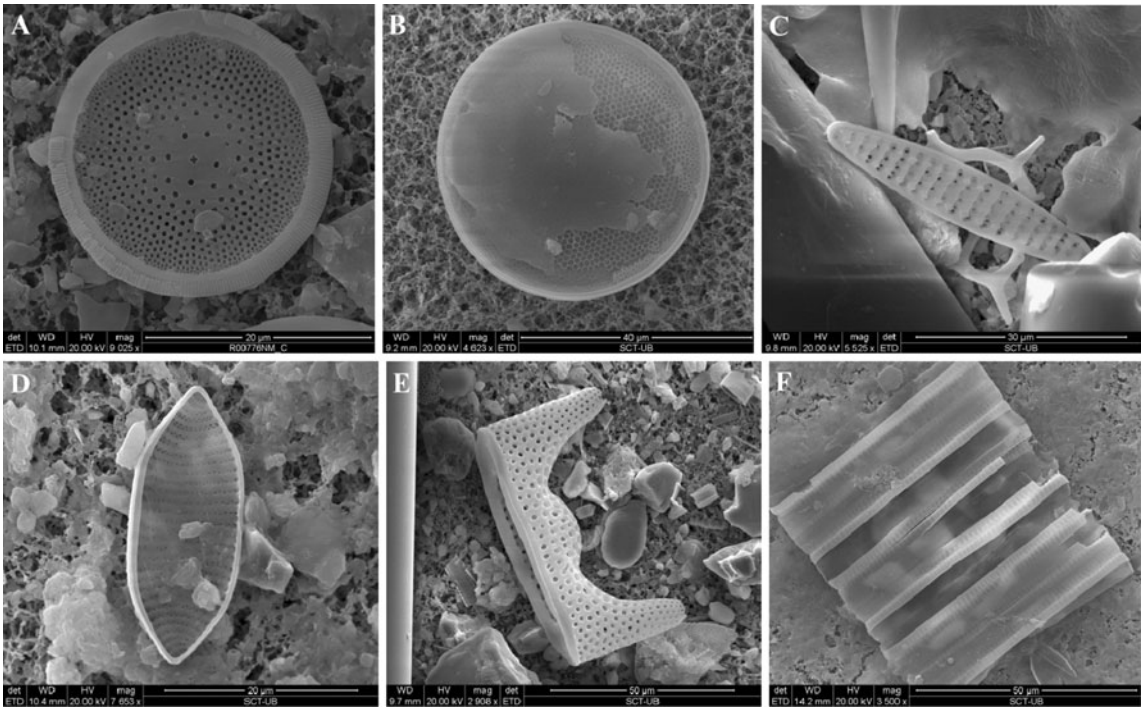

Fig. 2 Diatoms found in Antarctic hexactinellids and desmosponges. Large centric diatoms **a** *Actinocyclus actinochilus* and **b** *Thalassiosira lentiginosa* specimens. **c** Elongated pennate *Fragilariopsis kerguelensis* with a silicoflagellate *Dictyocha speculum* behind and **d** *F. rhombica*. **e** *Eucampia antarctica*. **f** A chain of diatoms of the species *F. kerguelensis*

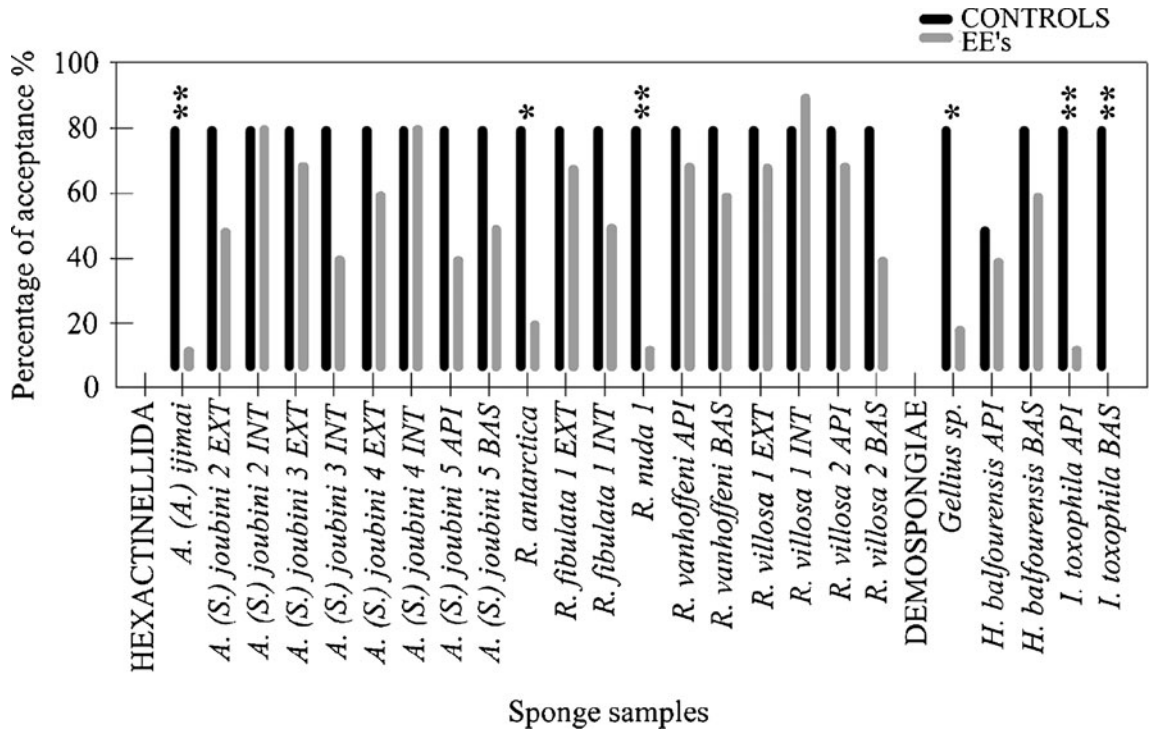

Fig. 3 *Bar diagrams* for feeding repellence assays with lipophilic fractions from Antarctic hexactinellids and demosponges against the sea star *Odontaster validus*, showing the paired results of control and extract treated shrimp cubes for each test, expressed as the percentage of acceptance. Significant differences: $p<0.05$* and $p<0.01$** with control as the preferred food (Fisher's exact test)

demosponges, *Gellius sp*. and *Isodictya toxophila*. The remaining 18 samples, 16 from hexactinellids and apical and basal regions of *Homaxinella balfourensis* were accepted by the sea star (Fig. 3). As for the isolated compounds, 5α(H)-cholestan-3-one resulted strongly deterrent to the asteroid ($p<0.001$**) at 2.5 mg g^{-1} dry weight, while the glycoceramide mixture 2 yielded no unpalatable activity ($p=0.07$).

Feeding preference assays with mesograzers

A total of 15 diethyl ether fractions were tested at their natural concentration towards the amphipod *C. femoratus*, 13 from glass sponges and 2 from *I. toxophila*. Both apical and basal extracts from the demosponge along with 11 lipophilic fractions from 6 rossellid sponges exhibited unpalatability. Only the apical and basal extracts from *A*. (*S*.) *joubini* 1 were inactive against the mesograzer (Fig. 4).

Isolation and identification of 5α(H)-cholestan-3-one (1) and glycoceramide mixture 2

A preliminary screening of the diethyl ether fractions from Antarctic sponges revealed the presence of an apparent yellowish band, moderately UV–visible, at R_f 0.57 (petroleum ether/diethyl ether 8/2) with $CeSO_4$ reaction in most of the hexactinellids. Also the presence of a blatant UV–visible violet band at R_f 0.73 (chloroform/methanol 8/2) with $CeSO_4$ and positive α-naftol reaction occurred in all of them, showing in all these glass sponges a very similar pattern. These bands corresponded with the fractions containing products 1 and 2a and 2b (Fig. 1) and were absent in demosponges.

Chemical purifications were then performed in some extracts, in particular in those coming from *R. antarctica*, *R. nuda* 1 and *A*. (*S*.) *joubini* 2 and 4, revealing that these species did not seem to contain peculiar secondary metabolites, which are typical of many other poriferan species. Therefore, we analysed specifically the lipidic metabolites. Among the most abundant lipophilic metabolites (fatty acids and sterols), significant amounts of a selected keto-steroid, the well-known 5α(H)-cholestan-3-one (1) (Breitmaier and Voelter 1989), were recovered from three of these samples (all except *A*. (*S*.) *joubini* 4). In addition, the sphingolipid fraction was formed by two components (2a and 2b), which were analysed as a mixture. The detailed information on this analysis are described in the Online Resource 1.

2D HSQC NMR-identification of 1 and 2, and detection of 2 by LC-ESIMS analysis

The presence of ketosteroid 1 and ceramide 2 was evaluated by 2D HSQC NMR (Fig. 5) in all the ether fractions, except for that obtained from the internal part of *Rossella villosa* 1. While mixture 2 exists in all the glass sponge fractions, compound 1 was present only in some of them (Table 1).

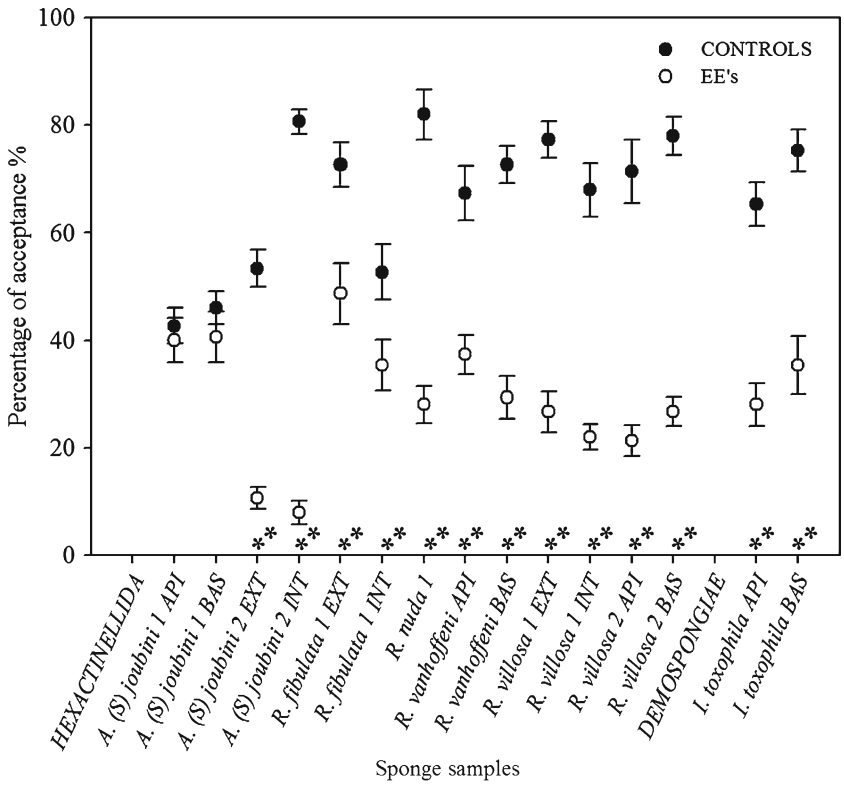

Fig. 4 Scatter plot diagrams for feeding preference bioassays with lipophilic fractions from Antarctic hexactinellids and demosponges towards the amphipod *Cheirimedon femoratus*, showing the paired results of control and extract treated foods with the mean percentage of acceptance and standard error bars. Significant differences: $p<0.01$** with control as preferred food (Exact Wilcoxon test)

LC-ESIMS analysis evidenced the occurrence of glycoceramides 2a and 2b in all the extracts from Antarctic hexactinellid samples (Fig. 6). Moreover, it is remarkable that 2a and 2b were found to be the only components of the sphingolipid fraction. Analogously with 1, these compounds (2a and 2b) were completely absent in the five demosponges analysed (Table 1). The same analyses were carried out on non-Antarctic hexactinellids (Table 1), revealing that the Arctic sponge *C.* (*C.*) *arcticus* contained compounds 1, 2a and 2b. These metabolites were not detected in the Canadian *A. vastus* neither in the Mediterranean *O. minuta*. However, in *O. minuta* the presence of a different sphingolipid was suggested by both LC-ESIMS and NMR analysis performed on the crude extract. More details on this are available in the Online Resource 1.

Discussion

Unpalatable activities in sponge extracts towards asteroids and amphipods

Hexactinellids are believed to have a poor secondary metabolism and to suffer low predation for living in deep seas. This, along with a poor nutritional quality (10% dry mass of organic material), makes them apparently not requiring defensive chemistry (Barthel 1995; Leys et al. 2007). However, this does not quite describe the real scheme of Antarctic benthos, where glass sponges may live in shallow waters and be intensely foraged by macroinvertebrates and associated mesofauna (isopods, amphipods, polychaetes and others; Dayton et al. 1974; Dayton 1979; Barthel and Tendal 1994; Kunzmann 1996; McClintock et al. 2005). For instance, *Rossella racovitzae* samples were collected with feeding *Austrodoris kerguelenensis* nudibranchs on them (author's personal observation). Hence, Antarctic hexactinellids must possess some sort of protection. Most experimental evidence indicates that the primary function of spicules is skeletal support (Jones et al. 2005). However, even if spicules are not the main deterrents for fishes, grazing amphipods, nor sea stars, synergistic interactions (through mechanical protection or by reducing the nutritional quality of sponge tissue) with chemical defenses could exist (Chanas and Pawlik 1995; Waddell and Pawlik 2000; Jones et al. 2005). Actually, some defensive metabolites are more (or only) effective when combined with poor quality foods (Duffy and Paul 1992; Barthel and Tendal 1994; Cruz-Rivera and Hay 2003; Sotka et al. 2009). This synergistic effect might be masked in assays using attractive diets, such as in the sea star test, in which shrimp cubes were treated with sponge extracts. In our assays, hexactinellid samples exhibited low lipophilic chemical protection towards the asteroid *O. validus*, with only three extracts from *Anoxycalyx* (*A.*) *ijiami*, *R. antarctica* and *R. nuda* being significantly repellent. However, *R. nuda* and *R. racovitzae* have been observed to be predated by this echinoderm and other spongivores, which opportunistically forage on the most

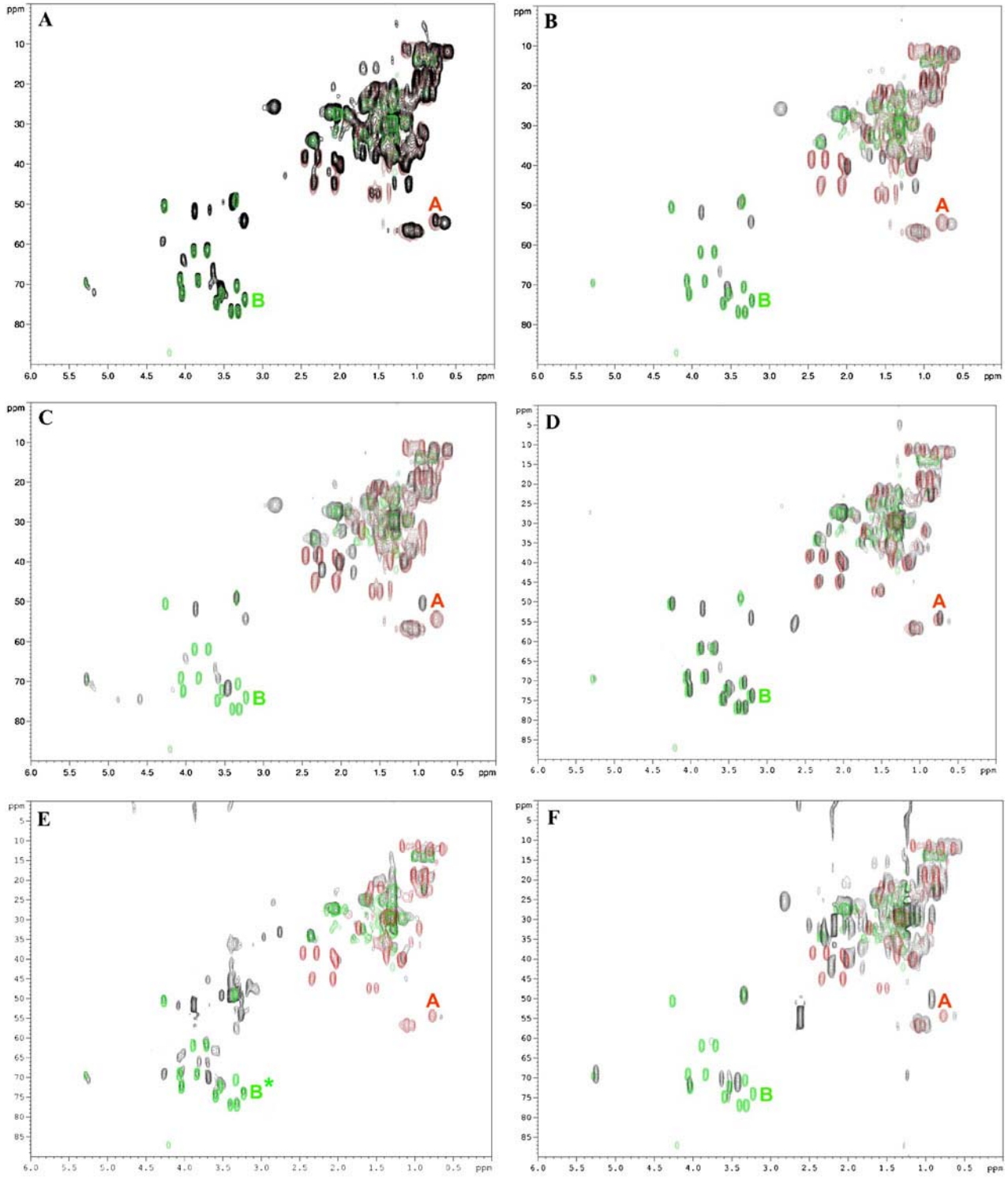

Fig. 5 Combined 2D HSQC NMR spectra of the two isolated compounds 5α-cholestan-3-one (1) in *red* with its selected peak for the recognition "A" (ppm (1H/13C)=0.76/53.94), and glycoceramides (2a and 2b) in *green* and their diagnostic peak named "B" common also to other GSL (ppm (1H/13C)=3.22/73.74), and spectra of crude lipophilic fractions in black. **a** An Antarctic rossellid extract (*Anoxycalyx* (*S.*) *joubini* 2 INT) containing both metabolites (presence of peaks *A* and *B*). **b** An Antarctic rossellid extract (*Anoxycalyx* (*S.*) *joubini* 4 INT) containing 2a and 2b, but lacking 1 (only peak *B* present). **c** An Antarctic demosponge extract (*Homaxinella balfourensis* API) lacking both metabolites (absence of peaks *A* and *B*). **d** The Arctic *C.* (*C.*) *arcticus* extract possessing both metabolites (presence of peaks *A* and *B*). **e** The Mediterranean *O. minuta* extract containing an unidentified GSL, and lacking 1 (only peak *B* present). **f** The Canadian *A. vastus* extract lacking both metabolites (peaks *A* and *B* absent)

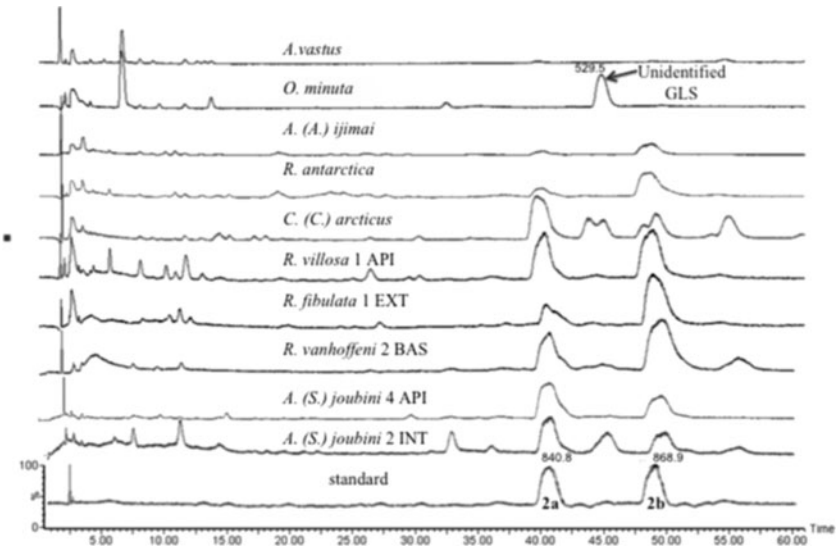

Fig. 6 Reverse phase LC-MS profile of some representative sponge lipophilic fractions analysed. *Numbers above peaks* indicate the molecular ion $(M–H)^-$ as determined by ESI^- ionization, for ceramides 2a (peak near 40 min; 840.8 MW) and 2b (peak near 48 min; 868.9 MW). In *Oopsacas minuta*, none of these peaks appear, and there is a peak at 44.8 min; 529.5 MW from an unidentified GSL

abundant species (McClintock 1987). Regarding the large vase volcano sponge, *A*. (*S*.) *joubini*, which yielded suitable fractions here (Fig. 3), previous studies described strong tube-foot retractions towards *Perknaster fuscus*, although this asteroid rarely eats any sponge other than *Mycale acerata* (Dayton et al. 1974; McClintock et al. 2000). Whereas little bioactivity has been reported in hexactinellid extracts against sea star feeding (our data; McClintock 1987), our samples displayed strong unpalatability towards the amphipod *C. femoratus*. Only the fractions from *A*. (*S*.) *joubini* 1 were accepted. Instead, *A*. (*S*.) *joubini* 2, with richer lipophilic fractions, was deterrent. Maybe the fact that samples came from different locations with different conditions and predation pressures, produced diverse metabolite profiles and/or concentrations (Table 1; Fig. 4). Actually, levels of deterrence in sponge extracts can vary among conspecifics (Jones et al. 2005), perhaps due to chemical defense induction, saving metabolic energy by keeping defensive products low and increasing them in response to predation episodes (Thoms et al. 2007).

Glass sponges reveal effective recovery from wounds (Leys and Lauzon 1998). Energy expenditure for regeneration is proposed to detract from that for chemical defense (Walters and Pawlik 2005; Leong and Pawlik 2010), and secondary metabolites always entail costs. Wound healing has been hypothesized to be typical of space holders with low recruitment rates, long life spans, massive growth and high predation exposition (Ayling 1983; Wulff 2010). Thus, synergism between low chemical defense and high spicule content might exist in Antarctic hexactinellids (Barthel 1995), favoring increased levels of energy available for regenerating after predator attacks. Yet defensive agents may be present in hydrophilic fractions not tested here. This indeed will be the subject of further studies.

Hexactinellids represent potential prey and substrata for omnivorous sedentary amphipods, which may use sponge tissues directly or indirectly while grazing on associated microbiota, such as diatoms. Spongicolous amphipods occur in large abundances and diversity, with no obligate associations (Kunzmann 1996; De Broyer et al. 2007; Amsler et al. 2009), and may exert larger localized predation pressures than more wandering asteroids, thus favoring the production of chemical defense (Toth et al. 2007). *C. femoratus* is an opportunistic bottom-dweller with reduced swimming. Sponges constitute rich and accessible resources of sterols for crustaceans (Blumenberg et al. 2002) that are unable to *de novo* biosynthesize vital steroids, such as ecdysteroid hormones for molting (Goad 1981). Furthermore, amphipods seem more susceptible to lipidic defenses (Cruz-Rivera and Hay 2003; Aumack et al. 2010), along with being more discriminative for unpalatabilities when comparing both assays (Núñez-Pons et al., unpublished results). All these facts may explain the larger deterrent activities found towards *C. femoratus* respect to *O. validus* using lipophilic sponge extracts. The only demosponge with inactive extracts was the fast growing *H. balfourensis*, which has already proved to lack defensive agents (for a review Avila et al. 2008). Finally, the ODT, which predicted defenses to accumulate in external layers due to sea stars extruding the cardiac stomach against the sponge pinacoderm, was not sustained (Rhoades 1979; McClintock 1994; Furrow et al. 2003). Our results, instead, showed similar activities for inner and outer fractions, suggesting no allocation of lipidic defenses (Figs. 3 and 4), similarly to some findings from other studies (Peters et al. 2009). This may be due to the fact that small biting grazers get to inner regions too, especially in hexactinellids with large oscula.

Chemical analysis of Antarctic sponges

Poriferans have been extensively investigated for their associations with microorganisms, sometimes considered as microbial fermenters (Hentschel et al. 2006), and for being the most prolific marine producers of natural compounds including: terpenoids, alkaloids, peptides and polyketides, as well as unique sterols and sphingolipids with remarkable chemodiversity (Blunt et al. 2011). Nonetheless, there is a growing suspicion that many bioactive chemicals found in sponges could be symbiont-derived, mainly from bacteria and cyanobacteria (Jayatilake et al. 1996; Sarà et al. 1998; Taylor et al. 2007a; Sabdono and Radjasa 2008). Hexactinellids, instead, have not yielded natural products so far (Blunt et al. 2011), and rarely present procariotic symbionts, since their negligible mesohyl does not provide a good substrate for cell migration or microorganism growth as do cellular sponges (Sarà et al. 1998; Leys et al. 2007; Taylor et al. 2007b). Still, glass sponges are understudied, even if their chemistry could provide an insight of the biosynthesis in early metazoan evolution (Thiel et al. 2002). According to current thoughts, no distinctive secondary metabolites characterizing other poriferan species were present in the lipophilic fractions examined here from several Antarctic hexactinellids. Instead, the chemical analysis resulted in the detection of two selected lipids with broad presence within our samples. The steroid derivative 5α(H)-cholestan-3-one (1) was present in almost all the extracts, whereas the peculiar glycoceramide mixture 2 characterized all samples (Fig. 1; Table 1). These lipids were not detected in the three Antarctic demosponges analysed. The 3-keto-5α(H)-derivatives, including 5α(H)-cholestan-3-one, were described as a group of natural products from the Antarctic poriferan *Artemisina apollinis*, but as far as we know there have been no further cites of demosponges possessing this steroid (Bergquist et al. 1980; Seldes et al. 1990b).

Regarding the presence of diatoms, the specimens found in our sponges belong to the most abundant species forming summer blooms along the Argentinian shelf, Drake Passage and Weddell Sea (Olguín and Alder 2011; Table 1; Fig. 2). Sinking microalgae are crucial in pelagic–benthic coupling constituting the main source of hydrocarbons to filter feeding communities (Hayakawa et al. 1996). Diatoms are incorporated alive, but eventually die inside the sponge, accumulating silica frustules, which are presumably dissolved for spicule formation (Cerrano et al. 2004a, 2004b). Indeed many marine animals have C_{26} sterols from planktonic origin (Seldes et al. 1990a). However, products 1 and 2 did not seem to derive directly from a specific diatom provision, since hexactinellids and demosponges shared similar diatom profile but had different lipidic composition. Furthermore, these molecules appeared also in our samples of the Northern Hemisphere glass sponges, which probably feed on different diatom species.

Even if most of the samples were quite voluminous, the extracted material was never a large amount and the concentration of metabolites was relatively low, especially in glass sponges, in agrrement with previous studies (Guella et al. 1988; Barthel 1995). Sterols have been stated to make up about 0.04% to 5% of total lipids, and sponges contain 0.5% to 7% lipids in relation to dry weight, corresponding to 0.002 to 3.5 mg sterols per gram of dry sponge. Nevertheless, absolute concentrations of lipids vary considerably among specimens and/or area of the sponge (Bergquist et al. 1991). Blumenberg et al. (2002) analysed sterols from 20 hexactinellid species by GC-MS obtaining cholesterol (cholest-5-en-3β-ol) and/or its saturated derivative 5α(H)-cholestan-3β-ol along with C-24-alkylated homologues. The 5α(H)-stanols co-occurred with their 3-keto-5α(H)-derivatives in some of the samples, similarly to our findings. From small fractions of specimens, they recorded significant concentrations of 5α(H)-cholestan-3-one (0.29–4.32 mg g^{-1} dry sponge), but their study did not focus on absolute concentrations (Blumenberg et al. 2002). Our approximative whole-sponge concentrations for steroid 1 do agree with their calculations though.

The presence of glycoceramides 2 in hexactinellids is noteworthy. Even if GSL are common in sponges (Muralidhar et al. 2003; Tan and Chen 2003), the composition of the ceramide mixture in the species analysed is quite characteristic. In particular, all our samples contained only two main GSL –C24 and C22 fatty acid homologues, thus suggesting a possible chemotaxonomical value (see below).

Bioactivities of 5α(H)-cholestan-3-one (1) and ceramide mixture 2

The ketosteroid 5α(H)-cholestan-3-one (1) displayed potent unpalatable activity against *O. validus* at the natural concentration. The 3 rejected hexactinellid fractions from *A.* (*A.*) *ijimai*, *R. antarctica* and *R. nuda*, all possessed compound 1, indicating that it could be responsible for the unpalatability. Nonetheless, the other 9 lipophilic extracts containing the steroid, failed in repelling the sea star from eating. This suggests that 5α(H)-cholestan-3-one might be present in different concentrations in the active samples. Unfortunately, isolated products could not be tested against the amphipod. However, the only two palatable fractions for amphipods lacked 1 (Table 1; Fig. 3 and 4). Steroid 1 could play a more or less preponderant role as deterrent, in synergism with other co-occurring chemicals.

Secondary metabolites are usually considered responsible for feeding unpalatability (Paul 1992). But also sterols, usually considered primary metabolites, have shown deterrent activities in sponges and a sea spider (Bobzin and Faulkner 1992; Tomaschko 1994). Besides, the Antarctic soft coral *Alcyonium paessleri* exudes sterols including

cholesterol into the surrounding water causing long tube-feet retraction periods in *O. validus* (Slattery et al. 1997). Whether keto-steroids (cholest-4-en-3-one and 5α(H)-cholestan-3-one) have a discrete function, like chemical defense, or are just metabolic dead-ends is a matter of debate. They are formed when conventional sterols (cholesterol) are converted into stanols, as demonstrated for some microorganisms and sea stars (Smith et al. 1972; Taylor et al. 1981; Blumenberg et al. 2002). A dietary uptake of Δ^5-stenols in hexactinellids containing C_{27}-C_{29} Δ^5-sterols, with cholesterol generally predominating, has been suggested. These would be further transformed, by the sponges or by microbes, via 3-keto intermediates to 5α(H)-stanols (Blumenberg et al. 2002). In animal cellular membranes, cholesterol is predominant, but some sponges and holoturians, have unusual sterols. Biochemical coordination has been proposed to provide protection from own membranolitic toxins, with altered membrane steroid compositions correlated with defensive chemistry (Santalova et al. 2004, 2007). Actually, in many sponges, a symbiotic origin of unusual steroids and secondary metabolites is supported, while those containing conventional-type sterols are often devoid of procariotic symbionts (Lawson et al. 1988). This agrees with what is known for glass sponges, with cholesterol preponderance in the steroid mixture, along with a low secondary metabolite occurrence and poor bacterial symbiosis (Blumenberg et al. 2002; Leys et al. 2007).

The defensive role of the ceramide mixture (2a and 2b) was not confirmed, since it showed no repellency in the sea star bioassay and did not affect the unpalatability against the amphipod (Table 1; Figs. 3 and 4). The glucocerebrosides or GSL are primary metabolites, formed by a hexose (glucose) at C-1 and a ceramide moiety consisting of a sphingoid base (long-chain aminoalcohol) and an amide-linked fatty acid. They are typical integrants of cell membranes, along with intercalated cholesterol molecules and embedded proteins. They provide structural and texture support, and act as mediators in intracellular communication and cell recognition binding to lectins or other GSL on neighboring cells (Tan and Chen 2003; van Meer and Hoetzl 2010). But, in spite of their great potential for drug discovery (by themselves or their breakdown products) the actual roles of cerebrosides are poorly understood (for a review see Tan and Chen 2003; Padrón 2006). Glucocerebrosides (2a and 2b) presumably take part of the syncytial membrane, where hypotethically they could participate in critical functions of the trabecular syncityum. Their structure as glucosylceramides, closer to phytosphingosines from plants (two –OH after the amide) than to sphingosine from animals (one –OH), could suggest a vicinity to the Plantae kingdom, in accordance with glass sponges being considered the most basic metazoans. This, however, remains only as a highly speculative hypothesis, and many more studies are needed to sustain that. In fact, similar GSL also occur in other sponges (Muralidhar et al. 2003; Tan and Chen 2003). Ceramides 2a and 2b, reported here in sponges (Hexactinellids) for the first time, were described from the plant *Euphorbia biglandulosa*, and could perhaps be related to syncytial structures and their particular body organization (Falsone et al. 1987).

Chemotaxonomical remarks on Antarctic and non-Antarctic sponges

The taxonomic relationships within the phylum Porifera are still under discussion. Attending to spicule nature (Mg-calcite vs siliceous spicules) and larval development, Demospongiae and Hexactinellida are proposed to form a common taxon, Silicea, separated from Calcarea. Nonetheless, cell and soft body organization rather supports the separation of Hexactinellida (Symplasma) from the other two classes, joined into the subphylum Cellularia (Reiswig and Mackie 1983; Leys 2003). Complementary to the classical morphological and molecular biological approaches, few investigations on lipidic markers have been carried out to further contribute to sponge taxonomy, dealing with steroids or with fatty acids, attending to presence/absence or to relative proportions (Lawson et al. 1984; Thiel et al. 2002). Steroids were proposed for chemotaxonomy due to their resistance to degradation and the variety of structures (Bergquist et al. 1980, 1986, 1991). Nevertheless there are restrictions with sponges of different genera, and even order, sharing similar steroid composition (Seldes et al. 1986, 1990a, b). Hexactinellida, containing predominantly C_{27}–$C_{29}\Delta^0$ and C_{27}–$C_{29}\Delta^5$ sterols and their keto derivatives (Blumenberg et al. 2002), differ from calcarean sterol patterns, which partially overlap with some Demospongiae (Hagernann et al. 2008). On the contrary, demosponges possess unique membrane long chain fatty acids (>C_{24}) called demospongic acids, similarly found in glass sponges (Lawson et al. 1988), but absent in calcareous sponges. This contradicts the view of Calcarea and Demospongiae more closely related to each other than either of them to Hexactinellida (Thiel et al. 2002). Even if sample sizes were in some cases low, our findings suggest that the ceramide (2a and 2b) could be a chemotaxonomical tracer within the class Hexactinellida, in particular for the rossellids (Table 1; Fig. 7). Families of glass sponges could then be separated attending to GSL content, like Rossellidae, where all the studied species possessed glycoceramides 2 (2a and 2b). Leucopsacidae, instead, represented only by *O. minuta*, which contains other unidentified GSL. Moreover, the occurrence of these GSL might be a particularity of the order Lyssacinosida, since the only sponge pertaining to Hexactinosida (*A. vastus*) had no sphingolipid alike, thus allowing also a distinction between these two orders (Fig. 7). However, these are preliminar approximations and more species need to be examined to drawn further conclusions, even though the inaccessibility of glass sponges makes this difficult. Sphingolipids have been already used in chemotaxonomy for certain

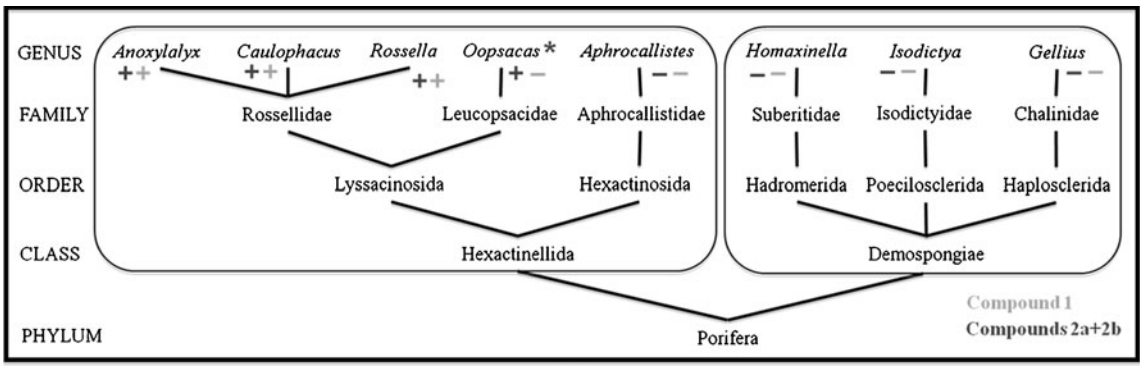

Fig. 7 Taxonomic relationships of the poriferan genera investigated, where the presence (+) or absence (−) of steroid 1 (5α-cholestan-3-one) in *light grey* and glycoceramides 2a and 2b in *dark grey* are shown for each representative sponge genus of the species analysed. In *Oopsacas*, the *positive dark grey sign* refers to the presence of a GSL similar to 2

microorganism genera (Takeuchi et al. 1995). In general, the taxonomy of Hexactinellida is incomplete and probably not even half the species are known to science yet. Furthermore, many species and even genera are described from just one, sometimes, fragmented specimen (Barthel 1992). Regarding the genus *Rossella* revisions are currently being performed (Göcken and Janussen 2011; Janussen, personal communication). For these reasons, chemotaxonomical studies may greatly contribute to glass sponge classification.

In summary, glass sponges, in spite of representing an unattractive meal, are readily attacked by some Antarctic benthic consumers. The sponges exhibited low incidence of lipophilic defenses to prevent sea star predation, contrasting with a high unpalatability against amphipod grazing. This may be explained by a higher localized pressure exerted by host opportunistic mesograzers, as well as by the greater discriminative potential of the amphipod test. Yet hydrophilic metabolites not assessed here may also be participating in defense towards asteroids. Antarctic hexactinellids could combine low nutritional value with defensive chemicals, allowing also an effective regeneration. However, this synergism might be disguised with richer diets in sea star assays. Contradicting previous convictions on glass sponges yielding inactive extracts, our fractions did exhibit significant bioactivity against omnivorous amphipods, suspected to derive from primary metabolism. Secondary metabolism is presumed to be poor in hexactinellids respect to other sponges, along with an insignificant procariotic symbiosis. This is consistent with our findings, at least for sponge typical products of lipophilic nature. The analysis of some fractions of glass sponges though, led to the isolation of two rich likely primary metabolites, absent in demosponges. These metabolites did not seem to be diatom derived, since demosponges and hexactinellids revealed the same profile of typical phytoplanktonic diatom species from Austral summer blooms. The steroid 5α(H)-cholestan-3-one (1) displayed deterrence against *O. validus* demonstrating, at least, a minor antipredatory role. The glycoceramides (2a and 2b), known from a superior plant and now here firstly reported in sponges, had no repellent activity. They were found in all Antarctic and in some non-Antarctic hexactinellids in a quite characteristic manner, representing the only components of the ceramide mixture, presuming a possible chemotaxonomical tool. These ceramides could likely play a role within the syncytial membrane of glass sponges, as similar ceramides do in plants, and could serve as molecular markers for the Rossellidae family. To some extent, similar kinds of GSL might be characteristic within the order Lyssacinosida. It will be intriguing to find out to what extent this type of GSL do spread throughout the class Hexactinellida, since they could contribute to the taxonomy of this unsettled class. Contrastingly, the keto-steroid (1) is undoubtly a transient functional metabolite not so useful for chemotaxonomy. A better understanding of the biology and chemistry of glass sponges and the functionalities of their metabolites within syncytial systems await for future research.

Acknowledgements We thank J. Vázquez, S. Taboada, B. Figuerola, M. Paone and E. Manzo for their precious help in the lab. Thanks are due to D. Janussen, S. Leys, T. Pérez and M. Bergmann (AWI) for kindly supplying hexactinellid samples from Northern latitudes. Also, we are grateful to W. Arntz and the crew of R/V Polarstern. UTM (CSIC), R/V "Las Palmas" and BAE "Gabriel de Castilla" crews provided logistic support. The "Centres Científics i Tecnològics" of the UB also provided technical support. Funding was provided by the Ministry of Science and Innovation of Spain (CGL2004-03356/ANT, CGL2007-65453/ANT and CGL2010-17415/ANT) and REDES Project (CGL2009/06185-E).

Ethical standards We declare that this research conforms to the legal requirements of the Spanish and Italian laws.

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ESM_1:

SUPPLEMENTARY MATERIAL (Online Resource 1)

Article title: **Chemo-ecological studies on hexactinellid sponges from the Southern Ocean**

Journal name: Naturwissenschaften

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Material and methods

*Isolation of 5α(H)-cholestan-3-one (**1**) and the ceramides **2a-b***

The diethyl ether extracts from *Rossella antarctica, R. nuda* 1 and *Anoxycalyx (Scolymastra) joubini* 2 and 4 (100, 345.7, 200 and 291.4 mg, respectively) were separately fractioned by silica gel chromatography using a gradient of light petroleum ether/diethyl ether. The fractions eluted with 10% of diethyl ether contained pure 5α(H)-cholestan-3-one (**1**) (except in *A. (S.) joubini* 4), whereas the 100% diethyl ether fraction provided a glycosphingolipid (GSL) mixture (**2**) (Fig. 1). The isolated metabolites were identified by spectroscopic analysis and comparison with literature data (Falsone et al. 1987; Breitmaier and Voelter 1989).

*5α(H)-cholestan-3-one (**1**):):*$[a]_D$ = 33.7 (*c* = 3.5, $CHCl_3$); selected ^{1}H NMR δ 1.00 (s, 3H, H_3-19), 0.90 (d, J_{H-H} = 6.6 Hz, 3H, H_3-21), 0.86 (d, J_{H-H} = 6.6 Hz, 6H, H_3-26 and H_3-27), 0.68 (3H, s, H_3-18); selected ^{13}C NMR δ 213.44 (C-3), 56.45 (C-17), 56.30 (C-14), 53.9 (C-9), 46.6 (C-5), 44.6 (C-4), 22.5 (C-26 and C-27), 18.6 (C-21), 11.9 (C-19), 11.4 (C-18). ESI-MS: m/z 409 $[M+Na]^+$.

*GSL mixture (**2**)*: selected ^{13}C NMR data (MeOD, 300 MHz) d = 177.1 (s, C"1), 130.8 (d), 104.7 (d, C-1'), 78.0 (d, C-3'), 77.9 (d, C-5'), 75.5 (d, C-4), 75.0 (d, C-2''), 73.0 (d, C-3), 72.5 (d, C-3), 71.6 (d, C-4'), 70.0 (t, C-1), 62.7 (t, C-6'), 51.6 (d, C-2), 28.2 (t, $\underline{C}H_2CH=CH$), 27.0 (t, $\underline{C}H_2CH=CH$), 14.5 (q, $(CH_2)_n$-$\underline{C}H_3$); selected ^{1}H NMR data (MeOD, 400 MHz) d = 5.37 (m, 4H, 2 CH=CH), 4.32 (d, J_{H-H} = 8 Hz, 1H, H-1'), 4.29 (m, 1H, H-2), 4.09 (m, 1H, H_2-1a), 4.06 (m, 1H, H-2''), 3.90 (dd, J_{H-H} = 12.0, 1.0 Hz, H_2-6'a), 3.84 (dd, J_{H-H} = 10.0, 4.0 Hz, 1H, H_2-1b),

3.70 (dd, $J_{H\text{-}H}$ = 110.0, 4.0 Hz, 1H, H_2-6'b), 3.65 (m, 1H, H-4), 3.55 (m, 1H, H-3), 3.37 (m, 1H, H-5'), 3.34 (m, 2H, H-3'and H-4'), 3.21 (m, 1H, H-2'), 2.06 (m, 8H, 4 $\underline{CH_2}CH=CH$), 0.93 (m, 6H, $(CH_2)_n$-$\underline{CH_3}$). ESIMS: $[M\text{-}Na]^-$ 864 and 892 *m/z*. HRESIMS calcd for $C_{48}H_{90}NO_{10}Na$: 864.6540. Found: 864.6572.

*LC–MS/MS analysis of **2***

Natural sample **2** was dissolved at a final concentration of 0.5 mg mL^{-1} (inj. 50 mg/25 mL) and analyzed on an RP-18 column (Kromasil, Phenomenex) using a MeOH/H_2O gradient elution from 95% to 100% MeOH in 40 min, holding at 100% MeOH for 30 min; flow: 1mL min^{-1}. The eluate was split after column and 9/10 was channeled to photodiode array detector: 1/10 to an ESI-QTof apparatus. In the order of elution, $[M\text{-}H]^-$ (Y_0, Z_0/K, Z_0/K-CH=CHCHO) *m/z*: 840 (678, 408, 353); 868 (706, 436, 381).

*Determination of the fatty-acid composition of **2***

A small amount (2.0 mg) of GSL mixture (**2**) was dissolved in 5% 1M HCl-MeOH, and the mixture was refluxed for 18 h at 80 °C. The reaction was then cooled and extracted with *n*-hexane. The hexane layer was separated and passed through a small silica gel column. The eluate was concentrated *in vacuo* and analyzed by GC-MS. Based on the results of GC-MS the major components of the methanolysis products were identified as methyl esters of (*Z*)-2-hydroxydocosenoic acid and of (Z)-2-hydroxytetracosenoic acid.

2D HSQC NMR and LC-MS identification of isolated compounds within the extracts

The keto-steroid (**1**) and ceramide **2** were identified within the diethyl ether extracts choosing representative peaks (A: ppm 1H/13C 0.76/53.94), in the 2D HSQC NMR spectrum discriminatory from other components of the fraction. Hence, the keto-steroid (**1**) was identified by peak A (ppm 1H/13C 0.76/53.94). Ceramide **2** instead, was represented in the 2D HSQC NMR spectra by a given diagnostic peak B (ppm 1H/13C 3.22/73.74), which was common for both ceramides comprising the mixture (**2a-b**), but also for other similar GSL, further corroborated by LC-MS (Fig. 2 and 3; Table 1). Sphingolipids like **2** are amphipatic molecules, which give viscous solutions in chloroform, and are not soluble in either hydro- or lipophilic solvents. Hence, crude fractions were dissolved in chloroform-methanol 1:1.

Results

*Isolation and identification of 5α(H)-cholestan-3-one (**1**) and glycoceramide mixture **2***

The ESI^+ mass spectrum of mixture **2** showed two pseudomolecular ion peaks $[M+Na]^+$ at 864 and 892 *m/z*, which suggested the presence of two homologues differing from each other in two

methylene units. A high-resolution measurement performed on the most abundant ion at *m/z* = 864.6572 indicated the molecular formula $C_{48}H_{91}NO_{10}$ for the dominant homologue.

The glycosphingolipid nature of **2** was immediately deduced by the presence in its NMR spectra of characteristic signals due to a sugar (an anomeric proton at d_H 4.32), an amide linkage (a nitrogenated methin proton at d_H 4.29 and a carbonyl at d_C 177.1), and long alkyl chains (terminal methyl and methylene protons at d_H 0.93 and d_H 1.24-1.38, respectively). In the ^{13}C NMR spectrum, the carbon resonances at d_C 62.7 (CH_2), 71.6 (CH), 75.0 (CH), 77.9 (CH), 78.0 (CH), and 104.7 (CH) indicated the presence of a b-glucopyranoside moiety. The coupling constant of the anomeric proton at d_H 4.30 (d, J_{H-H} = 8.0 Hz) further confirmed the b configuration of the glucose unit.

The ceramide scaffold of **2** resulted to be composed of a trihydroxyl monounsaturated sphinganine and a-hydroxy monounsaturated fatty acid residue. All the protons of the polar part of the sphinganine were assigned by analysis of the COSY spectrum of **2**, starting from the nitrogenated methin proton at d_H 4.29. The a-hydroxy substitution of the fatty acid residue was clearly revealed by the absence in the ^1H NMR spectrum of **2** of the typical triplet at d ≈ 2.3 due the fatty acid a-protons being replaced by a signal at δ = 4.06 ppm (H-2''). This proton was coupled with a methylene at δ = 1.83 ppm (H_2-3''), which was in turn correlated to the alkyl chain protons at δ = 1.24-1.38. The HMBC correlation observed between H_2-1' and C-1 of glucose unit clearly indicated the position of the sugar. The relative stereochemistry of the 2-N-acyl-1,3,4-trihydroxyl fragment of ceramide was suggested by comparing the d_C carbon values with those reported in the literature for several model compounds (Kawano et al. 1988; Higuchi et al. 1991; Honda et al. 1991) whereas the configuration of C-2''' remained undetermined.

The MS/MS analysis was applied to rapidly identify both fatty acid and sfinganine parts of the two components of the mixture. According to Cutignano *et al.* (2011), the diagnostic ceramide fragmentation allowed the straight identification of the fatty acid residue and, indirectly, of the nature of long chain-base. In the MS/MS ESI^- experiment performed on the ion at 840 *m/z* $[M-H]^-$, a successive rupture of the carbon bonds between C3–C4 and C1–C2 on the Y0 fragment ion at 678 *m/z* (M-163) generated the Z0/K fragment at 408 *m/z*. Further loss of CH=CHCHO from the ceramide residue (Z0/K-55 *m/z*) generated the ion due to the 2-hydroxy fatty acid moiety at 353 *m/z*. The same fragmentation pattern was observed for the ion at 840 *m/z*. MS/MS structural data obtained for both molecular ions were corroborated by GC–MS analysis of the fatty acid methyl derivatives obtained by methanolysis. The GC–MS profile revealed the occurrence of two types of 2-OH monounsaturated long chain fatty acids (C22 and C24), as predicted from LC–MS/MS data. The small amount of sample prevented further structural analysis to determine the position of the double bonds, whose geometries were however both assigned as *Z* on the basis of allylic carbon values at d 28.2. (Seki et al. 2001) Thus the structures of **2a** and **2b** were proposed as in the formula (Fig. 1). Glucosylceramides having

structural features as same **2a** and **2b**, that is a 1,3,4-trihydroxy monounsaturated C-20 base and an a-hydroxy monounsaturated fatty acid, have been previously reported from the plant *Euphorbia biglandulosa* (Falsone et al. 1987).

*2D HSQC NMR-identification of **1** and **2**, and detection of **2** by LC-ESIMS analysis*

In *O. minuta* the presence of a different sphingolipid was suggested by both LC-ESIMS and NMR analysis performed on the crude extract. In particular, in the LC-MS profile, a peak at rt 45 min. ($[M-H]^-$: 529 *m/z*) was detected, with the characteristic fragment Y_0 (M-163) observed at 367 *m/z* in the corresponding MS/MS spectrum. Accordingly, the presence of the amino alcohol fragment of a sphingosine unit was evidenced by analysis of HSQC and COSY experiments [d_H/d_C: 4.03, 3.82/ 69.0 (CH_2-1); 4.32/51.7 (CH-2); 3.59/74.0 (CH-3); 3.52/71.6 (CH-4)].

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Capítulo 3.3. Resumen en castellano de la Publicación III

Estudios quimio-ecológicos en esponjas hexactinélidas del Océano Austral

LAURA NÚÑEZ-PONS, MARIANNA CARBONE, DEBORA PARIS, DOMINIQUE MELCK, PILAR RÍOS, JAVIER CRISTOBO, FRANCESCO CASTELLUCCIO, MARGHERITA GAVAGNIN y CONXITA AVILA. 2012. *Naturwissenschaften* 99(5):353-368.

Resumen

Las hexactinélidas (o esponjas de cristal) son una clase de esponjas poco estudiadas, con una organización histológica de tipo sincitial y escasas asociaciones procarióticas, en las que se supone haya una carencia en metabolitos secundarios. Sin embargo, los poríferos son increíbles fuentes de compuestos bioactivos, aunque existe la creciente sospecha de que muchos de estos productos quizás deriven de simbiontes. En latitudes polares las esponjas están densamente invadidas por diatomeas, las cuáles podrían proporcionarles productos naturales. Las hexactinélidas son típicas de aguas profundas, pero en la Antártida dominan la plataforma continental superior, y aportan cobijo y alimento a muchos invertebrados oportunistas de mediano y pequeño tamaño, que a su vez ejercen una fuerte presión ecológica hacia ellas. Con el fin de evaluar la incidencia de estrategias defensivas contra la depredación en hexactinélidas, se llevaron a cabo experimentos de alimentación usando fracciones orgánicas lipofílicas. Extractos de esponjas hexactinélidas y demosponjas antárticas se probaron frente a la estrella de mar *Odontaster validus* y el anfípodo *Cheirimedon femoratus,* como posibles consumidores omnívoros simpátricos. Las hexactinélidas revelaron ser más activas contra la depredación por parte del anfípodo, y no mostraron distribuciones de las defensas químicas aparentes dentro de su anatomía. Tras realizar una serie de análisis químicos exhaustivos, las muestras de hexactinélidas reflejaron unos perfiles químicos muy parecidos entre ellas, y no se detectó la presencia de ningún metabolito secundario típico de otras esponjas . En cambio, se purificaron los compuestos lipídicos 5α(H)-cholestan-3-one, junto con dos glicoceramidas debido a su amplia presencia en nuestras muestras. Se probaron estos compuestos aislados en los experimentos con estrellas, y su presencia fue evaluada también con fines quimiotaxonómicos en todas las muestras de esponjas antárticas, además de en hexactinélidas de otras latitudes por medio de técnicas espectroscópicas de NMR y MS. Esto nos premite proponer que algunos tipos de esfingolípidos podrían ser marcadores químicos dentro de la clase Hexactinellida, y podrían contribuir a la clasificación de este grupo de esponjas todavía sometido a debates taxonómicos.

Capítol 3.3. Resum en català de la Publicació III

Estudis quimio-ecológics en esponges hexactinèl·lides de l'Oceàn Austral

LAURA NÚÑEZ-PONS, MARIANNA CARBONE, DEBORA PARIS, DOMINIQUE MELCK, PILAR RÍOS, JAVIER CRISTOBO, FRANCESCO CASTELLUCCIO, MARGHERITA GAVAGNIN i CONXITA AVILA. 2012. *Naturwissenschaften* 99(5):353-368.

Resum

Les hexactinèl·lides (o esponges de vidre) són una classe d'esponges poc estudiades, amb una organització histològica de tipus sincitial i escasses associacions procariòtiques, en les que es suposa una carència en metabòlits secundaris. Els porífers però, són increïbles fonts de compostos bioactius, malgrat que existeix la creixent sospita de que molts d'aquests productes potser deriven de simbionts. En latituds polars les esponges estan densament envaïdes per diatomees, les quals podrien proporcionar-les productes naturals. Les hexactinèl·lides són típiques d'aigües profundes, però a l'Antàrtida dominen la plataforma continental superior, i aporten aixopluc i aliment a mots invertebrats oportunistes de mida petita i mitjana, que al seu torn exerceixen una forta pressió ecològica cap a elles. Per tal d'avaluar la incidència d'estratègies defensives contra la predació en hexactinèl·lides, es varen realitzar experiments d'alimentació utilitzant fraccions orgàniques lipofíliques. Extractes d'esponges hexactinèl·lides i demosponges antàrtiques es varen provar front a l'estrella de mar *Odontaster validus* i l'amfípode *Cheirimedon femoratus,* com a possibles consumidors omnívors simpàtrics. Les hexactinèl·lides varen revelar ser més actives contra la predació per part de l'amfípode, i no varen mostrar distribucions de les defenses químiques aparents dins de la seua anatomia. Després de realitzar una sèrie d'anàlisis químics exhaustius, les mostres d'hexactinèl·lides varen reflectir uns perfils químics molt semblants entre elles, i no es va detectar la presència de cap metabòlit secundari típic d'altres esponges. En canvi, es varen purificar els compostos lipídics 5α(H)-cholestan-3-one, conjuntament amb dos glicoceramides, degut a llur àmplia presència en les nostres mostres. Es varen provar aquests compostos aïllats als experiments amb estrelles, i la seua presència fou avaluada també amb finalitats quimiotaxonòmiques en totes les mostres d'esponges antàrtiques, a més de en hexactinèl·lides d'altres latituds per mitjà de tècniques espectroscòpiques de NMR i MS. Açò ens permet proposar que alguns tipus d'esfingolípids podrien ser marcadors químics dins de la classe Hexactinellida, i podrien contribuir a la classificació d'aquest grup d'esponges encara sotmès a debats taxonòmics.

CHAPTER 3.4. PUBLICATION IV

NÚÑEZ-PONS L, CARBONE M, VÁZQUEZ J, GAVAGNIN M and AVILA C. 2012. Chemical ecology of *Alcyonium* soft corals from Antarctica. ***Journal of Chemical Ecology*** Submitted.

CHEMICAL ECOLOGY OF *Alcyonium* SOFT CORALS FROM ANTARCTICA

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Abstract – Alcyonacean soft corals lack protection from massive carbonate skeletons. Their minute, spiny sclerites, even if sometimes regarded as deterrents, are primarily structural, while their nematocysts are considered ineffective as defense. Indeed, soft corals majorly rely on the chemistry for protection. In Antarctic ecosystems predation is especially intense and mainly driven by invertebrate consumers. The genus *Alcyonium* is here represented by 8 species, some of them quite abundant. Aiming to investigate the notably understudied chemical ecology of Antarctic *Alcyonium* soft corals, six samples belonging to five species were assessed for the presence of lipid-soluble defensive agents. Feeding bioassays were performed using diethlyl ether extracts towards the sea star *Odontaster validus* and the amphipod *Cheirimedon femoratus* as putative sympatric predators. Striking repellent activities were observed towards both consumers in all but one of the samples assessed. Soft corals have shown to exude certain chemicals, of primary and secondary metabolism origin, which participate keeping potential feeders and pathogenic epibiosis away. Actually, corals usually lack heavy fouling, even if a rich associated microbial flora lives in the mucus surface layer. Three of our samples additionally displayed inhibition against a sympatric marine bacterium. Our results suggest that lipophilic chemical defense is a first line protection strategy in Antarctic *Alcyonium* soft corals against predation and fouling. The ether extracts afforded characteristic illudalane sesquiterpenoids in two of the samples, as well as particular wax esters fractions in all the samples analyzed. Both kinds of metabolites displayed significant deterrent activities when tested, thus demonstrating their defensive role.

Key Words - Chemical defense, illudalane sesquiterpenes, wax esters, deterrent metabolites, sea star *Odontaster validus*, amphipod *Cheirimedon femoratus*.

INTRODUCTION

Corals comprise about 5100 recognized species, and inhabit over a tremendous range of latitudes and depths, some reaching amazing longevities (Hughes et al., 1992). Soft corals (order Alcyonacea) are a group of octocorals, including the families Alcyoniidae, Nephtheidae, Nidaliidae and Xeniidae. They are made up of many polyps connected by a fleshy tissue (coenenchyme), lacking calcium carbonate massif skeletons. Instead, they have an assortment of internal minute spiky sclerites that render shape and structure (Brusca and Brusca, 2003) and are useful for taxonomy (Bayer et al., 1983). Shallow species live in association with photosynthetic zooxanthellae (Muscatine and Porter, 1977; Muscatine et al., 1981), while deep species, outside photic zones, lack algal symbionts. In general, soft corals represent food, host substrata and refuge for many symbiotic organisms, including animals, bacteria, fungi and algae, sharing food inputs and allelochemicals (Humes, 1990; Slattery et al., 1998; Avila et al., 1999; Kelecom, 2002; Barneah et al., 2004; Barneah et al., 2007). Colonies are polymorphic

and exhibit defensive and reproductive activities unevenly distributed (Harvell et al., 1988; Harvell and Fenical, 1989; Van Alstyne et al., 1992; Van Alstyne et al., 1994). Moreover, intracolonial genetic diversity is quite common (Jackson and Coates, 1986; Hughes et al., 1992), generating intraspecific variations evidenced in metabolic features (Harvell et al., 1993).

Despite their flabby aspect, missing safeguarding rigid skeletons, and their nutritious nature (La Barre et al., 1986b), no predators are known to be really deleterious to soft corals. Only specialist consumers (pycnogonids and opistobranchs) readily feed on them (Sammarco and Coll, 1992; Slattery et al., 1998; Avila et al., 1999). Hence, these sessile anthozoans must somehow prevent heavy generalist consumption. Defensive strategies may include nematocyst based protection (Stachowicz and Lindquist, 2000; Bullard and Hay, 2002; Hines and Pawlik, 2012), physical-mechanical protection provided by the thorny sclerites (Harvell and Fenical, 1989; Van Alstyne et al., 1992; Van Alstyne et al., 1994), or chemical defense through secondary (or primary) deterrent metabolites (La Barre et al., 1986b; Wylie and Paul, 1989; Sammarco and Coll, 1992; Hines and Pawlik, 2012). Many pelagic cnidarians, hydrozoans and scleractinian corals use diverse penetrating nematocysts that produce proteinaceous toxins for aggression (Sammarco and Coll, 1992; Stachowicz and Lindquist, 2000; Bullard and Hay, 2002; Hines and Pawlik, 2012). By contrast, Octocorallia have a weak nematocyst system lacking stinging devices (*i.e.* mastigophores), and have a low variety (basically a single type, *i.e.* rhabdoidic heteronemes) and density of cnidos (Schmidt, 1974; Brusca and Brusca, 2003). Structural defense achieved through sclerites is still being discussed (Harvell and Fenical, 1989; Sammarco and Coll, 1992; Van Alstyne et al., 1992; Slattery and McClintock, 1995; Kelman et al., 1999; O'Neal and Pawlik, 2002). Concentration and morphology of sclerites seem to be in fact determinant in their operability as protection. Indeed, sclerites are primarily necessary for structural support, since defense can be accomplished by repellent metabolites (Van Alstyne et al., 1992; Van Alstyne et al., 1994; Kelman et al., 1999).

Alcyonacea are rich in bioactive compounds which serve several ecological roles related to predator defense, competition for space, antifouling and reproduction enhancement (La Barre et al., 1986a; Coll et al., 1987; Mackie, 1987; Pass et al., 1989; Wylie and Paul, 1989; Sammarco and Coll, 1992; Kelman et al., 1999; Wang et al., 2008). Among these, terpenoids (di- and sesquiterpenes), many of them cytotoxic, and some particular sterols, predominate (see Blunt et al., 2012 and previous reviews). However, the specific molecules responsible for the defensive activities have rarely been determined (Mackie, 1987; Wylie and Paul, 1989; Sammarco and Coll, 1992; Miyamoto et al., 1994; Slattery et al., 1997a; Slattery et al., 1998; Slattery et al., 2001; Fleury et al., 2008; Wang et al., 2008). A high proportion of soft corals are ichthyotoxic and deterrent, although both properties seem to be no correlated, neither to derive from the same allelopathic agents (La Barre et al., 1986b). Actually, distastefulness rather than toxicity is most extended against predators (Paul, 1992). Quite often deterrent and antifouling properties are due

to several metabolites, which may act in additive or synergistic mode (Wylie and Paul, 1989; Van Alstyne et al., 1994; Kelman et al., 1998; Wang et al., 2008).

Corals generally stay free from evident epibiosis and resist detrimental microbial invasion of potential pathogens, and this is principally attributed to inhibitors (Coll et al., 1987; Slattery et al., 1995; Kelman et al., 1998; Wang et al., 2008). Yet, physico-chemical properties related to coral's mucosic surface, such as mucus sloughing or adhesiveness, are involved in protection too (Ducklow and Mitchell, 1979a; Rublee et al., 1980; Vrolijk et al., 1990). The surface of all living corals is covered with a complex muco-polysaccharide lipid material with antipredatory and antifouling characteristics (Miyamoto et al., 1994; Slattery et al., 1997a; Kelman et al., 1999), that provides a matrix for bacterial colonization. The established, associated microbial community is specific and confers beneficial nutritional, defensive, and/or antibiotic attributes (Ducklow and Mitchell, 1979a; Rublee et al., 1980; Ritchie, 2006; Shnit-Orland and Kushmaro, 2009). Among some of the substances exuded within the mucus of soft corals are sterols, wax esters, terpenic toxins, and also UV-absorbing compounds (Coll et al., 1982; Miyamoto et al., 1994; Slattery et al., 1997a; Brown and Bythell, 2005; Wang et al., 2008).

Lipidic energy reserves, in the form of wax esters and triglycerides, play a key role in polar marine organisms adapted to a fluctuant plankton depauperate system (Sargent et al., 1977). Moreover, vagile species of the Antarctic benthos, including keystone predators, acquire adaptative opportunistic habits, due to the discontinuous food supply (Arnaud, 1977). In deep habitats of the Weddell Sea, anthozoans are the third dominant taxon contributing most to the tridimensional structure of the system (Arnaud, 1977; Orejas, 2001). This includes *Alcyonium* soft corals, represented here by 8 Antarctic species, some of them very abundant. These communities, however, do not exhibit a depth zonation gradient and many species are both circumantarctic and eurybathic. Ergo, relevant Antarctic predators, such as voracious sea stars (Dayton et al., 1974; McClintock, 1994) and abundant amphipods (Bregazzi, 1972; De Broyer et al., 2007), and potential prey organisms usually share shallow and deep habitats (Dayton et al., 1974; Gutt et al., 2000). *Alcyonium antarcticum* and *A. haddoni* for instance, exhibit shallow (10-30 m), as well as deep-sea distributions (>300 m) in both Antarctica and South America (Slattery and McClintock, 1995; Casas et al., 1997; Van Ofwegen et al., 2007; author's unpublished data). In shallow Antarctic waters, soft corals are avoided as a prey, and only one pycnogonid species has been observed to feed on them (Slattery and McClintock, 1995; author's personal observations).

We hypothesized that Antarctic *Alcyonium* soft corals rely on defensive metabolites to elude predation and fouling. Up to date only the investigations of Slattery and co-workers (reviewed in Slattery and McClintock, 1997) have contributed to the knowledge on the chemical ecology of Antarctic soft corals, demonstrating an extended use of chemical defenses. Lipid-soluble extracts from soft corals and gorgonians frequently possess feeding deterrent properties against

generalist consumers (Wylie and Paul, 1989; Pawlik et al., 1987). Hence, we selected the lipid-soluble fraction of our samples of Antarctic *Alcyonium* soft corals to conduct feeding bioassays with the aim of assessing the presence of chemical defenses towards two influencing Antarctic predators, the sea star *Odontaster validus* and the amphipod *Cheirimedon femoratus*. Inhibitory activity against a sympatric marine bacterium was also tested. Furthermore, the same crude extracts led to the isolation of several terpenoid compounds and particular wax ester fractions, which revealed significant feeding repellency, but not antibiotic properties.

METHODS AND MATERIALS

Sample Collection and Chemical Organic Extractions. During the ANT XXI/2 cruise (November – January 2003 – 2004) on board R/V Polarstern (AWI, Bremerhaven, Germany) Antarctic soft corals of the genus *Alcyonium* were collected in the Eastern Weddell Sea by trawling between 308 - 622 m depth. Moreover, several specimens of the *A. haddoni* were collected at 9 m depth by diving in Deception Island (South Shetland Archipelago, Antarctica) during the ACTIQUIM-1 campaign (December - January 2008 - 2009). Colonial clumps of each species from a single collection site were grouped together as a single sample for further experimentation and analysis (Table 1). Pictures of fresh animals were taken on board and a voucher portion of each sample was conserved in 10% formaline for taxonomy. Sampling material was frozen at -20ºC, and sent to the University of Barcelona until processed. Samples were later identified to species level by using literature data (Verseveldt and Van Ofwegen, 1992; Casas et al., 1997; Van Ofwegen et al., 2007).

Every sample, consisting on several colonies, was exhaustively extracted in a mortar with acetone at room temperature. After removal of the solvent *in vacuo*, the residual water was partitioned into diethyl ether (three times) and butanol (once) fractions. The organic phases of these extraction were opportunely combined and evaporated under reduced pressure. The resulting dry crude fractions were weighted, providing the extract yields per dry mass. Sample tissue concentrations, hereafter referred to as “natural concentrations”, were calculated respect to the total dry weight (DWT = DW dry weight of the extracted sample + EE ethereal fraction weight + BE butanolic fraction weight). Ether partitions were further used for bioassays and chemical analysis, while butanolic fractions and aqueous residues were kept for future investigations (Table 2).

TABLE 1 *Alcyonium* soft coral samples collected in the Southern Ocean (Antarctica). AGT: Agassiz Trawl, BT: Bottom Trawl, RD: Rauschert Dredge, SD: Scuba diving

Species name and sample	Location	Latitude	Longitude	Gear	Depth (m)
Alcyonium antarcticum Wright & Studer, 1889	Weddell Sea	70° 56' S	10° 31’ W	BT	337.2
Alcyonium grandis Casas, Ramil & van Ofwegen, 1997	Weddell Sea	72° 51.43' S	19° 38.62' W	BT	597.6
Alcyonium haddoni Wright & Studer, 1889	Deception Island	62º 59.55' S	60º 33.68' W	SD	9
Alcyonium paucilobulatum Casas, Ramil & van Ofwegen, 1997	Weddell Sea	72º 49.99' S	19º 34.99' W	RD	622
Alcyonium roseum 1 van Ofwegen, Häussermann & Försterra, 2007	Weddell Sea	71º 17.1’ S	12º 36’ W	AGT	416
Alcyonium roseum 2 van Ofwegen, Häussermann & Försterra, 2007	Weddell Sea	71º 4' S	11º 31.99' W	BT	308.8

Chemical Purifications. Diethyl ether (Et_2O) extracts were transferred to the ICB-CNR (Pozzuoli, Napoli, Italia). They were preliminary screened by Thin Layer Chromatography (TLC), using Merck Kieselgel plates (20x10 cm and 0.25 mm thick), and light petroleum ether/ diethyl ether (1:0, 8:2, 1:1, 2:8, 0:1) and chloroform/methanol (8:2) as eluents. The plates were developed with $CeSO_4$. TLC analysis of *Acyonium grandis* showed the presence of a series of spots ranging R_f's; 0.35 and 0.75 (light petroleum ether/Et2O, 8:2), according to the nine known illudalane terpenoid containing fractions (**1**-**9**) (Carbone et al., 2009). *A. roseum* 1 manifested two bands at R_f's; 0.25 and 0.35 (light petroleum ether/Et2O, 8:2), coinciding with two previously unreported minoritary illudalane products (**10**-**11**). Moreover all *Alcyonium* samples revealed evident pinkish UV-visible bands at R_f's; 0.85 – 0.9 (light petroleum ether/Et2O, 9/1), which corresponded with fractions composed of two major wax ester compounds C34:1ω and C32:1ω (**12**-**13**). All extracts were submitted to purification steps with molecular exclusion, silica gel, and reversed-phase chromatography, using silica gel Merck Kieselgel 60 (0.063-0.200mm) and (0.040-0.063 mm) equilibrated with petroleum ether and Sephadex LH-20 columns with a gradient of petroleum ether/Et2O and chloroform/methanol 1:1. ^{1}H-NMR spectroscopic analyses were used to determine pure products or mixtures. Fractions composed of a mixture of molecules were further purified with TLC preparative (SiO_2) plates Merck Kiesegel 60 F_{254} (0.50 e 1.00 mm) and HPLC (Shimadzu with LC-10ADVP pump and SPD-10AVP UV detector) using reverse-phase semipreparative columns (Supelco Discovery® C_{18}, 25 cm x 46 mm, 5µm, and 250 10 mm, Phenomenex, Kromasil C_{18}) and water/acetonitrile and methanol/water 70:30 as solvent (flux 2 ml/min).

General Chemical Experimental Procedures. The isolated pure compounds were subjected to spectral analysis with NMR, UV, as well as MS spectrometry. Optical rotations were measured on a JASCO DIP 370 digital polarimeter. The UV spectra and CD curves were recorded on an Agilent 8453 spectrophotometer and a JASCO 710 spectropolarimeter, respectively. The IR spectra were taken on a Bio-Rad FTS 155 FT-IR spectrophotometer. 1H and 13C NMR spectra were recorded on DRX 600, Avance 400, and DPX 300 MHz Bruker spectrometers in CDCl3, with chemical shifts reported in ppm referred to CHCl3 as internal standard (δ 7.26 for proton and δ 77.0 for carbon). ESIMS and HRESIMS were measured on a Micromass Q-TOF Micro spectrometer coupled with a HPLC Waters Alliance 2695. The instrument was calibrated by using a PEG mixture from 200 to 1000 MW. Silica gel chromatography was performed using precoated Merck F254 plates and Merck Kieselgel 60 powder. HPLC purification was carried out on a Shimadzu LC-10AD liquid chromatograph equipped with a UV SPD-10A wavelength detector. The spectral data of compounds isolated were compared with the data reported in the literature (Palermo et al., 2000; Carbone et al., 2009; Annex I). The fractions containing the two types of wax esters (**12**-**13**) were subjected to methanolysis reactions: they were dissolved in

anhydrous MeOH (1 mL), and an excess of Na_2CO_3 was added. The solution was stirred at room temperature for 4 h, filtered, and the solvent evaporated. The crude products were purified on a Pasteur column (light petroleum ether/Et_2O), affording pure methyl esters and fatty acids, which were analyzed by mass spectrometry to determine the chain lengths (mainly C16:0 and C14:0-alcohol moieties and C18:1 fatty acids. The composition of the wax ester fractions (**12-13**) in all the samples was evaluated by LC-MS.

TABLE 2 Data of diethyl ether (Et_2O) extract yields and of the fraction containing wax esters (**12-13**) of the studied Antarctic *Alcyonium* soft coral samples. WW: wet weight of the sample, DW: total dry weight of the sample calculated as: DW = dry residue (DR) + dry diethyl ether extract (EE) + dry butanolic extract (BE). [N_{EE}]: Natural tissue concentration in mg of the dry Et_2O extract (EE) per g of the total dry weight (DW) of the sample; [$N_{(1\text{-}9)}$], [$N_{(10\text{-}11)}$] and [$N_{(12\text{-}13)}$]: Natural tissue concentrations in mg of the illudalane fractions (**1-9**) and (**10-11**), and the dry wax esters fractions (**12-13**) per g of the total dry weight (DW) of the sample

Species and sample	WW (g)	DW (g)	EE (mg)	[N_{EE}] (mg g^{-1} DW)	[$N_{(1\text{-}9)}$] (mg g^{-1} DW)	[$N_{(10\text{-}11)}$] (mg g^{-1} DW)	[$N_{(12\text{-}13)}$] (mg g^{-1} DW)
A. antarcticum	1.01	0.51	10.15	20.10	-	-	3.12
A. grandis	18.58	4.55	544.06	119.57	27.8	-	20.58
A. haddoni	118.9	17.65	813.41	46.09	-	-	2.88
A. paucilobulatum	1.25	0.33	15.95	47.89	-	-	8.06
A. roseum 1	9.32	1.66	59.21	35.67	-	3.9	1.34
A. roseum 2	1.56	0.47	17.78	38.00	-	-	4.49

Feeding Deterrence Assays with Asteroids. Experimental sea stars belonging to the eurybathic, ubiquitous Antarctic species *Odontaster validus*, with voracious omnivorous habits and circumpolar distribution (McClintock, 1994) were captured at Port Foster Bay in Deception Island, South Shetland Archipelago (62º 59.369' S, 60º 33.424' W). Collection was done during three campaigns: ECOQUIM-2 (January 2006), ACTIQUIM-1 (December 2008-January 2009) and ACTIQUIM-2 (January 2010), by scuba diving at 3 - 17 m depth (n>1500), with sea stars' diameter ranging 7 - 10.5 cm. Several Antarctic feeding bioassays used this asteroid as a model predator previously (for review see Avila et al., 2008). The detailed methodology is described in previous papers (Avila et al., 2000; Iken et al., 2002). Briefly, the sea stars were maintained in large tanks with fresh seawater at the Spanish Base BAE "Gabriel de Castilla" (Deception Island), and starved for five days. The tests included 10 replicates each. Thus 10 containers

filled with 2.5 L of seawater accommodated one sea star each. Each individual was offered one small shrimp food item (5x5x5 mm and 13.09 ± 3.43 mg of dry mass), and treatment and control experiments were run simultaneously. Control shrimp feeding cubes (12.4% protein, 9.1% carbohydrates and 1.5% lipids, and 17.8 KJ g^{-1} dry wt and 4.1 KJ g^{-1} wet wt, by Atwater factor system; Atwater and Benedict, 1902) were treated with solvent alone (Et_2O). Treatment cubes contained natural concentrations of pre-diluted lipophilic Et_2O extracts or sub-fractions from Antarctic *Alcyonium* soft coral samples (Table 2). The solvent was removed then under flow hood. Considering sea star extraoral feeding, extruding the cardiac stomach and bolting down the whole shrimp food cubes (McClintock, 1994), dry weight is a good approximation for assessing the "defense per feeding cube". Dry weight was chosen for eliminating the water content, which may produce remarkable deviations in marine samples, especially those with soft porous tissues that capture humidity. The illudalane mixture from *A. grandis* (**1**-**9**) as well as the wax ester fractions (**12**-**13**), common to all *Alcyonium* samples studied, were also assayed at their corresponding natural concentrations. Illudalanes **10**-**11** could not be assessed because there were not enough available quantities. For the illudalanes (**1**-**9**) the concentration used was 27.8 mg g^{-1} dry weight. The fractions containing the wax esters C34:1ω (**12**) and C32:1ω (**13**), obtained from various samples, were tested at several concentrations compressed within the range found in our samples (Table 2). The concentrations used were 1, 2.5, 5, 15 and 25 mg g^{-1} dry weight. After 24 hours the number of shrimp cubes eaten for each test were recorded, and the remaining (not eaten) were conserved. TLC screenings showed the permanence of the extracts or compounds in the food cubes. Products contained in diethyl ether fractions are not hydrophilic, hence theorically diffusion to the water column is implausible, especially in the cold (≈1ºC) Antarctic sea water. Feeding repellences were statistically evaluated with Fisher's Exact tests contrasting each treatment assay with the simultaneous control (Sokal and Rohlf, 1995). After experimentation asteroids were brought back to the sea.

Feeding Preference Assays with Amphipods. Lysianassoid amphipods of the abundant, eurybathic Antarctic species *Cheirimedon femoratus* were used in our experiments according to the protocol recently described (Núñez-Pons et al., 2012). This is an amphipod with devouring, omnivore-scavenger feeding habits and a circumpolar distribution (Bregazzi, 1972; De Broyer et al., 2007). Hundreds of individuals were captured in Port Foster Bay (Deception Island, South Shetland Archipelago; 62º 59.369' S, 60º 33.424' W) with fishing nets, between 2 to 7 m depth by scuba diving. Baited traps using canned sardines were also displayed along the BAE's coastline for this purpose during the campaign ACTIQUIM-2 (January 2010). Artificial caviar-textured food pearls were prepared with 10mg/mL alginate aqueous solution along with 66.7mg/mL of concentrated feeding stimulant (Phytoplan®; 19 KJ g^{-1} dry wt). The powdered dehydrated food was mixed into the cold alginate solution with a drop of green or red food

coloring (see below), and introduced into a syringe without needle. The mixture was then added drop-wise into a solution of 0.09 M (1%) $CaCl_2$ where it polymerized forming pearls 2.5 mm Ø (3.3% protein, 1.36% carbohydrates and 1.3% lipids, and 18 KJ g^{-1} dry wt and 1.5 KJ g^{-1} wet wt by Atwater factor system; Atwater and Benedict, 1902). For extract-treated pearls, *Alcyonium* Et_2O extracts at their natural concentration were pre-dissolved in diethyl ether, and the solvent was left to evaporate onto the dehydrated food (Table 2). Control pearls were prepared with solvent alone. Wax esters fractions were tested too. However, due to their limited amount, in this case three mean values within the range of the sample natural concentrations were chosen. These were 2.5, 5 and 10 mg g^{-1} dry weight (see above; Table 2). Illudalanes **1**-**9** and **10**-**11** could not be tested in this assay because the available quantities were too small. Amphipods were maintained in 8L aquariums and were starved for 1-2 days. Every assay consisted on 15 replicate containers filled with 500-mL of sea water and 15-20 amphipods each, which were offered a simultaneous choice of 10 treatment and 10 control pellets of different colorations (20 food pearls in total), green or red easily distinguished. The colors for treatment or control pearls were randomly swapt throughout the experimentation period, and previous trials confirmed the null effect of the different colorations in feeding preferences ($P > 0.1$, n.s.). The assays ended when approximately one-half or more of either food types had been consumed, or 4 hours after food presentation. The number of consumed and not consumed pearls of each color (control or treatment) was recorded for each replicate container. Since our feeding trials were short in time, autogenic alterations were avoided and there was no need to run "controls" in the absence of grazers for changes unrelated to consumption (Peterson and Renaud, 1989). Finally, statistics were calculated to determine feeding preferences of treated pearls respect to the paired controls to consequently establish unpalatable activities. For dealing with experiments with choice each replicate is represented by a paired result: treatments and controls. Since assumption of normality and homogeneity of variances were not met, data may be compared by non-parametric procedures. Thus, through R-command software, Exact Wilcoxon tests were applied. Uneaten treatment pearls were preserved for extraction and TLC analysis, to check for possible alterations in the extracts. No major changes were observed. Once testing was over the amphipods were returned to the sea.

Antibiotic Tests towards a Sympatric Marine Bacterium. Antibiotic activities towards an unidentified sympatric marine bacterium were assessed in the Et_2O soft coral extracts as well as in the purified wax esters fractions (**12**-**13**) by agar disc-diffusion method. Unfortunately, neither of the illudalane containing fractions (**1**-**9** and **10**-**11**) were available by the time this test was performed. The bacterium was obtained from a seawater sample collected at Crater 70, Deception Island (Antarctica). A 1mL alliquot of the seawater sample was added into Difco™ marine broth 2216 (Difco Laboratories), left for 24 hr at 18-20°C, and subsequently cultured in

Difco™ marine agar 2216 (Difco Laboratories). The resulting bacterial colonies were then isolated, and the strain exhibiting the best growth was chosen for the assays. A seawater subsample in 7% glycerol filtered-sterilized seawater, and a culture of the selected bacterium strain were frozen at -20°C and shipped to the University of Barcelona for further identification, which resulted unsuccessful. Rinse broth was then inoculated with pure cultures of the selected strain and incubated at 18-20ºC until optimal growth (turbidity corresponding to Nº0,5 McFarland scale; equivalent to 10^{-8} cfu/mL). A 0.1 mL suspension of bacterial culture was evenly spread onto marine agar plates. Each Petri dish was divided into 6 regions: 3 regions for testing the extracts or wax ester fractions (**12**-**13**) in triplicate; another one for the positive control with antibiotic activity; plus two regions for the negative controls, one with and one without solvent. The positive control was chloramphenicol, while negative controls consisted of 20µL solvent alone, in this case, diethyl ether. Paper antimicrobial assay disks (BBL Microbiology Systems) Ø 6 mm soaked with the corresponding testing Et_2O extracts or wax esters fractions (**12**-**13**) previously dissolved in 20µL solvent carrier, or control disks, were placed in the middle of each testing region in the inoculated Petri dishes. Extract amounts added to the disks were equivalent to the natural concentration on dry weight bases (Table 2), or mean values for the fraction of the wax esters (see above). After incubation for 1 day at 18-20°C, inhibition halos were measured to determine antibiotic activities. When the diameter of the inhibition was larger than 7 mm Ø, it was considered active (Mahon et al., 2003).

RESULTS

Soft Coral Organic Fractions. The *Alcyonium* soft corals studied here consisted on globular, massive, pale pinkish colonies with small white polyps, except for the shallower sample of *A. haddoni* with a more bright orange coloration and yellowish polyps. Colonies shape, polyp arrangement and sclerite morphological characterization (Verseveldt and Van Ofwegen, 1992; Casas et al., 1997; Van Ofwegen et al., 2007) allowed the identification of our samples as *A. antarcticum, A. grandis*, *A. haddoni, A. paucilobulatum* and *A. roseum* (Table 1). In total 6 samples, each consisting of several colonies, yielded 6 diethyl ether extracts that were used for ecological and chemical analysis (Table 2).

Chemical Analysis of the Natural Products. In our analysis for characteristic secondary metabolites, nine known sesquiterpenoids (**1**-**9**), members of the illudalane class, and belonging to the group of the alcyopterosins, were isolated from the Et_2O lipophilic fraction of the soft coral *Alcyonium grandis* (Fig. 1). These compounds were firstly reported in 2009 as part of our chemical research in Antarctic organisms (Carbone et al., 2009; Annex I). Two new illudalanes (**10**-**11**) were also recovered in the sample *A. roseum* 1 (Fig. 2). The conspecific sample *A. roseum* 2, instead, did not show to possess any illudalane-related terpenoid product. In addition,

the extracts coming from all the *Alcyonium* samples here analyzed yielded characteristic sub-fractions composed majorly of two wax ester compounds (**12**-**13**) at variable approximate concentrations ranging 1.3 and 21 mg g^{-1} dry weight (Table 2). Both products possess a fatty acid portion consisting on a C18-monounsaturated fatty acid (C18:1ω), in which the position of the double bond was not determined, esterified with an unsaturated alcohol. Hence, the two wax esters differ only in the alcoholic chain. Compound **12** has a C16-saturated alcohol (16:0), while **13** has a C14-saturated alcohol (14:0), thus producing a C34:1ω (**12**) and a C32:1ω (**13**) wax esters respectively (Fig. 3). Due to the limited amounts of illudalanes **10**-**11**, these compounds could not be assessed in the bioassays. However, the purified fraction of illudalanes **1**-**9** was tested in the sea star assay, and several wax ester fractions (**12**-**13**) were used at various natural sample concentrations in all assays of the present study.

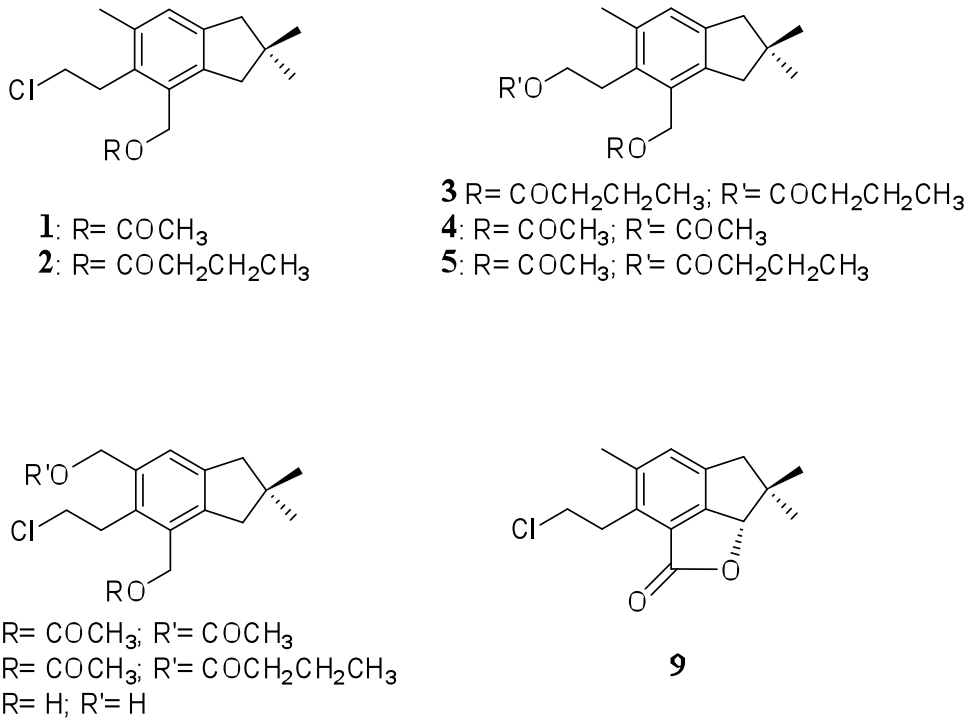


Fig. 1 Chemical structures of the nine illudalane compounds (**1**-**9**) purified from *Alcyonium grandis*

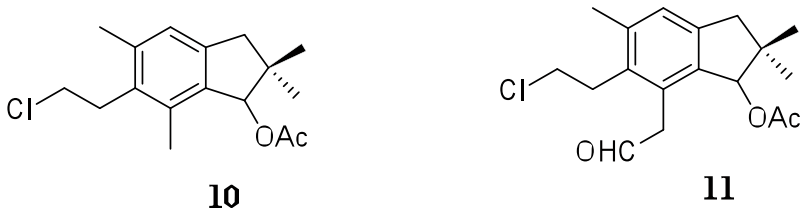
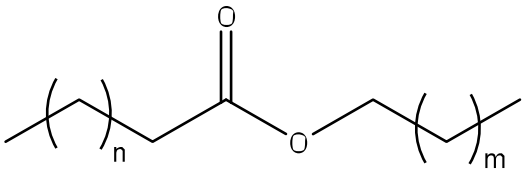


Fig. 2 Chemical structures of the two new illudalane compounds (**10**-**11**) purified from *Alcyonium roseum*

1



12: n = 15:1; m = 14
13: n = 15:1; m = 12

Fig. 3 Chemical structures of the two wax ester compounds (**12**-**13**) purified from all the *Alcyonium* soft corals of the present study

Feeding Deterrence Assays with Asteroids. The sea star *Odontaster validus* significantly rejected five out of the six soft coral lipophilic Et_2O fractions tested ($P<0.01$ in two cases and $P<0.05$ in three). This indicated that in all five *Alcyonium* species chemical defenses do exist. Nevertheless the sample *A. roseum* 2 did not cause significant ($P>0.1$) feeding repellence according to the Fisher's Exact test. Shrimp feeding control cubes impregnated with solvent alone produced an acceptance of eight to ten eaten cubes out of ten, whereas treatment food cubes provided a minimum rejection of five, except for those treated with the extract of *A. roseum* 2 (Fig. 4). The tests conducted with shrimp food cubes treated with the fraction containing the illudalane terpenoid mixture (**1**-**9**) reflected a potent deterrent activity against the asteroid ($P<0.05$), at their natural concentrations. Regarding the assays using the wax ester fractions (**12**-**13**), those performed in concentrations of 2.5, 5, 15 and 25 mg g^{-1} dry weight were significantly rejected ($P<0.05$, $P<0.05$, $P<0.01$ and $P<0.01$ respectively), while the 1 mg g^{-1} dry weight concentration did not provoke rejection ($P>0.1$). Similarly, the consumption ratio in the control tests was of eight or nine shrimp food cubes out of ten, respect to a maximum ingestion of 4 compound-treated cubes in the simultaneous treatment experiments. Only the feeding cubes containing the wax ester fraction (**12**-**13**) at the lowest testing concentration (1 mg g^{-1} dry weight) were ingested at a rate of nine cubes out of ten (Fig. 4).

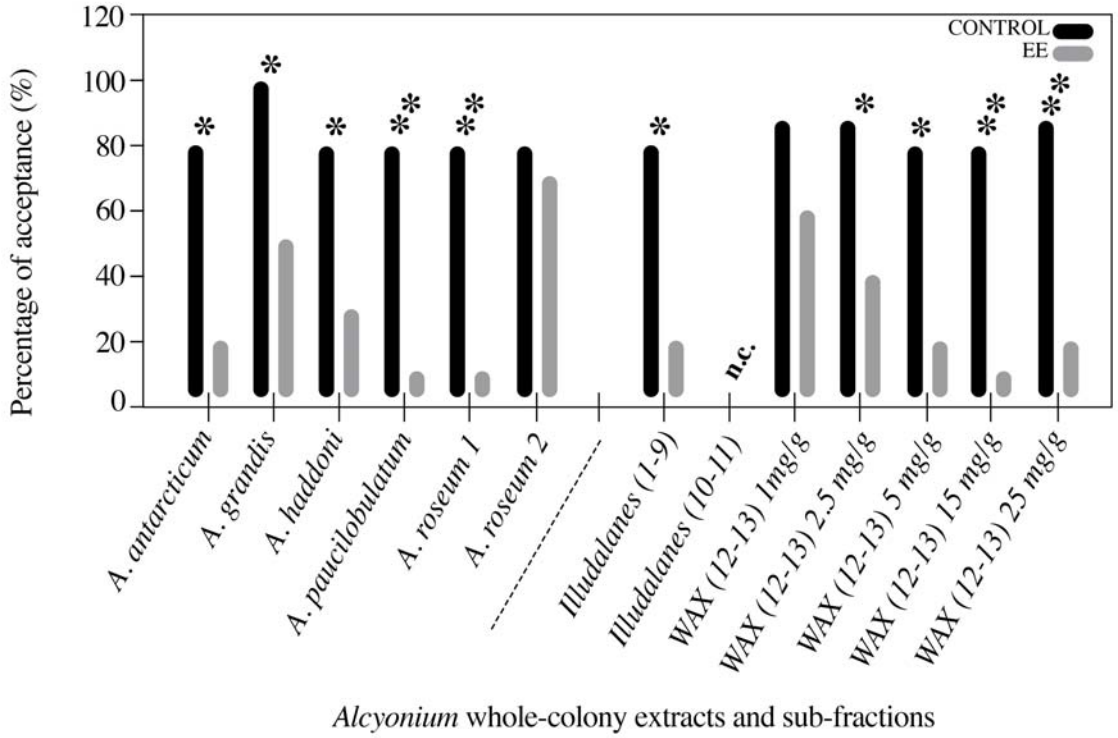


Fig. 4 Percentage of acceptance in the feeding repellence bioassays with the sea star *Odontaster validus* using whole-colony lipophilic Et_2O extracts from *Alcyonium* Antarctic soft corals, as well as sub-fractions of illudalanes (**1**-**9**) and wax esters WAX (**12**-**13**), the last ones at diverse concentrations. The paired results of control and extract treated shrimp cubes are shown for each test. *: significant differences ($p<0.05$), **: significant differences ($p<0.01$), with control as preferred food (Fisher's exact test); n.c.: test not done due to lack of enough material

Feeding Preference Assays with Amphipods. In these experiments only 3 species could be tested due to the lack of enough material to test. All three demonstrated to be remarkably unpalatable towards the amphipod *Cheirimedon femoratus* at their respective natural concentrations according to the Wilcoxon Exact test ($P<0.001$; Fig. 5). This amphipod is gregarious in its feeding habits, and was very voracious towards control food pearls. But in spite of this, repellent activities were very notable in the assays, and all extracts included in the alginate food pearls resulted in an almost nule consumption. Due to the lack of enough amounts of compounds, we could only test the wax ester fraction (**12-13)** at three mean concentrations. Food pearls containing the fraction of wax esters at 5 and 10 mg g^{-1} total dry weight were significantly rejected ($P<0.05$ and $P<0.01$ respectively) respect to the paired untreated control pearls, but concentrations of 2.5 mg g^{-1} total dry weight caused acceptance ($P>0.1$; Fig. 5).

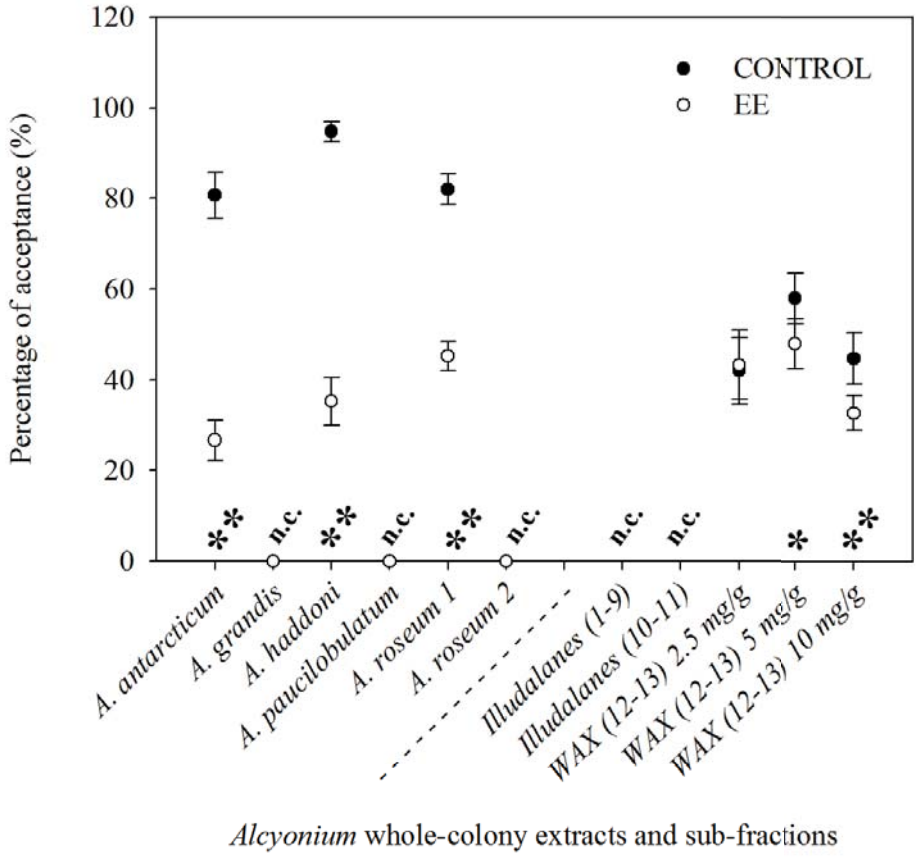


Fig. 5 Results of the feeding preference bioassays with the amphipod *Cheirimedon femoratus* conducted with whole-colony lipophilic Et_2O fractions from Antarctic *Alcyonium* soft corals, as well as with sub-fractions of wax esters WAX (**12**-**13**) at diverse concentrations. The paired results of control and extract treated food pearls are displayed for each test as the mean percentage of acceptance and standard error bars. *: significant differences ($p<0.05$), **: significant differences ($p<0.01$), with control as preferred food (Exact Wilcoxon test); n.c.: test not done due to lack of enough material

Antibiotic Tests against a Sympatric Marine Antarctic Bacterium. Unfortunately, due to conservation problems during the shipping of our samples, bacterium strains could not be identified. Only the Et_2O extracts from *Alcyonium antarcticum* and *A. paucilobulatum* caused remarkable growth inhibition on cultures of an unidentified sympatric marine bacterium (active (+ + +) in the 3 replicates, >10 mm Ø inhibition halo), as did the chloramphenicol positive controls. *A. haddoni* instead showed a mild antibiotic activity (only one active (+ - -) replicate). The other three *Alcyonium* extracts were inactive. Finally, neither of the three concentrations assayed (2.5, 5 and 10 mg g^{-1} dry weight) of the wax ester fractions (**12**-**13**) were effective inhibiting the bacterium in our antibacterial tests.

DISCUSSION

Even though soft corals are rich sources of protein, carbohydrate, and especially lipids, and are accessible (non-cryptic) prey, they thrive well in quite abundant numbers in areas with high levels of predation. Sclerites are proposed to be uneffective as deterrents against sea stars, considered Antarctic keystone predators, which predigest their prey externally (McClintock, 1994). Thus, the persistence of soft corals in many regions including the Antarctic is often attributed to the extended usage of deterrent metabolites (La Barre et al., 1986b; Wylie and Paul, 1989; Sammarco and Coll, 1992; Slattery and McClintock, 1995; Wang et al., 2008). Accordingly, the current study reports lipid-soluble deterrents towards *Odontaster validus* present in all five *Alcyonum* species tested, and in all but one of the six samples analyzed from Antarctic waters. Likewise, repellents within the same fractions were active against the amphipod *Cheirimedon femoratus* in the three species tested. Illudalanes (**1**-**9**) demonstrated to actively participate as chemical defenses against sea star predation, and wax esters (**12**-**13**) towards both types of consumers at natural whole-colony concentrations, revealing the identity of some of the involved metabolites.

Diterpenoids, sesquiterpenoids and sterols are the main compound classes accounting for ecological activities in soft corals and gorgonians. However, only extraordinarily have the specific noxious or antibiotic molecules been identified (Sammarco and Coll, 1992). Effectiveness of chemical defenses though, depends on the concentration, potency, and on the interactions among the different co-occurring metabolites, which needs further accurate investigations (Wylie and Paul, 1989; Van Alstyne et al., 1994; Kelman et al., 1998; Wang et al., 2008). In our case the illudalane terpenoids (**1**-**9**) and the wax ester compounds (**12**-**13**) seem to co-operate in predation avoidance in *A. grandis*. In *A. roseum* 1 both types of metabolites may as well collaborate in an additive way. Illudalanes (**10**-**11**) could not be assessed in the feeding assays, but due to the great resemblance with the other highly repellent illudalanes (**1**-**9**), we can expect them to also possess deterrent properties. Actually, *A. roseum* 1 containing illudalanes **10**-**11** showed significant unpalatability, whereas *A. roseum* 2 lacking these metabolites was palatable, even if both possessed wax esters. This also suggests that wax esters, in spite of being active as isolated fractions, might not be as effective in whole-colony antipredation without another co-occurring deterrents. In the rest of species studied here (*A. antarcticum*, *A. haddoni* and *A. paucilobulatum*), the synergistic effect of wax esters along with other unreported minor metabolites is likely responsible for their efficiency in predation deterrence. Deterrents are frequently described to appear in high concentrations (Paul, 1992), yet there are examples of minor components displaying antipredatory function (Fleury et al., 2008). The production of groups of metabolites that are potentially mimetic based on their similar structures could increase the concentration, and therefore the signal of the bioactive constituent (Slattery et al., 1997a). This may be exemplified in the illudalane fraction (**1**-**9**),

accounting for 27.8 mg g^{-1} dry weight.

Our *Alcyonium* corals displayed variable antibiotic activity against a marine Antarctic bacterium, with three samples showing some sort of inhibition. This could be due to different trace amounts and/or kinds of inhibitors in the different extracts. Antimicrobial activities in Antarctic and non-Antarctic soft corals are reported to affect co-occurring bacteria. However, mucoid surface-associated strains, distinct from those in the water column, are usually resistant (Ducklow and Mitchell, 1979a; Rublee et al., 1980; Slattery et al., 1995; Kelman et al., 1998; Ritchie, 2006). A few antibiotics have been isolated from soft corals, like sinulariolide, flexibilide, homarine and several steroids (Aceret et al., 1995; Slattery et al., 1997a).

As far as we know seven cnidarian species have been chemically studied up to date: *Alcyonium paessleri* (synonymized with *A. antarcticum* by Verseveldt & Ofwegen ;Verseveldt and Van Ofwegen, 1992)*, Clavularia frankliniana, Gersemia antarctica, Dasystenella acanthina, Ainigmaptilon antarcticus, Anthomastus bathyproctus* and *Alcyonium grandis* (reviewed in Avila et al., 2008; Carbone et al., 2009; Manzo et al., 2009). Of the five Antarctic soft coral species studied here, only *A. antarcticum*, had been previously investigated for its chemistry (Slattery et al., 1994; Slattery et al., 1997b; Palermo et al., 2000; Rodríguez-Brasco et al., 2001; Manzo et al., 2009) and chemical ecology (Slattery and McClintock, 1997). This species demonstrated to be ichthyotoxic, cytotoxic against sea urchin gametes (*Sterechinus neumayeri*), as well as noxious to sympatric asteroid and fish predators (Slattery et al., 1990; Slattery and McClintock, 1995; Slattery et al., 1997a). Moreover, it possesses antifouling agents against microbes and diatoms (Slattery et al., 1995), and agents able to induce tissue necrosis in the colonizer sponge *Mycale acerata*. All this suggested the existence bioactive products, working synergistically for several ecological functions. Indeed, *A. antarcticum* (before also *A. paessleri*) seems to possess an inconsistent secondary metabolite arsenal, which has probably prevented the identification of the responsible metabolites in the past (Slattery and McClintock, 1997). Actually in our study of *A. antarcticum* none of the different previously reported terpenoids were detected (Palermo et al., 2000; Rodríguez-Brasco et al., 2001; Manzo et al., 2009). This variability could respond to different reasons, among which: an interspecific variability, chemical defense induction, or symbiotic origin of certain metabolites.

The relatively common intracolonial genetic diversity described in corals is due to allogenic fusibility of colonies of distinct genotypes coalescing into coral chimeras, or by somatic mutations favored in long lived specimens (Jackson and Coates, 1986; Hughes et al., 1992). This drives to diversification in the secondary metabolism of conspecifics. Actually in the Caribbean gorgonian *Briareum asbestium* genetic differences cause different qualitative secondary metabolite profile in populations over small spatial scale, while environmental changes provoke changes at a quantitative level (Harvell et al., 1993). Variation of defensive chemicals is also described in soft corals as a response to predation episodes (Slattery et al.,

2001; Hoover et al., 2008). All these facts could also explain the different chemical profiles and bioactivities found in our *A. roseum* samples from distant locations. Illudalanes of the alcyopterosin series are a unique set of products rarelly obtained from marine sources, which have been afforded by the Antarctic deep sea soft corals *A. paessleri* (*A. antarcticum*) and *A. grandis* (Palermo et al., 2000; Carbone et al., 2009), and now here also by *A. roseum*. They are a group of compounds modestly distributed in nature, typically found in fungi and ferns (Gribble, 1996; Suzuki et al., 2005), with interesting DNA-binding, as well as cytotoxic and antispasmodic properties (Palermo et al., 2000; Finkielsztein et al., 2006). Even if seldom proved, a number of bioactive metabolites are suspected to derive from associated microorganisms, and both sesqui- and diterpenes are obtained from marine microbes (Kelecom, 2002). Hence, a symbiotic origin of illudalanes (**1**-**11**), along with other soft coral terpenoids should be considered. In fact, some bioactive terpenes have been isolated from various species and genera, and from different geographic areas (Blunt et al., 2012 and previous reviews; Wang et al., 2008). As an example, pukalide is present in several Pacific *Sinularia* species (Wylie and Paul, 1989; Van Alstyne et al., 1994; Slattery et al., 2001), and was also obtained from the Antarctic *A. antarcticum* (Manzo et al., 2009). This suggests a broad evolutionary retention of such products for the beneficial ecological properties they possess, but also a possible symbiotic origin, and consequent retention of the biotic association for the profitable bioactivities provided. This matter deserves further studies.

Secondary metabolites are usually seen as responsible for defensive activities (Paul, 1992), but also sterols, from the primary metabolism, provide antifouling and antipredation protections in *A. antarcticum* (Slattery et al., 1997a), as well as in other soft corals, sponges and sea spiders (Bobzin and Faulkner, 1992; Tomaschko, 1994; Fleury et al., 2008; Núñez-Pons et al., 2012). Under the assumption that resources are limited, trade-offs arise in organisms for the energy addressed for key physiological tasks, including growth, damage repair, reproduction and defense. There are costs associated with the production of allelochemicals (Rhoades and Gates, 1976), but this expenditure might be offset by the use of primary metabolites for ecological roles. In soft corals, wax esters are stored energy reserves, which decrease in concentrations after competitive interactions at expenses of the costs for the production of secondary metabolites (terpenoids) (Fleury et al., 2004). Hence, if wax esters would serve as defensive metabolites, as reported our results, this could allow the optimization of the available metabolic energy in the organisms by using such products also for protection.

Lipids make up to 30% of the dry matter in soft corals, while wax esters, the main storage lipids, account for >10% of the total lipid. Their composition depends on environmental conditions, food availability, symbiont composition, and others, making them difficult subjects for chemosystematics. Actually, **12**-**13** seem to be common wax esters in Antarctic anthozoans. They were similarly obtained from several Antarctic gorgonians of our collections, taking part

of more complex mixtures though (unpublished results from the authors). With the exception of corals and anemones, most marine animals rich in wax esters are pelagic. Antarctic corals are polytrophic, profiting simultaneously many food sources, including different classes of plankton and organic detritus, as well as phototrophic and heterotrophic supplies from symbionts (Orejas et al., 2001; Orejas et al., 2003). Fatty acids from nutrition, transformed into wax esters by esterification with a long chain alcohol, are indicative of external food sources (Imbs and Dautova, 2008). The two main wax esters isolated from our *Alcyonium* samples (**12**-**13**) contained the unsaturated 16:0 and 18:0 alcohols and the monosaturated 18:1 fatty acid, which are among the most abundant constituents. In fact, a typical marine wax is palmitoyl oleate, 16:0/18:1, palmitoyl alcohol (16:0) esterified to oleic acid (18:1) (Sargent et al., 1977). Usually, within the octadecanoic acid (18:1) content, there is a high oleic acid 18:1(n-9) to *cis*-vaccenic acid 18:1(n-7) ratio. High concentrations in *cis*-vaccenic acid 18:1(n-7) is an indicator of bacterial input, maybe living in the mucus or other coral tissues (Imbs et al., 2009). Actually, a rich array of microsymbionts has been described on the surface of *A. antarcticum* (Ritchie, 2006). The double bond was not localized in our fatty acid moiety, hence we cannot argue much about the origin.

Lipidic energy reserves consist more often in triglycerides than in wax esters, however these might have evolved in corals as an alternative for providing further advantages, like defense against predation. Wax esters are indigestible (Benson et al., 1978; Place, 1992), and as observed in the present study, they can confer unpalatability to the otherwise accessible and energy-rich coral tissues and mucus. Corals have large amounts of wax, and very few predators can metabolize it, which has allowed their flourishment. Only crown-of-thorns starfishes (*Acanthaster spp*) have the ability to voraciously feed on living corals because of a unique adaptation: a wax-digesting enzyme system (Benson et al., 1975). From our results, amphipods were deterred by wax fractions (**12**-**13**) at a certain concentration (5 mg g^{-1} dry weight), but they were less sensitive than asteroids, that rejected lower amounts (2.5 mg g^{-1} dry weight). This fact could be explained because Antarctic amphipods make use of wax esters as energy reserve, while sea stars do not possess such compounds (Sargent et al., 1977).

Our soft coral extracts were composed of a complex mixture of ether-soluble substances (primary and secondary metabolites), obtained from internal tissue but also mucus. Even if not specifically analyzed in this study, mucus plays a very important role in protective processes for the underlying coral tissues that must be considered. Soft coral mucus contains wax esters (about 60% of the mucolipid composition), sterols, and seldom mucus-borne terpenes, serving as a medium into which allelochemicals are exuded for defense against predation, fouling and competition (Ducklow and Mitchell, 1979b; Coll et al., 1982; Miyamoto et al., 1994; Slattery et al., 1997a; Wang et al., 2008). The bioactive illudalane terpenoids (**1**-**11**) described here, as well as the wax esters (**12**-**13**) may likely be secreted as part of the mucus in the living species here

analyzed. Wax esters, moreover, confer some impermeability (Patel et al., 2001; Brown and Bythell, 2005), thus reducing the loss of ecologically active chemicals into the surrounding water, keeping the activities of these metabolites near the coral's surface. Coral mucus secretion is variable, increasing for instance after disturbance. This modificates the relative metabolite composition, and may explain the diversity of concentrations found in the wax ester fractions (**12**-**13**). The nutritious coral mucus excretions are distasteful to most enemies (Coles and Strathma, 1973; Benson and Muscatine, 1974; Ducklow and Mitchell, 1979b; Coffroth, 1984; Miyamoto et al., 1994), and despite being energetically costly, their relevant ecological roles possibly compensate for the cost (Brown and Bythell, 2005; Slattery et al., 1995, 1997a; Kelman et al., 1998; Brown and Bythell, 2005).

A latitudinal cline with a higher diversity in octocoral secondary metabolites in the tropics than in temperate regions was proposed (Blunt et al., 2012 and previous reviews). Regarding polar waters, the research effort has been much lower, and therefore, it is not possible to make any final conclusion yet. Nonetheless, many Antarctic organisms, including cnidarians, are now yielding a notable number of new natural products, many of them with interesting bioactivities (Avila et al., 2008). We believe that the ecological success of soft corals in Antarctic communities is probably related to the presence of noxious feeding repellents and antifouling compounds, derived from both primary and secondary metabolism. As far as we know, this is one of the very few studies in which ecologically relevant metabolites have been identified in Antarctic *Alcyonium* soft corals. Additional studies are needed though, both on their biotic interactions and their defensive mechanisms.

Acknowledgements We thank F. Castelluccio, M. Rodríguez-Arias, M. Paone, S. Taboada, J. Cristobo, B. Figuerola, C. Angulo and J. Moles for their precious support and help in the lab. Thanks are due to S. Catazine for the artwork. Also we are grateful to W. Arntz and the crew of R/V Polarstern, UTM (CSIC), "Las Palmas" and BAE "Gabriel de Castilla" crews for logistic support. Funding was provided by the Ministry of Science and Innovation of Spain (CGL/2004-03356, ANT, CGL2007-65453/ANT and CGL2010-17415/ANT).

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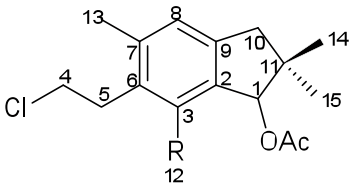
Supplementary material

NMR data for the new illudalanes **10**-**11** obtained from *Alcyonium roseum* 1



10: R= CH_3
11: R= CHO

Compound 10: ^{1}H NMR ($CDCl_3$) d_H 6.92 (1H, s, H-8), 5.96 (1H, s, H-1), 3.53 (2H, m, H_2-4), 3.12 (2H, m, H_2-5), 2.94 (1H, d, *J* = 16 Hz, H-10a), 2.54 (1H, d, *J* = 16 Hz, H-10b), 2.34 (3H, s, H_3-13), 2.23 (3H, s, H_3-12), 2.07 (3H, s, -$COCH_3$), 1.13 (3H, s, H_3-15 or H_3-14), 1.07 (3H, s, H_3-14 or H_3-15); ^{13}C NMR ($CDCl_3$) d_C 170.7 (-<u>CO</u>CH_3), 143.7 (C-2), 138.2 (C-9), 134.6 (C-6 and C-3), 133.2 (C-7), 124.8 (C-8), 83.2 (-1), 45.8 (C-10), 43.8 (C-11), 42.2 (C-4), 33.1 (C-5), 27.6 (C-14 or C-15), 22.4 (C-15 or C-14), 21.0 (-CO<u>CH</u>$_3$), 20.4 (C-13), 14.1 (C-12). HRESIMS $(M+Na)^+$ *m/z* 317.1296 (calcd for $C_{17}H_{23}O_2ClNa$, 317.1284).

Compound 11: $[a]_D$ +5.4 (*c* = 0.07, $CHCl_3$); ^{1}H NMR ($CDCl_3$) d_H 10.35 (1H, s, H-12), 7.32 (1H, s, H-8), 6.31 (1H, s, H-1), 3.65 (1H, m, H_2-4) 3.46 (2H, m, H_2-5), 2.95 (1H, d, *J* = 16 Hz, H-10a), 2.58 (1H, d, *J* = 16 Hz, H-10b), 2.42 (3H, s, H_3-13), 2.05 (3H, s, -$COCH_3$), 1.25 (3H, s, H_3-15 or H_3-14), 1.17 (3H, s, H_3-14 or H_3-15); ^{13}C NMR ($CDCl_3$) d_C 191.7 (C-12), 170.7 (-<u>CO</u>CH_3), 144.9 (C-2), 142.7 (C-9), 139.5 (C-7), 136.8 (C-6), 132.6 (C-8), 131.6 (C-3), 81.4 (C-1), 44.8 (C-10), 44.1 (C-11), 43.6 (C-4), 33.2 (C-5), 27.5 (C-14 or C-15), 22.4 (C-15 or C-14), 20.9 (C-13), 20.1 (-CO<u>CH</u>$_3$). HRESIMS $(M+Na)^+$ *m/z* 331.1073 (calcd for $C_{17}H_{21}O_3ClNa$, 331.1077).

Table 1.

Irradiated proton	significant n.O.e. observed	
	10	**11**
H-1	H_3-12; H_3-15 or H_3-14	H-12; H_3-14 or H_3-15
H-8	H_3-13	H-13
H_3-12 (in **10**); H-12 (in **11**)	H-1; H_2-4; H_2-5	H-1; H_2-5
H_3-13	H-8; H_2-4; H_2-5	H-8; H_2-4; H_2-5
-$COCH_3$	H-1	H-1

Capítulo 3.4. Resumen en castellano de la Publicación VI

Ecología química de corales blandos antárticos del género *Alcyonium*

LAURA NÚÑEZ-PONS, MARIANNA CARBONE, JENNIFER VÁZQUEZ, MARGHERITA GAVAGNIN y CONXITA AVILA. 2012. *Journal of Chemical Ecology* Submitted.

Resumen

Los corales blandos del grupo de los alcionáceos carecen de la protección proporcionada por esqueletos masivos de carbonato cálcico. Sus diminutos y espinosos escleritos, a veces considerados como repelentes, son principalmente estructurales, mientras que sus nematocistos, se consideran inefectivos como defensa. De hecho, los corales blandos recurren generalmente a la química como medio de protección. En los ecosistemas antárticos, la depredación es particularmente intensa y causada en su mayor parte por invertebrados. El género *Alcyonium* está representado en estas aguas por 8 especies, algunas de ellas muy abundantes. Con el propósito de investigar la ecología química de este género de corales tan escasamente estudiado en el Polo Sur, seis muestras pertenecientes a cinco especies diferentes fueron evaluadas para determinar la presencia de agentes defensivos liposolubles. Los experimentos de repelencia alimentaria se hicieron probando los extractos etéreos de estos corales contra la estrella de mar *Odontaster validus* y el anfípodo *Cheirimedon femoratus* como posibles depredadores simpátricos. Se observaron actividades muy marcadas contra ambos consumidores en todas las muestras excepto en una. Los corales blandos generalmente exudan sustancias derivadas tanto del metabolismo primario como del secundario, que ayudan a mantener a los depredadores alejados, así como a reducir la epibiosis por patógenos. De hecho, la superficie de los corales suele estar libre de recubrimiento evidente causado por epibiontes, aunque existe una rica microflora asociada en el mucus superficial. Tres de nuestras muestras exhibieron además actividad inhibitoria contra una cepa de bacteria marina simpátrica. Nuestros resultados sugieren que las defensas químicas lipofílicas son el principal mecanismo de protección ante la depredación y el recubrimiento en corales antárticos del género *Alcyonium*. Dos de las muestras contenían varios iludalanos sesquiterpenoides, y también se obtuvieron subfracciones características de ésteres de ceras en todas las muestras analizadas. Ambos tipos de metabolitos exhibieron repelencia, demostrando así su papel defensivo.

Capítol 3.4. Resum en català de la Publicació VI

Ecologia química de corals tous antàrtics del gènere *Alcyonium*

LAURA NÚÑEZ-PONS, MARIANNA CARBONE, JENNIFER VÁZQUEZ, MARGHERITA GAVAGNIN i CONXITA AVILA. 2012. *Journal of Chemical Ecology* Submitted.

Resum

Els coralls tous del grup dels alcionacis manquen de la protecció proporcionada per esquelets massius de carbonat càlcic. Llurs diminuts i espinosos esclerits, sovint considerats com repel·lents, són principalment estructurals, mentre que llurs nematocists, es consideren inefectius com a defensa. De fet, els coralls tous recorren generalment a la química com a mitjà de protecció. Als ecosistemes antàrtics, la predació és particularment intensa i causada en major part per invertebrats. El gènere *Alcyonium* està representat en aquestes aigües per vuit espècies, algunes d'elles molt abundants. Amb el propòsit d'investigar l'ecologia química d'aquest gènere de coralls tan escassament estudiat al Pol Sud, sis mostres pertanyents a cinc espècies diferents varen ser avaluades per determinar la presència d'agents defensius liposolubles. Els experiments de repel·lència alimentaria es varen fer provant els extractes eteris d'aquests coralls contra l'estrella de mar *Odontaster validus* i l'amfípode *Cheirimedon femoratus* com a possibles predadors simpàtrics. Es varen observar activitats molt marcades contra ambdós consumidors en totes les mostres excepte en una. Els coralls tous generalment exsuden substàncies derivades tant del metabolisme primari com del secundari, que ajuden a mantindre els predadors allunyats, així com reduint l'epibiòsi per patògens. De fet, la superfície dels coralls sol estar lliure de recobriment evident, malgrat que existeix una rica microflora associada al mucus superficial. Tres de les nostres mostres varen exhibir, a més, activitat inhibitòria contra una soca de bactèria marina simpàtrica. Els nostres resultats suggereixen que les defenses químiques lipofíliques són el principal mecanisme de protecció envers la predació i el recobriment en coralls antàrtics del gènere *Alcyonium*. Dos de les mostres contenien varis iludalans sesquiterpenoides, i també es varen obtindre subfraccions característiques d'èsters de ceres en totes les mostres analitzades. Ambdós tipus de metabòlits varen exhibir repel·lència, demostrant així llur paper defensiu.

CHAPTER 3.5. PUBLICATION V

NÚÑEZ-PONS L, FORESTIERI R, NIETO RM, VARELA M, NAPPO M, RODRÍGUEZ J, JIMÉNEZ C, CASTELLUCCIO F, CARBONE M, RAMOS-ESPLÁ A, GAVAGNIN M, and AVILA C. 2010. Chemical defenses of tunicates of the genus *Aplidium* from the Weddell Sea (Antarctica). ***Polar Biology*** 33(10):1319-1329.

Polar Biol (2010) 33:1319–1329
DOI 10.1007/s00300-010-0819-7

ORIGINAL PAPER

Chemical defenses of tunicates of the genus *Aplidium* from the Weddell Sea (Antarctica)

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Received: 27 October 2009 / Accepted: 29 April 2010 / Published online: 22 May 2010

Abstract Predation and competition are important factors structuring Antarctic benthic communities and are expected to promote the production of chemical defenses. Tunicates are subject to little predation, and this is often attributed to chemical compounds, although their defensive activity has been poorly demonstrated against sympatric predators. In fact, these animals, particularly the genus *Aplidium*, are rich sources of bioactive metabolites. In this study, we report the natural products, distribution and ecological activity of two *Aplidium* ascidian species from the Weddell Sea (Antarctica). In our investigation, organic extracts obtained from external and internal tissues of specimens of *A. falklandicum* demonstrated to contain deterrent agents that caused repellency against the Antarctic omnivorous predator, the sea star *Odontaster validus*. Chemical analysis performed with Antarctic colonial ascidians *Aplidium meridianum* and *Aplidium falklandicum* allowed the purification of a group of known bioactive indole alkaloids, meridianins A-G. These isolated compounds proved to be responsible for the deterrent activity.

Keywords Chemical defense · Antarctic tunicates · Indole alkaloids · Deterrent activity · *Aplidium* species · *Odontaster validus*

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Introduction

Antarctic benthos is characterized by stable environmental conditions and abundant faunal communities, which are considered to be structured mainly by biological factors (Gutt and Starmans 1998; Arntz et al. 2005). However, perturbations are quite common in shallow areas where ice disturbance can be an important factor (Gutt 2000). Antarctic invertebrate communities are affected by intense predation by other macroinvertebrates (such as sea stars) rather than fish, in contrast to what is common in other geographic areas (Dayton et al. 1974; Dearborn 1977; Bakus et al. 1986; McClintock et al. 1994). These circumstances may favor the evolution of chemical defenses. In fact, bioactivity detected in sessile Antarctic marine organisms has been shown to be very abundant, commensurable with temperate, and perhaps even tropical marine environments (McClintock 1989; Baker et al. 1993; Amsler et al. 2000; McClintock and Baker 2001; Avila 2006; Lebar et al. 2007; Avila et al. 2008; Peters et al. 2009). In spite of this, the

Southern Ocean remains understudied, and only 1.7% of all marine natural products reported so far come from Antarctic organisms (Marin Lit Database).

Many common benthic Ascidians (Chordata, Tunicata, Ascidiacea) lack strong structural elements, such as spicules or a tough tunic, as physical defenses against predators; however, they are relatively free from predation by generalists (Millar 1971; Goodbody and Gibson 1974; López-Legentil et al. 2006). This suggests that chemicals may be responsible for protecting them. In fact, a combination of factors including low caloric content, low digestibility and the presence of chemical defenses, such as high vanadium concentrations, low pH in tunic tissues and, especially, natural products, may be responsible for repellence against predators (Carliste 1968; Stoecker 1980b, a; Pisut and Pawlik 2002; Paul et al. 2008; Koplovitz et al. 2009). Tunicates, especially colonial species, appear to be protected against epibiosis as well, since fouling is rarely observed on them (Tatián et al. 1998; Davis et al. 2002). In general, ascidians are considered rich sources of bioactive natural products (Marchant et al. 1991; Faulkner 2000; Blunt et al. 2009), which may deter invertebrate and fish predators, as well as inhibit the growth of microorganisms (Tarjuelo et al. 2002; McClintock et al. 2004; López-Legentil et al. 2006).

Tunicates have the potential to yield novel compounds of ecological, chemical and also biomedical interest (Davis and Bremner 1999; Blunt et al. 2009; Paul et al. 2008). In particular, the cosmopolitan genus *Aplidium* is renowned for the variability of its metabolites (Zubía et al. 2005). A large variety of alkaloids have been isolated from this group (Arabshahi and Schmitz 1988; Zubía et al. 2005), such as piperidins, tetracyclic alkaloids and indoles, which display potent bioactivities (Table 1). However, even though a wide range of natural products has been isolated from tunicates, little is known about the ecological roles of most of these metabolites and their allocation within ascidian tissues (Paul et al. 1990, 2008; McClintock et al. 1991; Vervoort et al. 1998; Pisut and Pawlik 2002; Avila et al. 2008). According to the Optimal Defense Theory, defensive compounds should be located in areas that are most vital for survival and fitness (Rhoades 1979). In the case of predation by sea stars, for example, we would expect to find chemical defenses in the external body parts.

Ascidians are conspicuous members of Antarctic marine benthic communities (Gutt and Starmans 1998; Gili et al. 2000; Arntz et al. 2005, 2006). However, only seven species of Antarctic tunicates have been studied for their natural products so far (McClintock et al. 1992; Paul et al. 2008; Lebar et al. 2007; Avila et al. 2008) and fourteen species for their chemical ecology (Koplovitz et al. 2009). Among these, we emphasize the potent cytotoxic properties described for aplicyanins from *Aplidium cyaneum* (Reyes et al. 2008), meridianins from *Aplidium meridianum* (Gompel et al. 2004) and palmerolide from *Synoicum adareanum* (Diyabalanage et al. 2006), with both ecological and biomedical potential. Antarctic colonial ascidians, and more precisely those belonging to the genus *Aplidium*, are indeed a little-studied group of animals that are often observed apparently free from obvious macrofouling and predation (personal observations by the authors). For these reasons, they are expected to possess ecologically active compounds, as described for other congeners from other latitudes (Avila et al. 2008; Blunt et al. 2009).

Aplidium is a common genus among Antarctic tunicates, and it is represented by approximately 40 Antarctic and/or Subantarctic species (Varela 2007). *Aplidium falklandicum* Millar, 1960 is a common Antarctic ascidian that forms typically intense lemon-yellow colonies when alive (Tatián 1999). *A. meridianum* (Sluiter 1906) has a variable coloration, often forming gray colonies when alive (Varela 2007).

Table 1 Alkaloid compounds isolated from *Aplidium* species

Aplidium spp.	Compound	Type of Alkaloid	Geographical area	Activity	Reference
A. conicum	Conicamin	Indole alkaloid	Mediterranean	Histamine antagonist	Aiello et al. (2003)
A. cyaneum	Aplicyanins A-F	Indole alkaloid	Weddell Sea, Antarctica	Cytotoxic/antitumoral	Reyes et al. (2008)
A. haouarianum	Haouamines A, B	Alkaloid	Tarifa Island, Spain	Cytotoxic/antitumoral	Garrido et al. (2003)
A. meridianum	Meridianins A-G	Indole alkaloid	South Georgia I., Antarctica	Cytotoxic/antitumoral	Hernández Franco et al. (1998), Gompel et al. (2004), Seldes et al. (2007)
A. pantherinum	Pantherinine	Tetracyclic alkaloid	Australia	Cytotoxic	Kim et al. (1993)
A. tabascum	Lepadins F, G, H	Decahydroquinoline	Great Barrier Reef, Australia	Antiplasmodial, antitrypanosomal	Davis et al. (2002)
A. uouo	Uouamines A, B	Piperidin	Maui, Hawaii	–	McCoy and Faulkner (2001)
Aplidium sp	Aplidites A-G	Macrocyclic alkaloid	Australia	–	Murray et al. (1995)
Aplidium sp1 and *sp2*	3 compounds	Iodinated alkaloid	Australia	Cytotoxic	Carroll et al. (1993)

Both these produce short-lived lecithotrophic larvae throughout the year (Sahade et al. 2003; Tatián et al. 2005) and have a typical Antarctic-Subantarctic distribution (Ramos-Esplá et al. 2005; Primo and Vázquez 2007).

The aim of this study was to establish the presence and location of defensive natural products in Antarctic tunicates of the genus *Aplidium* collected from the Weddell Sea. This geographically remote area was totally unexplored with respect to the chemical ecology of tunicates, until recently. A first analysis of *Aplidium cyaneum* from this area revealed very interesting new metabolites: the aplicyanins (Reyes et al. 2008). Here, we report results for another two Antarctic *Aplidium* species: *A. falklandicum* and *A. meridianum*. Crude extracts were tested for ecological activity against a sympatric generalist predator, the Antarctic sea star *Odontaster validus*. The isolation of some compounds provided the opportunity to evaluate their repellent properties and their antimicrobial activity in laboratory assays against cosmopolitan bacteria and yeasts.

Methods and materials

Collection of samples

Antarctic tunicates of the species *Aplidium falklandicum* and *A. meridianum* were collected in the Eastern Weddell Sea between 280 m and 340 m depth during the ANT XXI/2 cruise of R/V Polarstern (AWI, Bremerhaven, Germany), from November 2003 to January 2004, using Bottom and Agassiz Trawls. Individuals of each species from a single collection site were grouped together as a single sample for experimental analyses (Table 2). A part of each sample was conserved, and pictures of living animals were taken on board for further taxonomical identification at the University of Alicante (Spain). The remaining material was frozen at −20°C and transported to the laboratory in Spain. Later, each sample was dissected into two parts: the tunic or external part and the internal part (visceral tissues), except for samples #4 and #5, which were separately processed as a whole. In total, therefore, eight samples, each consisting of several colonies (see Table 2), were used for chemical analysis (#1int, #1ext, #2int, #2ext, #3int, #3ext, #4 and #5). Sample #5 was processed differently in order to obtain the fraction containing all the meridianins together, for testing the deterrent activity of this mixture, without separating the different compounds.

Organic extractions

Each sample was separately extracted with acetone and sequentially partitioned into diethyl ether and butanol fractions (except for #4 which was processed with different chemical techniques as reported below). Each step was repeated three times, except for butanol which was only done once, and the solvents were then evaporated under reduced pressure, resulting in dry extracts later used for both bioassays and chemical analysis (Table 3). Sample #4 was extracted with hexane, dichloromethane and butanol and was exclusively used for analyzing its chemistry. In addition, a voucher of each sample was extracted with dichloromethane, methanol and water, and the dichloromethane fractions were further used for detailed chemical relative quantification analysis. The detailed description of the extraction procedure has been reported elsewhere (Avila et al. 2000; Iken et al. 2002). Butanolic extracts and water residues were kept for further analysis on compounds with different polarities and are not reported here.

Purifications and chemical analysis

Diethyl ether extracts were screened by thin layer chromatography (TLC), using Merck Kieselgel plates (20 × 10 cm and 0.25 mm thick), and light petroleum ether/diethyl ether (1:0, 8:2, 1:1, 2:8, 0:1) and chloroform/methanol (8:2) as eluents. The plates were developed with $CeSO_4$. A conspicuous UV–Visible band at R_f 0.63 (chloroform/methanol 9/1) with $CeSO_4$ reaction was observed in all samples. Extracts were further fractioned by molecular exclusion chromatography, using Sephadex LH-20 columns with chloroform/methanol 1:1. ^{1}H-NMR spectroscopic analyses were done to determine pure products or mixtures in the fractions obtained. Fractions composed of a mixture of molecules were further purified with HPLC techniques (Shimadzu with LC-10ADVP pump and SPD-10AVP UV

Table 2 Data of the *Aplidium* samples collected in the Weddell Sea

Species name	Sample code	Number of colonies	Latitude	Longitude	Depth (m)
A. falklandicum	1	5	70° 55.92′ S	010° 32.37′ W	288
A. falklandicum	2	2	70° 56.67′ S	010° 32.05′ W	302.4
A. falklandicum	3	14	70° 52.16′ S	010° 43.69′ W	290.8
A. meridianum	4	13	70° 57.11′ S	010° 33.52′ W	337.2
A. falklandicum	5	1	70° 56.67′ S	010° 32.37′ W	296.4

Table 3 Extracts and weights of the different samples of *Aplidium* spp

Species name	Sample code	WW (g)	DW (g)	EE (mg)	% [N]
A. falklandicum	1 int	13.5	0.6	34.1	7.97
	1 ext	12.3	1.3	74.4	5.64
A. falklandicum	2 int	61.9	0.5	64.2	12.64
	2 ext	84.6	4.0	190.4	4.81
A. falklandicum	3 int	62.8	2.0	38.8	1.97
	3 ext	192.3	5.0	119.0	2.40
A. meridianum	4	331	NA	NA	NA
A. falklandicum	5	9.38	0.4	26.1	6.53

WW wet weight of the sample, *DW* dry weight of the sample, *EE* dry weight of the diethyl ether extract; *% [N]* natural concentration of the ether extract in the sample. % [N] is calculated by dividing the dry weight of the ether extract (EE) by the dry weight of the whole sample (DW). *NA* not available

Table 4 Presence of the different meridianins in the diethyl ether extracts (EE) and dichloromethane extract (DCME) of the two analyzed species of *Aplidium*, *A. falklandicum* and *A. meridianum*

Sample code	Mer A	Mer B	Mer C	Mer D	Mer E	Mer F	Mer G
A. falklandicum							
EE 1 int	+	+	+	−	+	−	−
EE 1 ext	+	+	+	−	+	−	+
EE 2 int	+	+	+	−	+	−	+
EE 2 ext	+	+	+	−	+	+	−
EE 3 int	+	+	+	−	+	+	+
EE 3 ext	+	+	+	−	+	+	−
A. meridianum							
DCME 4	+	+	+	+	+	+	+

Mer, Meridianin; (+), present; (−), absent. Sample codes refer to the sample number, kind of extract and body part (int, internal; ext, external)

Meridianins were detected by ^{1}H NMR (600 MHz). Lowest level of detection was about 1 μM

detector) using a semipreparative column in reverse phase (Supelco Discovery® C_{18}, 25 cm × 46 mm, 5 μm) and water/acetonitrile as solvent.

Spectral analysis of the natural products

The isolated pure compounds were subjected to spectral analysis using both NMR and UV spectroscopy as well as MS spectrometry. The ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker Avance DRX-400, Bruker DRX-600 equipped with in inverse TCI CryoProbe and Bruker DRX-300 spectrometers. The ESIMS and EIMS spectra were obtained on a Micromass Q-TOF Micro™ spectrometer connected to a Waters Alliance 2695 HPLC chromatograph and on a HP-GC 5890 series II spectrometer, respectively. The UV spectra were recorded on an Agilent 8453 spectrophotometer. The spectral data of compounds (Table 4) isolated were compared with the data reported in the literature (Hernández Franco et al. 1998; Gompel et al. 2004; Seldes et al. 2007). NMR spectra of meridianins F and G were also recorded in dimethylsulfoxide (DMSO); the reported δ values are referred to the solvent peaks (2.54 ppm for proton and 40.4 ppm for carbon).

HPLC–MS relative quantification

Relative percentages of each meridianin (A-G) within the total meridianin mixture in the dichloromethane extracts from *Aplidium* samples were quantified in an Orbitrap-MS spectrometer connected to a Thermo Accela-HPLC. Liquid chromatographic separations were performed in a C18 column using a MeOH:water gradient. Meridianins C/D were jointly quantified due to their isomeric nature ($[M+H]^+$ peaks at *m/z* 289.0083), and the same was done for meridianins B/E ($[M+H]^+$ *m/z* 305.0032). In order to quantify ion-counting in the mass spectrometer, the number of ions of 50 μg of flumequine diluted in 1 mL of methanol were used as standard (Table 5).

Feeding-deterrent experiments

Individuals of the Antarctic omnivorous predator, the sea star *Odontaster validus,* were collected in the South Shetland Islands (Livingston and Deception) on board of B/O

Table 5 Relative percentages among meridianins in dichloromethane extracts from the five different collections of Antarctic *Aplidium* tunicates

Meridianins	Sample #1 (*A. falklandicum*)	Sample #2 (*A. falklandicum*)	Sample #3 (*A. falklandicum*)	Sample #4 (*A. meridianum*)	Sample #5 (*A. falklandicum*)
A	17.8	5.7	18.7	13.7	19.1
G	3.6	2.2	3.3	1.3	2.8
C/D	34.8	26.8	35.5	21.6	35.4
B/E	40.2	62.6	39.1	61.1	38.5
F	3.6	2.6	3.3	2.3	4.2

Meridianins C/D and B/E were jointly quantified in pairs due to their isomeric nature. As explained in the text, for some samples (#1, #2, #3 and probably #5) the percentage values of meridianins C/D are only attributable to meridianin C

Hespérides in January 2006 for feeding-repellence assays. They were kept alive with fresh sea water for the experiments and placed back at the sea at the same location after testing. The experiments took place at the Spanish Base "Gabriel de Castilla" in Deception Island, Antarctica. Dry diethyl ether extracts from the samples #1int, #1ext, #2int, #2ext, #3int, #3ext (*A. falklandicum)* were transported frozen from Spain to the Base "Gabriel de Castilla", where they were diluted in diethyl ether and coated into shrimp pieces, which were then presented to the sea stars. The methodology has been already explained with detail elsewhere (Avila et al. 2000; Iken et al. 2002). Each test consisted of 10 containers filled with 2.5 l of sea water with one sea star and one piece of coated shrimp per container. Shrimp coating was either extract or just the solvent in the control tests. Extracts were applied at their natural tissue concentrations in the assays (Table 3). Dry weight was selected for calculating natural concentrations according to sea star extraoral feeding habits. The extract or the solvent were totally impregnated into the shrimp cube in the coating process, since the size of the cubes was sufficiently small ($5 \times 5 \times 5$ mm) and their dry mass was 13.09 ± 3.43 mg. Solvent was evaporated under flow hood before starting the test. Feeding repellence for the shrimp coatings was evaluated after 24 h exposure, by counting the number of shrimp eaten for each test (Avila et al. 2000; Iken et al. 2002). The remaining shrimp pieces (not eaten) were frozen and later extracted and checked on a TLC, for ensuring the presence of the extracts or compounds on the shrimp after 24 h, which was always the case. Statistical analyses were carried out for each experiment respect to the control run simultaneously using Fisher's exact tests (Sokal and Rohlf 1995).

In a further Antarctic expedition at the Spanish base "Gabriel de Castilla" during the austral summer of 2008–2009, several mixtures of the isolated meridianins were assayed at their natural concentrations in palatability tests following the procedure previously explained and using methanol and diethyl ether as solvents. The meridianin mixtures selected were those abundant enough to do the tests at natural concentrations, and these were samples: #1int, #2int, #2ext and #5 (Table 6). This time the specimens of *O. validus* for testing were collected by scuba diving down to 15 m depth at Whalers Bay (Deception Island) on December 2008. The sea stars were treated as reported above for previous assays.

Antibacterial and antifungal tests

These assays were intended to assess general antibiotic properties of the isolated compounds. Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterial colonies and yeasts (*Candida albicans*) were cultured in LB medium (Luria–Bertani broth) for one night under agitation at 37°C. Cultures were then diluted at 1:1000 volume in LB medium (=10^8 cfu/ml), and 1 ml of each solution was mixed homogeneously with agar and placed onto Petri dishes. Each Petri dish was divided into $n + 1$ regions, being "n" the number of substances to be tested, corresponding to the 6 meridianins tested, plus one region for the positive control with antibiotic activity and one for the negative control. Positive controls were chloramphenicol (10 µg) for Gram-positive and Gram-negative bacteria, and fluconazol (20 µg) for yeasts, while negative controls consisted of solvent only, in this case, methanol. Paper disks (Ø 5/6 mm) soaked with 20 µl (equivalent to 100 µg) of the corresponding testing pure products (meridianins A, B, C, E, F, G) previously dissolved in methanol at 5 mg/ml, or control disks, were placed in the middle of each testing region in the Petri dishes. After 18–24 h at 37°C, inhibition halos were measured to determine antibiotic activity. When the diameter of the inhibition zones was larger than 11 mm Ø, it was considered active.

Table 6 Data and weights of different samples and fractions of *Aplidium falklandicum* and their meridianin mixtures tested in repellency assays

Species name	Sample code	Meridianin Mix	WMer (mg)	% [N]
A. falklandicum	1 int	A, B, C, E	6.3	0.97
A. falklandicum	2 int	A, C, E, G	0.4	0.073
	2 int	A, B	0.8	0.146
A. falklandicum	2 ext	A, C, E, F	22.03	0.545
	2 ext	A, B, C	9.42	0.233
	2 ext	B, C	4.95	0.122
A. falklandicum	5	A-G	9.7	2.425

Meridianin mix, meridianins contained in the mixed fraction; WMer, dry weight of the meridianin mix; % [N], natural concentration of the meridianin mixture in the sample. % [N] is calculated by dividing the dry weight of the ether extract (EE) by the dry weight of the whole sample (DW); EE and DW for each sample are already provided in Table 3. For sample #5, the precise meridianins present in the mixture (A-G) are not known

Results

Morphological characterization of colonies, zooid individuals and larvae allowed the identification of samples #1, #2, #3 and #5 as *A. falklandicum* and sample #4 as *A. meridianum*.

A total of seven aromatic alkaloids, meridianins A-G (Fig. 1), which had been previously reported only in *A. meridianum* from the South Atlantic Ocean (Hernández Franco et al. 1998; Seldes et al. 2007), were isolated from distinct individuals of these two Antarctic species. All

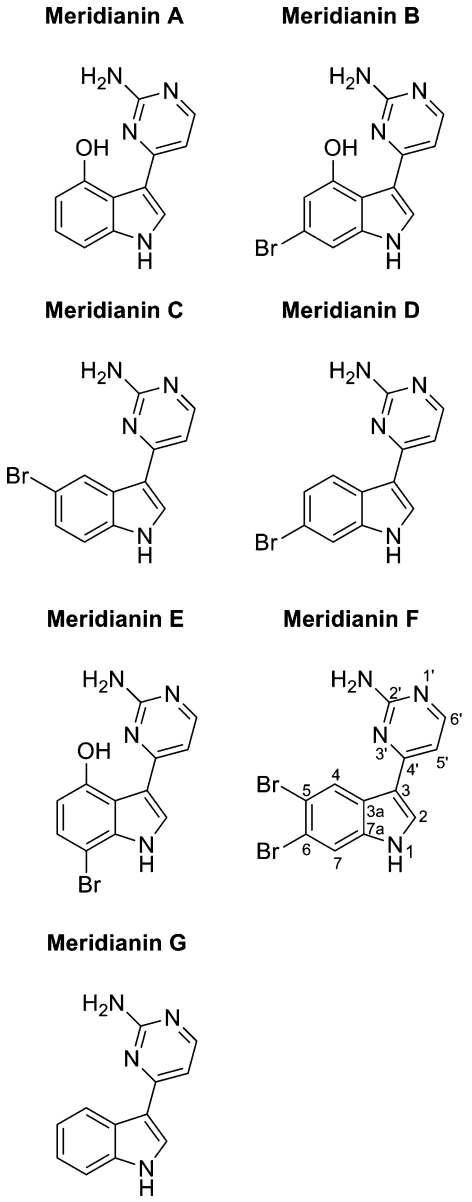

Fig. 1 Chemical structures of the seven indole alkaloids, meridianins A-G, found in one or both of the studied species, *A. falklandicum* and *A. meridianum*

compounds were identified by comparison with their spectral data (^{1}H- and ^{13}C-NMR, UV and MS) with the literature (Hernández Franco et al. 1998; Gompel et al. 2004; Seldes et al. 2007). NMR spectra of meridianins F and G that had been described in methanol (Seldes et al. 2007) were also recorded here in dimethylsulfoxide-d_6 (DMSO-d_6), the same solvent used for the other meridianins (Hernández Franco et al. 1998). All carbon and proton values of meridianins F and G were assigned as reported below.

Meridianin F: ^{1}H-NMR (600 MHz, DMSO-d_6) δ 11.93 (1H, br s, H-1), 8.95 (1H, s, H-4), 8.30 (1H, br s, H-2), 8.12 (1H, d, J = 5.3 Hz, H-6′), 7.83 (1H, s, H-7), 7.00 (1H, d, J = 5.3 Hz, H-5′), 6.53 (s, -NH_2); ^{13}C-NMR (300 MHz, DMSO-D_6) δ 163.4 (s, C-2′), 161.7 (s, C-4′), 157.2 (d, C-6′), 136.7 (s, C-7a), 130.4 (d, C-2), 126.4 (d, C-4), 126.1 (s, C-3a), 116.4 (d, C-7), 116.0 (s, C-5), 114.6 (s, C-6), 113.1 (s, C-3), 105.1 (d, C-5′).

Meridianin G: ^{1}H-NMR (600 MHz, DMSO-d_6) δ 11.93 (1H, br s, H-1), 8.56 (1H, d, J = 7.8 Hz, H-4), 8.17 (1H, d, J = 2.4 Hz, H-2), 8.08 (1H, d, J = 5.3 Hz, H-6′), 7.42 (1H, d, J = 7.9 Hz, H-7), 7.16 (1H, t, J = 6.8 Hz, H-5), 7.10 (1H, t, J = 6.8 Hz, H-6), 7.00 (1H, d, J = 5.3 Hz, H-5′), 6.38 (s, -NH_2); ^{13}C-NMR (300 MHz, DMSO) δ 157.0 (d, C-6′), 137.0 (s, C-3a), 128.2 (d, C-2), 125.2 (s, C-7a), 122.4 (d, C-5), 121.9 (d, C-4), 120.2 (d, C-6), 113.2 (s, C-3), 111.8 (d, C-7), 105.3 (d, C-5′).

Meridianins are structurally characterized by the presence of an indolic nucleus connected to an amino-pyrimidinic moiety through a C-3/C-4′ linkage. With the exception of meridianin A and meridianin G, all meridianins contain one or two bromine atoms in their structure (Fig. 1). Meridianins were detected in the diethyl ether fractions of both the internal and the external parts of *A. falklandicum* (samples #1int, #1ext, #2int, #2ext, #3int, #3ext and #5) and in the dichloromethane extract of specimens of *A. meridianum* (sample #4) (Table 4). The distribution of the diverse meridianins in the samples analyzed was quite homogeneous, especially for meridianins A, B, C and E, which were present in the liposoluble fractions of all samples (Table 4). Meridianin D was detected only in *A. meridianum* (sample #4), while meridianins F and G were found in some samples but not in others (Table 4).

Relative quantification of the secondary metabolites by means of HPLC–MS using an internal standard revealed that meridianins B/E were the major joint compounds, followed by meridianins C/D and meridianin A. As for meridianins F and G, similar values of much smaller range were detected in all samples (Table 5). Meridianins B/E were detected in significantly major quantities in *Aplidium meridianum* (sample #4), and A and C/D presented similarly higher percentages than in other samples.

All the ether extracts from the tunic (external) and the internal parts of the three tested samples (#1, #2, #3) of *A. falklandicum* caused significant (P = 0.01) feeding repellence against the sea star *O. validus* at natural concentrations according to the Fisher's exact test. Control assays conducted using only the solvent, diethyl ether, as shrimp coating, showed a minimum consumption of six pieces of shrimp out of ten. Pieces of shrimp coated with diethyl ether fractions at natural concentrations from the samples tested were never consumed by the sea stars (Fig. 2). Regarding the tests conducted with meridianin mixtures (Table 6), control tests (with methanol or diethyl ether only) were eaten at a ratio of 8 pieces out of ten, while experiments treated with meridianin mixture coatings at natural concentrations showed significant ($P < 0.05$*)

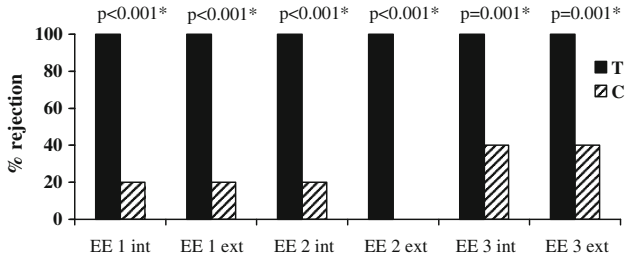

Fig. 2 Results of the repellence experiments with shrimp pieces coated with ether extracts of samples of *A. falklandicum*, using the sea star *Odontaster validus* as a predator. Tests were done using natural concentrations. Controls consisted of coating only the solvent (diethyl ether). Each test was compared to the control run simultaneously. *P**: significant differences from the controls according to Fisher's exact test; T, treatment; C, control

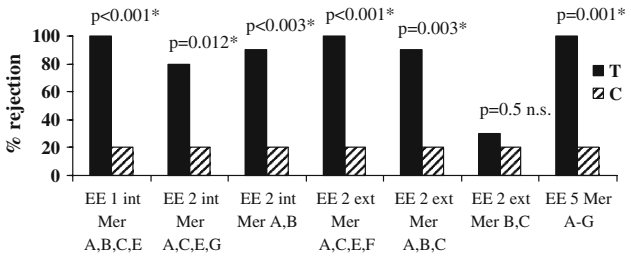

Fig. 3 Results of the repellence experiments with shrimp pieces coated with fractions of meridianin mixtures from samples of *A. falklandicum*, using the sea star *Odontaster validus* as a predator. Tests were done using natural concentrations. Controls consisted of coating only the solvent (diethyl ether). Each test was compared to the control run simultaneously. *P**: significant differences from the controls according to Fisher's exact test; n.s. not significant; T, treatment; C, control. For sample #5, the precise meridianins present in the mixture (A-G) are not known

deterrency against the sea star, with a maximum consumption of 2 out of ten pieces of coated shrimp (except for fraction #2ext mer B, C) (Fig. 3).

None of the isolated meridianins from *Aplidium* species caused growth inhibition on cultures of cosmopolitan yeasts and Gram-negative and Gram-positive bacteria (with the exception of meridianin D which was not tested because there was not enough material). The same was observed in the solvent control. Positive controls (chloramphenicol and fluconazol) significantly inhibited Gram-positive and Gram-negative bacteria and yeasts, respectively. Therefore, no antifungal or antibacterial activity has been detected in meridianins A, B, C, E, F and G in our laboratory assays.

Discussion

Meridianins from Antarctic colonial ascidians of the genus *Aplidium* were shown to provide protection from predation by a sympatric macroinvertebrate. The common Antarctic sea star, *O. validus*, is a voracious omnivorous predator that feeds on a wide range of prey, even on conspecifics (Dearborn 1977; McClintock 1994). Previous trials and experiments, as well as controls in the present study, show how specimens of this sea star avidly consume pieces of shrimp in laboratory assays (Avila et al. 2000; Iken et al. 2002). However, when individuals of *O. validus* are presented with pieces of shrimp treated with coatings of ether extracts from inner or outer parts of specimens of *A. falklandicum* at natural concentrations, no consumption was detected (Fig. 2). In some cases, the sea stars were even observed to move quickly away from the shrimp piece (personal observations). Natural concentrations were calculated according to the total dry mass, since sea stars usually extrude their stomach out against their prey. Sea stars pre-digest their food externally by enzymatic processes, rather that biting and performing internal digestion, as is usual in other predators such as fish. Therefore, one or more deterrent compounds must be present in the lipophilic fractions from *A. falklandicum*, causing the unpalatability to the coated shrimp pieces, and rejection from the sea stars. The meridianins (Fig. 1) isolated from diethyl ether extracts of *A. flaklandicum* (Table 6) were shown to be the agents responsible for the repellent activity, since shrimp pieces coated with several meridianin mixtures proved to be significantly unpalatable to the sea stars (Fig. 3). This deterrent property cannot be attributed to a specific meridianin but to the mixture of these alkaloids. The only fraction not active in the assays was fraction #2ext mer B, C (Fig. 3), and this could be due to a problem during the coating procedure resulting in not enough extract being coated onto the shrimp pieces in that particular experiment.

The total meridianin mixture represents an important proportion within the total dry mass of the lipophilic fraction of the animal, e.g., 37.2% taking as an example sample #5 (Table 6). Considering the fact that these molecules are secondary metabolites, they must play an important role for the tunicate's integrity to appear in such high concentrations. Even though scarce, there are a few studies on Antarctic tunicates containing anti-predatory defenses in the literature: the colonial ascidian *Distaplia cylindrica* (McClintock et al. 2004) and the solitary ascidian *Cnemidocarpa verrucosa* (McClintock et al. 1991; McClintock and Baker 1997), as well as a recent study of fourteen species of tunicates (Koplovitz et al. 2009), although the repellence has not been traced to any particular metabolites. In this last study, however, Koplovitz et al. (2009) did not detect activity in extracts of an unidentified *Aplidium* sp. near Palmer Station (Antarctic Peninsula). In other geographical areas, however, some compounds have been described as providing ascidians with antipredator chemical defense (Paul 1992; Blunt et al. 2009). For instance, tambjamines (Paul et al. 1990), 15′ didemnin B and nordidemnin B, and patellamide C (Paul 1992) have icthyodeterrent

properties. Also, tambjamines and ecteinascidin alkaloids, extracted from two different tunicates, confer chemical protection to their larvae (Young and Bingham 1987). Furthermore, defensive mechanisms often act at different levels, such as fouling avoidance or space competition (Stoecker 1980a; Becerro et al. 1997; Davis and Bremner 1999; López-Legentil et al. 2006). Similarly, the alkaloid eudistomins isolated from a colonial tunicate (*Eudistoma olivaceum*) exhibit potent antiviral, antimicrobial and antifouling properties (Davis et al. 2002). Our tests with meridianins, however, did not show apparent antimicrobial activity against cosmopolitan bacteria or yeasts in laboratory assays. Further experiments using sympatric marine bacteria or fouling organisms should be conducted in order to evaluate other possible defensive activities with ecological relevance for these compounds.

It was suggested that tunicates use both physical (spicules, tunic toughness) (Lambert and Lambert 1987) and chemical (natural products, acidity, heavy metals, vanadium) strategies to defend themselves (Stoecker 1980b, a; Parry 1984; Pawlik 1993; Davis et al. 2002; Tarjuelo et al. 2002; López-Legentil et al. 2006; Koplovitz et al. 2009). Nonetheless, assays performed using silicious (Pawlik et al. 1995; Chanas and Pawlik 1995, 1996) or calcareous spicules and sclerites (Lindquist and Hay 1996; Pawlik et al. 1995; Puglisi et al. 2002) failed to demonstrate rejection by fish, suggesting that natural products are the primary means of defense against predators, even if occasionally combined with other defensive systems (Stoecker 1980a; Pisut and Pawlik 2002). According to the Theory of Optimal Defense (Rhoades 1979), an ascidian would be expected to store organic or inorganic chemical defenses in body regions that maximize fitness, considering its potential predators' habits. In the case of Antarctic ecosystems with sea stars as frequent predators, protection would be most useful in outer regions. Chemical defenses are thus expected to accumulate in the tunic for adult protection. However, if defending larval stages leads to a higher survival of the species, then internal body tissues, such as the gonads, are likely to be protected as well (Rhoades 1979; Young and Bingham 1987; Lindquist et al. 1992; Lindquist and Hay 1996; Pisut and Pawlik 2002). Tunics are commonly less attractive to predators since they have very little nutritive value, whereas visceral mass and gonads contain the bulk of usable protein and lipid (McClintock et al. 1991). This could explain why a number of ascidians have undefended tunics (Pisut and Pawlik 2002). Defenses stored in the gonads would not seem to protect solitary adult ascidians, since a predator would need to open the tunic to encounter such localized defenses, resulting in the death of the tunicate. In contrast, for clonal ascidians, a predator could attack and kill a single (or a few) zooid(s) and then be deterred from further feeding without killing the whole colony (Stoecker 1980b). For this reason, allocation of defensive metabolites may not be as necessary in colonial ascidians as it is in solitary ascidians. From our study, we conclude that the colonial tunicate *A. falklandicum* contains chemical defenses which are not concentrated in specific tissues. Meridianins are distributed throughout the inner parts, as well as in the tunic, and provide protection from sympatric predators, such as the sea star *O. validus* (Tables 4; Figs. 2, 3). Isolated meridianins from both *A. falklandicum* and *A. meridianum* were shown to repel the sea star. This is the first example of characterized secondary metabolites from Antarctic tunicates with ecological activity.

Ascidians produce many nitrogen-containing metabolites, almost all derived from amino acids (Davidson 1993). In our study, meridianins were found in *A. falklandicum* and *A. meridianum*. The presence of meridianins in *A. falklandicum* is reported here for the first time. Previously, meridianins had been reported in *A. meridianum* from South Georgia Islands (Hernández Franco et al. 1998; Gompel et al. 2004; Seldes et al. 2007). To date, a total of seven of these metabolites have been described: meridanins A, B, C, D, E, F and G, and they frequently appear together as a mixture of indolic alkaloids. Meridianins F and G are two of the least common compounds in the mixture (Hernández Franco et al. 1998). The carbon and proton values of meridianins F and G in DMSO were assigned in our study for the first time. These molecules are composed of a brominated and/or hydroxylated indole nucleus with a 2-aminopyrimidine substituent at C3.

Meridianin composition in external and internal lipophilic extracts of *A. falklandicum* varied slightly among samples and compared to *A. meridianum*. Specimens of *A. meridianum* (sample #4) contained all seven meridianins (A-G). This is not surprising, since this was the organism from which these metabolites were originally described. All the samples analyzed, of both species, contained meridianins A, B, C and E. Meridianin D was only found in *A. meridianum* and meridianins F and G had a peculiar distribution, appearing in some samples but not in others (Table 4). Differences in meridianin composition could be due to interspecific variability, and thus, the different species might have characteristic meridianin profiles. Also the variability could have a geographic component, since animals living in separate habitats, under different ecological and/or environmental conditions, may produce different compounds. Another hypothesis is the presence of diverse symbiotic organisms in the samples that produce different metabolites. However, a more plausible explanation for the absence of meridianins F and G in some of the samples is the small amounts in which they appear; this makes detection difficult. This is probably the cause of their apparent absence in some extracts analyzed in the past (Seldes et al.

2007). However, the absence of meridianin D in *A. falklandicum* samples must have a different explanation, since it is not considered a minor metabolite in the indolic mixture. Meridianin D could be a characteristic metabolite of the species *A. meridianum*, although more data are needed to support this hypothesis.

From the relative chemical quantification, we can confirm that B/E are the most common meridianins followed by C/D and then by A. Meridianins F and G are clearly minor compounds in the mixture and appear at constant ratios in all samples. Similar ratios are also observed for meridianins C/D, except for a slight increase in sample #4 with respect to the rest. Meridianins B/E and A show more variable relative percentages (Table 5). Meridianins B/E and C/D constitute two isomeric couples which appear jointly in the chromatographic peaks, and so the contribution of each isomer to the ratio of the isomeric pair is impossible to calculate using this method. Nonetheless, for samples #1, #2 and #3 (*A. falklandicum*), the relative ratio recorded for the couple C/D is all due to meridianin C, since meridianin D was never detected. For sample #5, although it also corresponds to *A. falklandicum*, we cannot draw any conclusion, since we did not demonstrate the absence of meridianin D. The increased ratio of the couple C/D detected in *A. meridianum* (sample #4) could be due to meridianin D.

It is also noteworthy that the species studied here are currently subject to intensive taxonomic studies, due to their high intraspecific variability, which is very common in colonial ascidians (Tatián 1999; Varela 2007). In fact, it has been suggested that *A. meridianum* and *A. falklandicum* might be synonymous species and could be considered as two morphotypes of the same species. However, more detailed morphogenetic studies are needed to confirm this (Varela 2007). In that case, *A. meridianum* could be a morphotype containing meridianin D while *A. falklandicum* lacks it. Finding meridianins in both species is also remarkable for this reason; however, these metabolites have also been reported in collections of the related tunicate *Synoicum* sp. from Palmer Station, Antarctica (Lebar et al. 2007; Ankisetty and Baker, unpublished). Further studies are needed to explain this variability.

Indole alkaloids, frequently isolated from tunicates and sponges, are important potential antitumoral natural products (Davidson 1993). Some of them display interesting ecological defensive activities, as demonstrated here for the meridianins. Moreover, meridianins have been reported to exhibit protein kinase inhibitory properties, as well as a moderate cytotoxicity toward human tumor cell lines. Meridianins B and E are the most potent inhibitors (Hernández Franco et al. 1998; Gompel et al. 2004; Seldes et al. 2007). The interesting bioactivities found in meridianins make these compounds a promising scaffold for pharmacological anticancer research (Gompel et al. 2004). The biological activity of organic products extracted from marine organisms has generated considerable pharmacological interest, but the ecological roles of most of these metabolites remain unclear and much experimental research is still needed (Fenical 2007; Avila et al. 2008; Taboada et al. 2010). To date, more than 18,000 compounds have been reported from marine sources; however, only about 300 marine natural products originate from organisms collected in Antarctic habitats (MarineLit Database; Munro and Blunt 2009; Lebar et al. 2007; Avila et al. 2008). Thus, cold-water marine habitats represent a source of natural products that has yet to be fully explored.

Acknowledgments We wish to thank W. Arntz and the R/V Polarstern crew for their help and support during the ANT XXI/2 cruise, as well as the BIO Hespérides and the BAE "Gabriel de Castilla" teams during the ECOQUIM cruise. Funding was provided by the Ministry of Science and Education of Spain through the ECOQUIM Projects (REN2003-00545, REN2002-12006E ANT and CGL2004-03356/ANT). Also thanks are due to S. Taboada for his laboratory support, as well as in the field work. We are thankful to J. Vázquez, B. Figuerola and D. Melck for helping in the laboratory and in the preparation of the experiments and to F. J. Cristobo, J. L. Moya and M. Ballesteros and the Bentart team for their help in collecting the sea stars in Deception Island during the ECOQUIM 2006 cruise. Thanks are also due to "Servizo de Apoio a Investigación (SAI-UDC)" for instrumental support. L. Núñez-Pons was consecutively supported by PharmaMar S.A., an I3P (CSIC) grant and a FPU Fellowship from the Ministry of Education (MEC) during this study. Finally, we wish to thank the reviewers for their helpful comments and the Serveis Lingüístics of the UB for reviewing our English.

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Capítulo 3.5. Resumen en castellano de la Publicación V

Defensas químicas en tunicados del género *Aplidium* del Mar de Weddell (Antártida)

LAURA NÚÑEZ-PONS, ROBERTO FORESTIERI, ROSA Mª NIETO, Mª MERCEDES VARELA, MICHELA NAPPO, JAIME RODRÍGUEZ, CARLOS JIMÉNEZ, FRANCESCO CASTELLUCCIO, MARIANNA CARBONE, ALFONSO RAMOS-ESPLÁ, MARGHERITA GAVAGNIN, y CONXITA AVILA. 2012. *Polar Biology* 33(10):1319-1329.

Resumen

La depredación y la competencia son factores relevantes en la estructuración de las comunidades bentónicas antárticas, y por ello promueven la producción de defensas químicas. Los tunicados están sujetos a relativamente poca depredación, y esto se atribuye frecuentemente a compuestos químicos. A pesar de ello, su defensa química contra depredadores simpátricos se ha demostrado en contadas ocasiones. De hecho estos animales, en particular aquellos pertenecientes al género *Aplidium*, son prolíferas fuentes de metabolitos bioactivos. En el presente estudio, describimos los productos naturales, su distribución y la actividad ecológica de dos especies de ascidias del género *Aplidium* del Mar de Weddell (Antártida). En nuestra investigación, los extractos orgánicos obtenidos a partir de los tejidos externos e internos de especímenes de las especies *A. falklandicum* demostraron contener agentes repelentes que causaron rechazo hacia la estrella omnívora y voraz depredadora antártica *Odontaster validus*. Los análisis químicos realizados con ascidias coloniales antárticas de las especies *Aplidium meridianum* y *Aplidium falklandicum* permitieron purificar un grupo de alkaloides indólicos bioactivos ya conocidos, las meridianinas A-G. Estos compuestos aislados revelaron ser responsables de la actividad defensiva repelente.

Capítol 3.5. Resum en català de la Publicació V

Defenses químiques en tunicats del gènere *Aplidium* del Mar de Weddell (Antàrtida)

LAURA NÚÑEZ-PONS, ROBERTO FORESTIERI, ROSA Mª NIETO, Mª MERCEDES VARELA, MICHELA NAPPO, JAIME RODRÍGUEZ, CARLOS JIMÉNEZ, FRANCESCO CASTELLUCCIO, MARIANNA CARBONE, ALFONSO RAMOS-ESPLÁ, MARGHERITA GAVAGNIN, i CONXITA AVILA. 2012. *Polar Biology* 33(10):1319-1329.

Resum

La predació i la competència són factors rellevants en l'estructuració de les comunitats bentòniques antàrtiques, i per aquest motiu promouen la producció de defenses químiques. Els tunicats estan subjectes a relativament poca predació, fet que sol atribuir-se a compostos químics. Malgrat això, la seua defensa química contra predadors simpàtrics s'ha demostrat en comptades ocasions. De fet aquests animals, en particular aquells pertanyents al gènere *Aplidium*, són prolíferes fonts de metabòlits bioactius. En el present estudi, descrivim els productes naturals, llur distribució i l'activitat ecològica de dues espècies d'ascídies del gènere *Aplidium* del Mar de Weddell (Antàrtida). En la nostra recerca, els extractes orgànics obtinguts a partir dels teixits externs i interns d'espècimens de les espècies *A. falklandicum* varen demostrar contindre agents repel·lents que varen causar rebuig envers l'estrella omnívora i voraç depredadora antàrtica *Odontaster validus*. Les anàlisis químiques realitzades amb ascídies colonials antàrtiques de les espècies *Aplidium meridianum* i *Aplidium falklandicum* varen permetre purificar un grup d'alcaloides indòlics bioactius ja coneguts, les meridianines A-G. Aquests composts aïllats varen demostrar ser responsables de l'activitat defensiva repel·lent.

CHAPTER 3.6. PUBLICATION VI

NÚÑEZ-PONS L, CARBONE M, VÁZQUEZ J, RODRÍGUEZ J, NIETO RM, VARELA M, GAVAGNIN M and AVILA C. 2012. Natural products from Antarctic colonial ascidians of the genera *Aplidium* and *Synoicum*: variability and defensive role. ***Marine Drugs*** Submitted.

Mar. Drugs **2012**, *10*, 1-x manuscripts; doi:10.3390/md100x000x

OPEN ACCESS

Marine Drugs

ISSN 1660-3397

www.mdpi.com/journal/marinedrugs

Article

Natural Products from Antarctic Colonial Ascidians of the Genera *Aplidium* and *Synoicum*: Variability and Defensive Role

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Received: / Accepted: / Published:

Abstract: Ascidians have developed multiple defensive strategies mostly related to physical, nutritional or chemical properties of the tunic. One of such is chemical defense based on secondary metabolites. We analyzed a series of colonial Antarctic ascidians belonging to the genera *Aplidium* and *Synoicum* to evaluate the incidence of organic deterrents and their variability. The ether fractions from 15 samples including specimens of the species *A. falklandicum, A. fuegiense, A. meridianum, A. millari* and *S. adareanum* were subjected to feeding assays towards two relevant sympatric predators: the starfish *Odontaster validus*, and the amphipod *Cheirimedon femoratus*. All samples revealed repellency. Nonetheless, some colonies tended to

concentrate defensive chemicals more in internal body-regions rather than in the tunic. Four ascidian-derived meroterpenoids, rossinones B-E, and the indole alkaloids meridianins A-G, along with other minoritary meridianin compounds were isolated from several samples. Some purified metabolites were tested in feeding assays exhibiting potent unpalatabilities, thus revealing their role in predation avoidance. Ascidian extracts and purified compound-fractions were further assessed in antibacterial tests against a marine Antarctic bacterium. Only the meridianins showed inhibition activity, demonstrating a multifunctional defensive role. According to their occurrence in nature and within our colonial specimens, the possible origin of both types of metabolites is discussed.

Keywords: Antarctic colonial tunicates; deterrent activity; sea star *Odontaster validus*; amphipod *Cheirimedon femoratus*; antibacterial activity.

1. Introduction

Ascidians are exclusively marine animals, occurring in all oceans, with > 2800 described species [1]. They may be solitarian, or constitute social groups of individuals connected by the base, or be compound (colonial), with many clonal zooids embedded in a gelatinous matrix sharing the external tunic [2]. This outer integumentary tissue, harbors diverse cell types, including symbionts in some cases, and is multifunctional, exhibiting variable consistency, from gelatinous to leathery [3]. Ascidians are sessile ciliary-mucus filter feeders, which natural dispersal is exclusive of gamete and larval stages. This is not usually more than a few meters, especially in colonial species producing fewer but larger eggs rich in vitelum (lecitotrophic) that are brooded until released as tadpoles [1, 2].

A great variety of predators feed on ascidians and many mechanisms have evolved to prevent predation, most related to properties (physical or chemical) of the tunic [1, 4]. Tough tunics occur in some colonial ascidians, but they are mainly found in solitarian ascidians [5]. Besides, calcium carbonate spicules embedded within the tunics of certain species may serve to avoid consumption [6-8]. Occasionally, palatability is more related to the nutritional value [4]. However, defensive chemistry is likely the first line of protection adopted by most ascidians. This may include the accumulation of heavy metals like vanadium, or sulfuric and (or) hydrochloric acid in tunic bladder cells [9-12]. But the production of deterrent natural products is a common strategy too [8, 12, 13]. In certain species these compounds are transferred from adults to larvae and eggs to confer protection, especially in compound ascidians where the investment in reproduction is particularly valuable [11, 14, 15]. Redundancy of several defensive mechanisms can operate either against diverse enemies, or also at different life stages [4, 8, 11, 12, 16]. Indeed, inducible distasteful chemicals are more typical of clonal organisms, consisting of clumps of genetically identical, but independent individuals, than of solitary (aclonal) ones that less likely recover from a significant loss of tissue [17]. Furthermore,

colonial ascidians tend to maintain a clean, unfouled surface, an indication of antifouling properties. Most of these mechanisms block initial bacteriofilms, impeding further biofouling, epibiosis and infections [18]. Instead, a number of solitary species become heavily fouled and cryptic, which is a proposed tactic towards enemies [1, 9, 19].

It 1974, Fenical isolated the first ascidian bioactive metabolite, geranyl hydroquinone from *Aplidium* sp. Since then, ascidians have yielded numerous compounds with remarkable bioactivities, including the first marine natural product to enter human clinical trials, didemnin B [reviewed in 20]. Ascidians mostly possess nitrogen-bearing metabolites particularly aromatic heterocycles, like peptides, alkaloids, and amino acid derived products, but also in lesser amount non-nitrogenous compounds, such as lactones, terpenoids or quinones [21, and previous reviews]. Although the ecological function of most of these metabolites remains undetermined, it is known that at least some of them are used as predator deterrents [8, 13, 15, 22, 23] and antifoulants [24]. A number of bioactive natural products have been obtained from Antarctic ascidians, such as palmerolide A, a group of ecdysteroids, meridianins, aplicyanins and rossinones [25-29]. It is often unclear if the animals are the true producers of the molecules [*i. e.* 30-32] or if associated microbes play a role in the secondary metabolism [*i. e.* 33, and reviewed in 34, 35]. Indeed, microsymbiotic origin of ascidian metabolites has received much less attention [*i. e.* 36] respect to compounds from sponges [reviewed in 37].

While the vast majority of ascidian metabolites have been isolated from whole-body extractions, several compounds were obtained from specific tissues, physiological fluids or cells [20, 31, 38-40]. If these products would result to possess ecological defensive function, then this particular location should be contrasted with the Optimal Defense Theory (ODT). The ODT predicts effective allocation of defensive compounds in most valuable/exposed body-regions of liable prey organisms, attending to the metabolic costs secondary metabolism entails [41]. Localization of defenses to specific regions has been observed in some sponges [42], gorgonians [43], etc… Ascidians possess a complex, organized body-plan and circulatory system, which may allow them to encapsulate bioactive compounds to fulfill ecological roles avoiding autotoxocity [44].

In Antarctic benthic ecosystems, invertebrate predators, mainly asteroids but also dense populations of amphipods, have replaced fish as principal predators [45-47]. Sea stars feed by extruding their cardiac stomachs over their prey, and initiating digestion from the outer layers [48], while amphipods bestow superficial bites. Hence in most Antarctic organisms chemical defenses should likely be stored externally to benefit survival.

Ascidiacea is one of the principal taxons structuring Antarctic-shelf filter-feeding communities [49]. The ascidiofauna here is very homogeneous and endemic [50]. Within the Family Polyclinidae, one of the most prolific genera is *Aplidium* with 40 species described from the Southern Ocean. *Synoicum* instead is represented by 8 Antarctic and subantarctic species. *Synoicum adareanum* produces pedunculated colonies of variable colorations, whereas those of *Aplidium* are usually globular, with *A. falklandicum* being characteristically bright yellow, *A. fuegiense* pink-orange, *A. meridianum* gray, green, or brownish but with bright yellowish reflexes, and *A. millari* being mostly pink [51].

In this study, we aim to evaluate the defensive potential based on the lipophilic secondary metabolism of several Antarctic ascidian species of the genera *Aplidium* and *Synoicum* to fight against sympatric predation and bacterial fouling. For this purpose we conducted feeding assays with the ether fractions of selected ascidian samples, using the asteroid *Odontaster validus* and the amphipod *Cheirimedon femoratus* as putative consumers, and considering the presumptions of the ODT in terms of intra-colonial defense allocation. Moreover, the antibiotic activity towards an Antarctic marine bacterium was also assessed. Finally, chemical analysis carried out in some of the samples led to the purification of several characteristic compounds, which were similarly tested for their defensive ecological activities.

2. Methods and Materials

2.1. Collection of Samples.

Antarctic tunicates of the genera *Aplidium* and *Synoicum* were collected in the Eastern Weddell Sea between 280 m and 340 m depth during the ANT XXI/2 cruise of R/V Polarstern (AWI, Bremerhaven, Germany), from November 2003 to January 2004, by using Bottom and Agassiz Trawls. Individual colonies of each species from a single collection site and trawl were grouped together as a single sample for further experimentation and analysis (Table 1). A portion of each sample was conserved and pictures of living animals were taken on board for further taxonomical identification at the University of Alicante (Spain). The remaining material was frozen at -20ºC, and transported to the laboratory at the University of Barcelona until processed.

Table 1. Ascidian samples collected during the Antarctic cruise on board the R/V Polarstern (ANT XXI/2) in 2003 in the Eastern Weddell Sea (Antarctica). B&W: Black & White, O: Orange, Br: Brown morphs; AGT: Agassiz Trawl, BT: Bottom Trawl.

Ascidian species name and code number	Latitude	Longitude	Gear	Depth (m)
Aplidium falklandicum Millar, 1960 (1)	70° 57.00' S	10° 33.02' W	BT	332.8
Aplidium falklandicum Millar, 1960 (2)	70º 55.92’ S	10º 32.37’ W	AGT	288
Aplidium falklandicum Millar, 1960 (3)	70º 56.67’ S	10º 32.05’ W	BT	302.4
Aplidium falklandicum Millar, 1960 (4)	70º 57.11’ S	10º 33.52’ W	BT	337.2
Aplidium fuegiense Cunningham, 1871	71° 7' S	11° 26' W	AGT	228.4
Aplidium meridianum (Sluiter, 1906) (1)	70° 56.42' S	10° 31.61' W	BT	284.4
Aplidium meridianum (Sluiter, 1906) (2)	71° 04.30' S	01° 33.92' W	BT	308.8
Aplidium millari Monniot & Monniot, 1994	71° 04.30' S	01° 33.92' W	BT	308.8
Synoicum adareanum (B&W) (Herdman, 1902) (1)	70° 56' S	10° 32' W	BT	337.2
Synoicum adareanum (B&W) (Herdman, 1902) (2)	70° 55.92' S	10° 32.37' W	AGT	288.0
Synoicum adareanum (B&W) (Herdman, 1902) (3)	70° 56.42' S	10° 31.61' W	BT	284.4
Synoicum adareanum (Br) (Herdman, 1902)	71° 06.44' S	11° 27.76' W	AGT	277.2
Synoicum adareanum (O) (Herdman, 1902) (1 and 3)	70° 55.92' S	10° 32.37' W	AGT	288.0

Synoicum adareanum (O) (Herdman, 1902) (2)	70° 56' S	10° 32' W	BT	337.2

2.2. Organic Extractions.

When possible, colonial tunicates were dissected into external/internal (tunic/visceral), and in one case apical, regions, in order to allocate chemical defenses or particular compounds. Each ascidian sample was exhaustively extracted with acetone at room temperature. After removal of the solvent *in vacuo*, the obtained extract was partitioned into diethyl ether (three times) and butanol (once) fractions. The organic phases of each extraction were dried and weighted, providing the yield of extract per dry mass. The natural tissue concentrations were calculated respect to the total dry weight (DWT = DW dry weight of the extracted sample + EE ethereal fraction weight + BE butanolic fraction weight). Ether extracts were further used for bioassays and chemical analysis, and butanolic fractions and water residues were kept for future studies (Table 2).

2.3. Purifications and Chemical Analysis.

Diethyl ether (Et_2O) extracts were screened by Thin Layer Chromatography (TLC), using Merck Kieselgel plates (20x10 cm and 0.25 mm thick), and light petroleum ether/ diethyl ether (1:0, 8:2, 1:1, 2:8, 0:1) and chloroform/methanol (8:2) as eluents. The plates were developed with $CeSO_4$. Four conspicuous UV-visible bands at R_f's; 0.65, 0.57, 0.45 and 0.21 (light petroleum ether/ diethyl ether 2/8) with $CeSO_4$ reaction were observed in the *Aplidium fuegiense* INT sample, coinciding with the four meroterpenoid containing fractions. Moreover all fractions pertaining to samples from the species *A. falklandicum* and *A. meridianum* from internal and external regions revealed a yellowish blatant UV-visible band at R_f's; 0.63 (chloroform/methanol 8/2) with $CeSO_4$ reaction, which corresponded with the fraction composed of the alkaloid mixture of meridianins A-G. Extract were further fractionated on both Sephadex LH-20 and silica gel (Merck Kieselgel 60, 0.063-0.200) columns by using chloroform/methanol 1:1 and a gradient of petroleumether/diethyl ether as eluent respectively. ^{1}H-NMR spectroscopic analyses were carried out to determine pure products or mixtures. Fractions composed of a mixture of molecules were further purified with TLC using preparative (SiO_2) plates Merck Kiesegel 60 F_{254} (0.50 e 1.00 mm) and HPLC (Shimadzu with LC-10ADVP pump and SPD-10AVP UV detector) using reverse-phase semipreparative columns (Supelco Discovery® C_{18}, 25 cm x 46 mm, 5µm, and 250 10 mm, Phenomenex, Kromasil C_{18}) and water/acetonitrile and methanol/water 70:30 as solvent (flux 2 ml/min). Subfractions from *A. falklandicum* 1 were additionally passed through an Orbitrap LC-MS/MS manifesting the presence of minoritary derivative meridianins.

2.4. Spectral Analysis of the Natural Products.

The isolated pure compounds were subjected to spectral analysis with NMR, UV, as well as MS spectrometry. Optical rotation measurements were performed on a Jasco DIP-370 polarimeter, using a 10 cm lon cell. The ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker Avance DRX-400, Bruker DRX-600 equipped with in inverse TCI CryoProbe, and Bruker DRX-300 spectrometers. Chemical shifts were reported in ppm and refered to $CDCl_3$ and CD_3OD as internal standard (δ 7.26 e 77.0 ppm for $CDCl_3$ e δ 3.34 and 49.0 ppm for CD_3OD). The ESIMS and EIMS spectra were obtained on a Micromass Q-TOF Micro™ spectrometer connected to a Waters Alliance 2695 HPLC chromatograph, on a Thermo LTQ-Orbitrap Discovery connected on a Accela Thermo Fischer HPLC system, and on a HP-GC 5890 series II spectrometer, respectively. The IR and UV spectra were recorded on a Bio-Rad FTS 155 FTIR and an Agilent 8453 spectrophotometer respectively. The spectral data of compounds isolated were compared with the data reported in the literature [25, 28, 29]. More detailed data on the chemical procedures may be consulted elsewhere [13, 40, and Rodríguez et al., unpublished; Annex I].

2.5. Feeding Deterrence Assays with Sea Stars.

Alive individuals of the voracious, eurybathic, Antarctic sea star *Odontaster validus*, with omnivorous habits and a circumpolar distribution [46], were captured for bioassays at Port Foster Bay in Deception Island, South Shetland Islands (62º 59.369' S, 60º 33.424' W). Captures took place during three campaigns: ECOQUIM-2 (January 2006), ACTIQUIM-1 (December 2008-January 2009) and ACTIQUIM-2 (January 2010). Collection was done by scuba diving from 3 to 17 m depth (n>1300), with the sea stars sizing between 4.5 and 10.5 cm diameter. This asteroid is a model macropredator in many Antarctic feeding deterrence studies [for review see 52]. The sea stars were maintained alive in large tanks with fresh seawater at the Spanish Base BAE "Gabriel de Castilla" (Deception Island), and starved for five days. The bioassays included 10 replicates each hence, 10 containers filled with 2.5 L of seawater, accomodating one sea star individual. Each asteroid was offered one shrimp food item (5x5x5 mm and 13.09 ± 3.43 mg of dry mass) that could be fully gobbled, and treatment and control experiments were ran simultaneously. This methodology is described in previous papers [53, 54]. Control shrimp feeding cubes (12.4% protein, 9.1% carbohydrates and 1.5% lipids, and 17.8 KJ g^{-1} dry wt and 4.1 KJ g^{-1} wet wt, by Atwater factor system [55]) were treated with solvent alone, whereas treatment ones contained natural concentrations of lipophilic Et_2O extracts or isolated compounds from Antarctic ascidians (Table 2). The extracts or isolated compounds were previously diluted in diethyl ether, removing always the solvent under flow hood. Previous feeding acceptability studies with asdicians have used several parameters to normalize natural concentrations: volume [56], wet or dry biomass, for biting and no-biting predators [12, 13]. In our study, considering the sea star extraoral feeding habits, extruding the cardiac stomach and bolting down whole shrimp pieces [48], dry weight seems a good approximation for assessing the "defense per shrimp feeding cube". We chose dry weight because the water content may produce remarkable deviations.

Furthermore, the isolated compound rossinone B and a fraction containing the mixture of meridianins A-G were also assayed at their corresponding sample natural concentrations, which were 4.8 and 19.11 mg g^{-1} dry weight respectively. After 24 h, the number of shrimp items eaten for each test were recorded, and the remaining (not eaten) were frozen for extraction and checked by TLC to ensure the presence of the extracts or compounds, which was always the case. Products contained in diethyl ether extracts are not hydrophilic, hence diffusion to the water column is theoretically implausible, especially in the cold (<1ºC) Antarctic water. Feeding repellence was statistically evaluated with Fisher's Exact tests for each treatment assay referred to the simultaneous control [57]. After experimentation the stars were returned to the sea.

2.6. Feeding Preference Assays with Amphipods.

The abundant, eurybathic Antarctic lysianassoid amphipod *Cheirimedon femoratus*, with devouring omnivore-scavenger feeding habits and circumpolar distribution [47], was used for our experiments according to our recently described protocol [58]. Hundreds of individuals were captured between 2 to 7 m depth by scuba diving with fishing nets, and also by displaying baited traps with canned sardines along the coastline of the Antarctic Spanish Base (BAE) during the campaign ACTIQUIM-2 in January 2010. Artificial caviar-textured foods (pearls) were prepared with 10mg/mL alginate aqueous solution along with 66.7mg/mL of concentrated feeding stimulant (Phytoplan®; 19 KJ g^{-1} dry wt). The powdered food was mixed into the cold alginate solution with a drop of green or red food coloring (see below), and introduced into a syringe without needle. The mixture was then added drop-wise into a solution of 0.09 M (1%) $CaCl_2$ solution where it polymerized forming pellets 2.5 mm Ø (3.3% protein, 1.36% carbohydrates and 1.3% lipids, and 18 KJ g^{-1} dry wt and 1.5 KJ g^{-1} wet wt by Atwater factor system [55]). For extract-treated pearls, ascidian Et_2O extracts at their natural concentration were dissolved in a minimum volume of diethyl ether to totally wet the dehydrated food, and the solvent was left to evaporate (Table 2). Control pearls were prepared similarly with solvent alone. Rossinone B and the meridianin mixture were tested too at their sample natural concentrations (see above).

Alive organisms were maintained in 8L aquariums and were starved for 1-2 days. Every assay consisted on 15 replicate containers filled with 500-mL of sea water and 15 amphipods each, which were offered a simultaneous choice of 10 treatment and 10 control pellets of different colorations (20 food pearls in total), green or red easily distinguished. The colors for treatment or control pearls were randomly swapt throughout the experimentation period, and previous trials confirmed the null effect of the different colorations in feeding preferences (P = 0.4688, n.s.). The assays ended when approximately one-half or more of either food types had been consumed, or 4 h after food presentation. The number of consumed and not consumed pearls of each color (control or treatment) was recorded for each replicate container. Since our feeding trials were short in time, autogenic alterations were avoided and there was no need to run "controls" in the absence of grazers for changes unrelated to consumption [58, 59].

Statistics were calculated to determine feeding preference of treated pearls respect to the paired controls to consequently establish unpalatable activities. Exact Wilcoxon tests were applied using R-command software. Uneaten treatment pearls were preserved for extraction and TLC analysis, to check for possible alterations in the extracts. No major changes were observed. Once testing was over the amphipods were returned to the sea.

2.7. Antibacterial Tests against a Sympatric Marine Antarctic Bacterium.

These assays were intended to assess antibiotic properties within the ascidian extract, as well as that of the purified compounds rossinone B and the meridianin mixture (A-G) towards an unidentified sympatric marine bacterium. The bacterium was isolated from a seawater sample collected at Crater 70 area, in Port Foster Bay, Deception Island (Antarctica). A 1 mL alliquot of the seawater sample was transferred into Difco™ marine broth 2216 (Difco Laboratories), grown for 24 hr at 18-20°C, and subsequently cultured in Difco™ marine agar 2216 (Difco Laboratories). The obtained individual bacterial colonies were then isolated, and the strain exhibiting the best growth was chosen for our experiments. A seawater subsample in 7% glycerol filtered-sterilized seawater, as well as a culture of the selected bacterium strain were frozen at -20°C and shipped to the University of Barcelona for further identification, which unfortunately was unsuccessful. Rinse broth was then inoculated with pure cultures of the selected strain and incubated at 18-20ºC until optimal growth (slight turbidity corresponding to Nº0,5 McFarland scale; equivalent to 10^{-8} cfu/mL). A 0.1 mL suspension of bacterial culture was spread evenly onto marine agar plates. Each Petri dish was divided into 6 regions: 3 regions for testing the extracts or isolated compounds in triplicate; another one for the positive control with antibiotic activity; plus two regions for the negative controls, one with and one without solvent. The positive control was chloramphenicol, while negative controls consisted of 20µL solvent alone, in this case, diethyl ether for the extracts and the rossinone B and methanol for the meridianin fraction. Paper antimicrobial assay disks (BBL Microbiology Systems) Ø 6 mm soaked with the corresponding testing extracts or pure products (rossinone B, meridianin mixture) previously dissolved in 20µL solvent carrier, or control disks, were placed in the middle of each testing region in the inoculated Petri dishes. Extract and compound concentrations added to the disks corrrespond to natural concentrations calculated as reported above (Table 2). After incubation for 1 day at 18-20°C, inhibition halos were measured to determine antibiotic activities. When the diameter of the inhibition zones was larger than 7 mm Ø, it was considered active [60].

3. Results

3.1. Ascidian Samples and Organic Extractions.

Colonies, zooid individuals and larval morphology allowed the identification of our samples as *A. falklandicum, A. fuegiense*, *A. meridianum, A. millari* and *Synoicum adareanum*. This last

species presented three different morphs referred to as: black and white (B&W), brown (Br) and orange (O), clearly distinguishable (Table 1). In total 15 tunicate samples, each consisting of several colonies, yielded 25 diethyl ether extracts that were used for ecological and chemical analysis (Table 2).

Table 2. Data of lipophilic Et_2O extracts and isolated metabolites from the studied Antarctic ascidian samples. [N_{EE}] Natural tissue concentration in mg of dry diethyl ether extract (EE) weight per g of the total dry weight (DW) of the sample; API: Apical part; EXT: External part; INT: Internal part. B&W: Black & White, O: Orange, Br: Brown morphs.

Species name, sample code and bodypart	[N_{EE}] (mg g^{-1} DW)	Isolated metabolites
Aplidium falklandicum 1	42,00	Meridianins (A-G)[a] + (I-U)[b]
Aplidium falklandicum 2 EXT	57,23	Meridianins (A-G)[a]
Aplidium falklandicum 2 INT	79,3	Meridianins (A-G)[a]
Aplidium falklandicum 3 EXT	47,60	Meridianins (A-G)[a]
Aplidium falklandicum 3 INT	128,40	Meridianins (A-G)[a]
Aplidium falklandicum 4 EXT	23,80	Meridianins (A-G)[a]
Aplidium falklandicum 4 INT	19,40	Meridianins (A-G)[a]
Aplidium fuegiense EXT	15,12	Rossinone B
Aplidium fuegiense INT	85,10	Rossinone B + (C-E)
Aplidium meridianum 1	128,51	Meridianins (A-G)[a]
Aplidium meridianum 2	79,36	Meridianins (A-G)[a]
Aplidium millari EXT	39,31	-
Aplidium millari INT	81,60	-
Synoicum adareanum (B&W) 1 EXT	20,04	-
Synoicum adareanum (B&W) 1 INT	33,09	-
Synoicum adareanum (B&W) 2 API	55,69	-
Synoicum adareanum (B&W) 2 EXT	18,12	-
Synoicum adareanum (B&W) 2 INT	27,31	-
Synoicum adareanum (B&W) 3	20,88	-
Synoicum adareanum (Br)	36,83	-
Synoicum adareanum (O) 1	20,41	-
Synoicum adareanum (O) 2 EXT	28,02	-
Synoicum adareanum (O) 2 INT	26,43	-
Synoicum adareanum (O) 3 EXT	30,71	-
Synoicum adareanum (O) 3 INT	66,04	-

[a] Meridianin mixtures A-G from our samples were not analyzed separately in the current study and are only indicative of the presence of the mixture.

[b] Meridianins I-U could be present in trace amounts in other meridianin-containing samples, which were not analyzed in more detail due to the lack of enough biological material

3.2. Chemical Analysis of the Natural Products.

Four meroterpene derivatives, of the class of the cyclic prenyl quinones, rossinones B-E (Fig. 1), were isolated from the Et_2O lipophilic internal fraction of the colonial Antarctic tunicate *Aplidium fuegiense* (*A. fuegiense* INT). Instead, the tunic of this sample (*Aplidium fuegiense* EXT) possessed very small quantities of Rossinone B, but lacked the other minoritary rossinone meroterpene-related products. Rossinone B, which was firstly reported in an *Aplidium sp.* ascidian from the Ross Sea, Antarctica [29], was the major metabolite of this family of compounds. Rossinones B-E were also recently described as part of our chemical investigations [40]. Furthermore, all the extracts from internal viscera and external regions from samples of the species *A. falklandicum* and *A. meridianum* revealed the presence of the known meridianins A-G (Fig. 2). The purified meridianin fraction from the sample *A. falklandicum* 1 was used in the sea star assay. Finally, a group of twelve unknown minoritary meridianins (I, J, J', L, O, P, Q, R, R', S, T and U) with combinations of bromide, chloride, and hydroxi groups, as well as two unknown dimeric derivates from the majoritary meridianins A and B (or E) were detected by means of an Orbitrap LC-HRMS-MS from the sample *A. falklandicum* 1 (see Electronic Supplementary Information 1). More details on the chemical characterization of these compounds are reported elsewhere [Rodríguez et al., unpublished; Annex I].

Figure 1. Chemical structures of the Rossinone compounds purified from *Aplidium fuegiense*: Rossinone B-E.

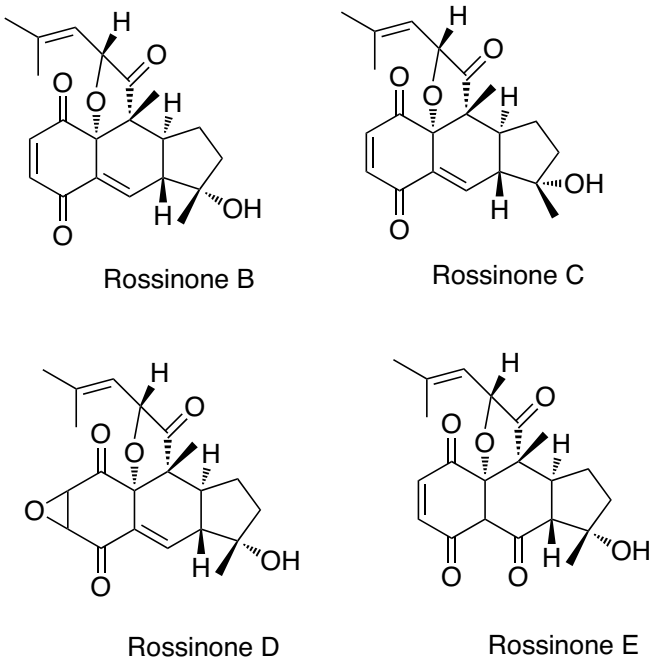
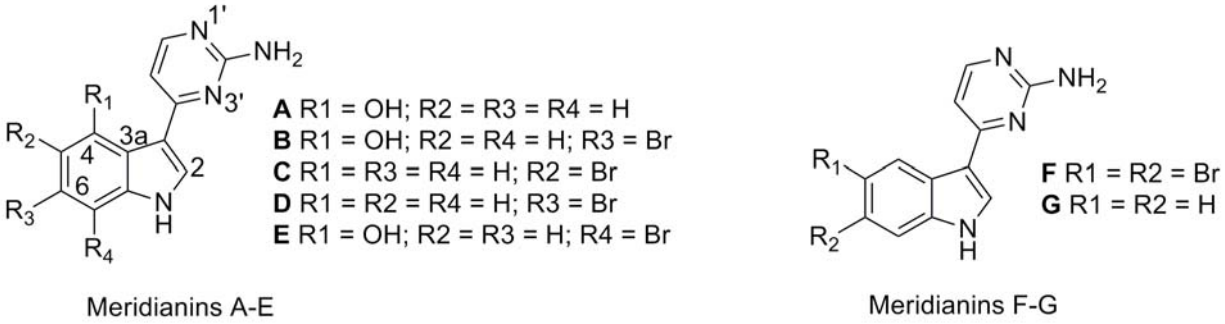


Figure 2. Chemical structures of the meridianin compounds (A-G) purified from *Aplidium falklandicum* and *A. meridianum.*



3.3. Feeding Deterrence Assays with Sea Stars.

All 5 ascidian species and 15 samples demonstrated the presence of chemical defenses. Twenty-one of the lipophilic Et_2O fractions tested caused significant (P = 0.01 or P = 0.05) feeding repellence against the sea star *O. validus* at natural concentrations according to the Fisher's Exact test. Control assays using shrimp feeding cubes impregnated with solvent alone displayed a minimum acceptance of eight cubes out of ten. In 14 experiments the lipophilic fractions provoked an absolute rejection by the sea stars (P = 0.01). On the other hand, only four samples coming from the external tunics of *Aplidium millari*, *Synoicum adareanum* (B&W) 1 and 2 and *S. adareanum* (O) 3 yielded edibility and were accepted (Fig. 3). Regarding the tests conducted with isolated metabolites, both the rossinone B (P<0.001), as well as the mixture of meridianins A-G (P<0.001) showed potent deterrency against the asteroid, when included in shrimp food items at their natural concentrations. In both cases the consumption was of 0 out of ten compound-treated cubes, whereas the simultaneous control tests had a ratio of 8 items eaten out of ten.

3.4. Feeding Preference Assays with Amphipods.

In the preference experiments towards the amphipod *Cheirimedon femoratus* the 4 species tested, represented by 9 samples showed to possess repellent compounds. In fact, all the fractions assayed except one (12 out of 13) revealed remarkable feeding unpalatable activity (P<0.01) at natural concentrations according to the Wilcoxon Exact test. The amphipod devored control food pearls at impressive high rates, and regardless of its gregarious behavior unpalatabilities were evident. Actually most of the extracts that yielded deterrency in this assay were strongly rejected and not ingested at all when they were presented included in alginate pellets. Only the apical ethereal fraction (API) from the ascidian *Synoicum adareanum* (B&W) 2, was palatable contrasting with basal-external and visceral extracts (EXT and INT), which were remarkably repellent (Fig. 4). In addition, the amphipod significantly rejected food pearls treated with rossinone B (P<0.01) or meridianin (A-G) mixture (P<0.001), respect to the controls.

Figure 3. Bar diagram displaying the results in the feeding repellence bioassays with the sea star *Odontaster validus* performed with lipophilic Et_2O extracts from Antarctic colonial ascidians, showing the paired results of control and extract treated shrimp cubes for each test and representing the percentage of acceptance. *: significant differences ($p<0.05$), **: significant differences ($p<0.01$), with control as preferred food (Fisher's exact test).

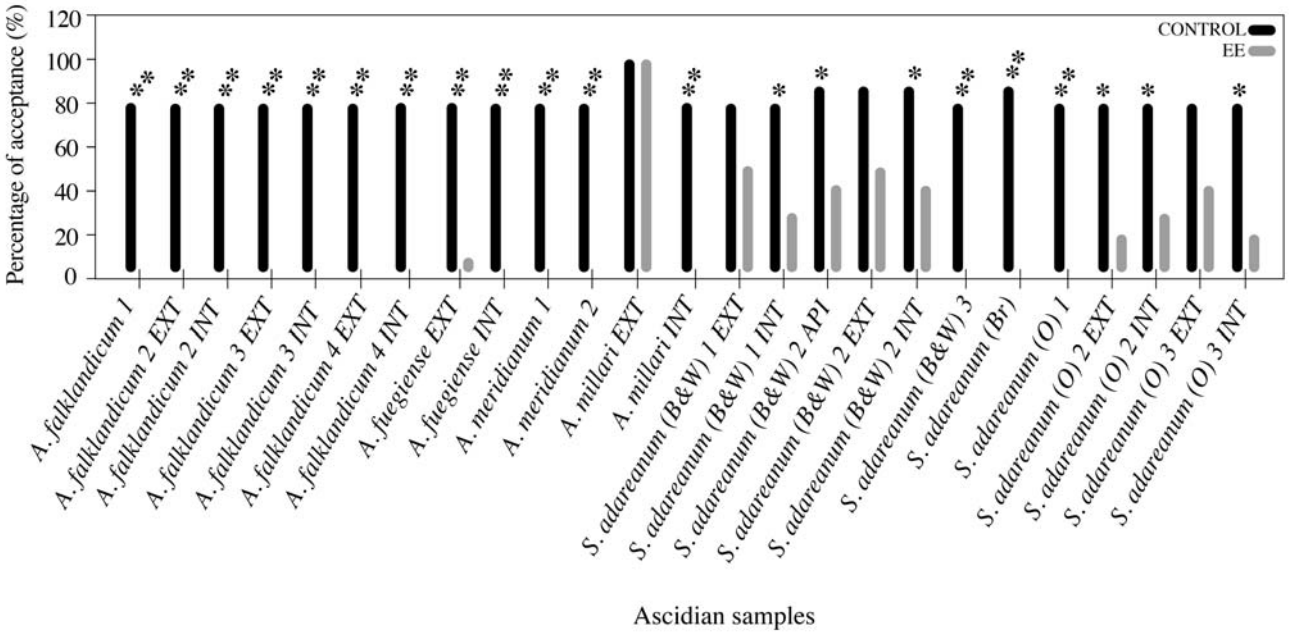
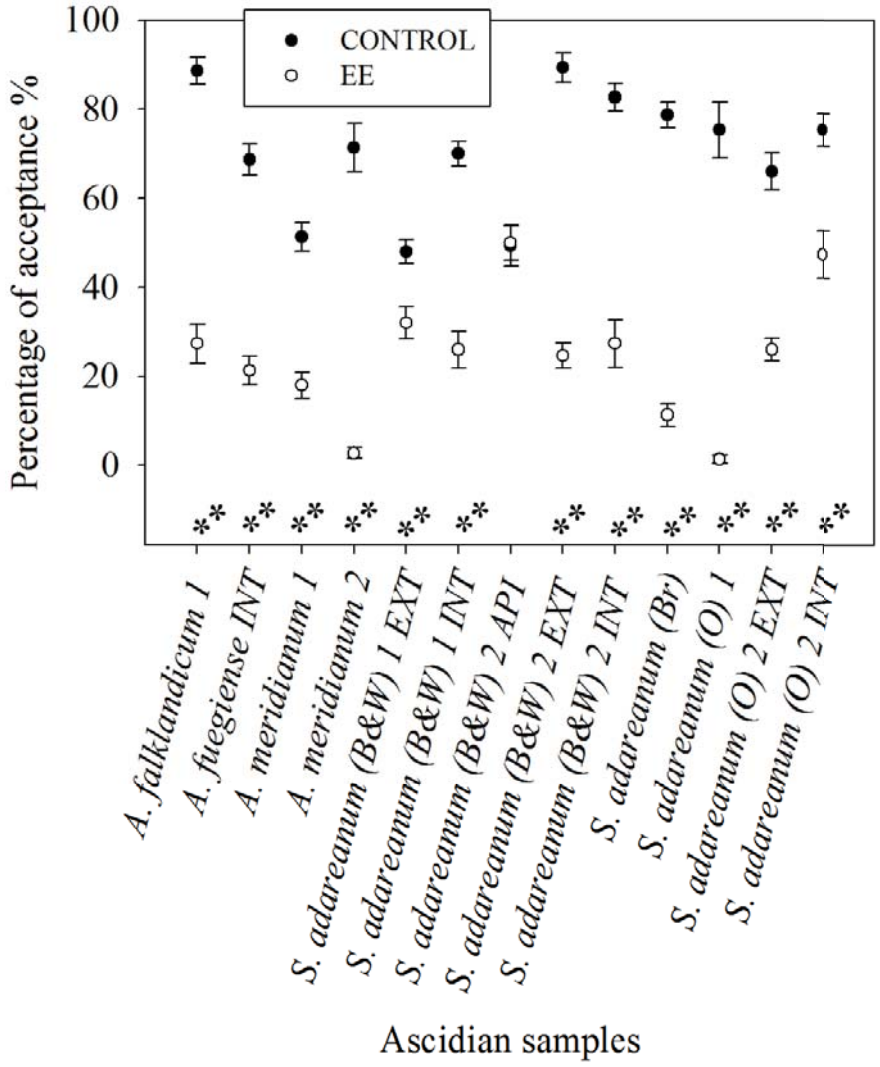


Figure 4. Scatter plot diagram showing the results in the feeding preference bioassays with the amphipod *Cheirimedon femoratus* conducted with lipophilic Et_2O fractions from Antarctic colonial ascidians. The paired results of control and extract treated food pearls are displayed for each test as the mean percentage of acceptance and standard error bars. **: significant differences ($p<0.01$) with control as preferred food (Exact Wilcoxon test).



3.5. Antibacterial Tests against a Sympatric Marine Antarctic Bacteriums.

The isolated mixture of meridianins from *Aplidium flaklandicum* 1 caused strong growth inhibition (active (+++) in the 3 replicates >10 mm Ø inhibition halo) on cultures of an unidentified sympatric Antarctic marine bacterium, as did the positive controls with chloramphenicol. Instead none of the extracts assessed from our ascidian samples, neither the rossinone B inhibited the bacterium in our laboratory assays, similarly to what was observed in the solvent negative controls.

4. Discussion

4.1. Incidence and allocation of chemical defenses against predation.

Antarctic ascidians thrive in environments where predation pressure, mostly driven by invertebrate consumers, is intense [1, 45]. Still, so far only seldom it has been demonstrated that natural products are responsible for chemical defense in ascidians [4, 56, 61]. Moreover, these animals exploit inorganic acids against sea star predators (especially colonial ascidians) and the protection afforded by a tough tunic (especially solitary ascidians) [12, 56, 62]. Our findings complete this map by showing that organic chemical defense is largely used in these ascidians, since all our samples possess repellent metabolites (Fig 3 and 4). The species analyzed in this study were free of evident epibionts and lacked mechanical protection [51, personal observations from the authors]. Likewise, bioaccumulation of acids or heavy metals has not been reported within their tunic, nor in closely related species of the family Polyclinidae [9, 10, 63], which in fact report absence of bladder cells [64]. These facts put forward a presumable protection based on organic chemistry. On the other hand, lipophilic partitions have proved to be more actively deterrent than hydrophilic ones in marine organisms [23, 52, 65], thus we focused our study on the ether fractions of our specimens. In the past though, only rarely have the chemicals responsible for the unpalatablity been identified. Yet some examples of deterrent metabolites in ascidians include the tambjamines C and F, didemnin B and nordidemnin B, patellamide C, ascididemin, and meridianins A-G [8, 13, 15, 22, 23].

Aplidium falklandicum and *A. meridianum* possess protective chemicals, the meridianins, which besides deterring the asteroid *Odontaster validus*, have now shown feeding repellence towards the amphipod *Cheirimedon femoratus*. Meridianins are present both in inner and outer tissues, even if they seem to be more concentrated in outer zones [13]. Apart from these two species, a seeming lack of within-specimen defense allocation was detected in *S. adareanum* (O) 2, as has been observed in other ascidians too [38]. Rossinone B proved to take part in the whole-colony chemical defense of *A. fuegiense*, repelling both sea stars and amphipods, but it was predominant in internal regions.

According to the ODT [41], tunics with low palatability (determined by a combination of energy content, digestibility, chemicals and, pH) are expected when protecting adult stages surpasses the benefits of defending larval ones [4]. In fact, in some colonial species bioactive alkaloid pigments are stored in tunic bladder and pigmentary cells, presumably acting as sunscreens or deterrents [31, 38, 39]. However, the presence of chemical defenses within the tissues of some Antarctic sponges and ascidians suggests that predators other than sea stars are also acting here, or that the assumptions of the ODT are inappropriate in such case [13, 66, 67]. Also, big complex eggs and larvae produced by most compound ascidians are often protected with noxious cyclic peptides and alkaloids, compensating the great investment assigned to reproduction [4, 14, 15, 30]. This outcome explains the presence of deterrents in inner tissues (gonads) in order to produce chemically defended larval stages [11, 15]. The predominant internal allocation of defenses in some of our samples is thus not fortuitous. Some tunics have

low caloric value respect to inner tissues, making them already less attractive to predators (McClintock [4, 11, 68]. Besides, colonial ascidians are often able to recover from wounds and fastly regenerate the damaged tunic [69]. This capacity would allow them to address less energy in defending not reproductive regions. Instead, solitary species may require better-protected tunics [68]. Pisut and Pawlik [11] found deterrents allocated in the gonads of solitarian species, yet whole-specimen extracts were palatable. This indicated the possession of thick tunics that diluted any deterrency found in viscera and gonads. Our compound asdidians, instead, had thin tunics accounting for a small fraction in the colony, and even if some samples had poorly (or no) defended tunics, whole-colony extracts were always deterrent. Tunics from *A. millari*, *S. adareanum* (B&W) and *S. adareanum* (O) seemed to be less (or not) chemically protected against predation from the sea star tests. However amphipod assays, probably due to a greater susceptibility of *C. femoratus* [unpublished results of the authors], do reflect the existence of deterrents in the tunics, presumably in fairly lesser amounts. The supposed low energetic value of the tunics, along with a weak chemical defense respect to inner regions may contribute to the overall protection of these colonies against heavy predation, complementing the remaining defensive mechanisms. The lower extract yields produced by most tunics respect to inner tissues likely reflect these facts (Table 2). Furthermore, this pattern of allocating deterrents more to the internal regions was also observed in the distribution of the defensive secondary metabolite rossinone B within the colonies of *A. fuegiense*.

4.2. Antibiotic activity towards marine bacteria.

Benthic organisms must combat pathogens as well as epibiosis by macro- and microorganisms. More commonly colonial rather than solitary ascidians, have revealed agents to prevent this [19, 24, 70-73]. Our Antarctic samples though, did not display significant inhibition against a sympatric bacterium strain. This agrees with other surveys of both Antarctic sponges and ascidians, which indicate a general lack of antibacterial chemistry. In Antarctic systems, diatom invasions apparently surpass that of bacteria, suggesting that there might be more selective pressure for chemical defenses against diatom fouling [62, 74-76]. It was also proposed that bacterial pathogens could be controlled through immune processes in asdicians [62, 65]. Furthermore, rossinone B, which was antimicrobial and antimycotic towards cosmopolitan strains [29], revealed no activity in our assays. Meridianins A, B, C, E, F and G, instead, caused no growth inhibition on allopatric microbes in the past [13]. However, in the present study the meridianin mixture revealed potent activity against an Antarctic marine bacterium suggesting a defensive role against pathogenic or fouling bacteria. Even if whole ascidian extracts seem inoquous, these are composed of a complex mixture of substances (primary and secondary metabolites, and nutrients) that may interfere with some bioactivities. However, in the case meridianins appeared allocated in compartments, which has not been proved so far, then they may fulfill this function too. Despite some biologically active marine natural products serve specific ecological roles [73], others such as the meridianins, appear to be multipurpose defenses.

4.3. Variability and origin of bioactive natural products.

Secondary metabolites are more typical of colonial than of solitary tunicates. Chemical analyses have been reported for six species of Antarctic ascidians, all of them colonial: *Synoicum sp., S. adareanum, Aplidium sp., A. falklandicum, A. meridianum* and *A. fuegiense* [21, and previous reviews in this series]. Diyabalanage and co-workers purified a cytotoxic macrolide, palmerolide A, from *S. adareanum* [26]. A dense microbial community was detected on the tunicate and a possible bacterial origin of this polyketide was proposed [77]. Several ecdysteroids (arthropod molting hormones) were also reported from *S. adareanum* [27]. Their presence suggested a potential to defend from arthropod predators through a strategy similar to that found in terrestrial plants, which elaborate ecdysteroids that short-wire molting in phytophagous insects. In our investigation we did not find these metabolites, however this species did exhibit amphipod feeding avoidance. We must pont out that intraspecific polymorphism in colonial ascidians is recurrent [51], and we found 3 morphotypes for *S. adareanum* among our samples. *S. adareanum* also occurred in two different morphs near Palmer Station revealing diverse bioactivities. Moreover, crude extracts of a *S. adareanum* from Anvers Island (western Antarctic Peninsula) lacked deterrency towards several sympatric consumers [56], as opposed to our results. The variable morphologies, bioactivities, and presence of some characteristic metabolites suggest a need for further taxonomical resolution in this species [62].

Acidians of the genus *Aplidium* are renowned for the variability of the metabolites that they present: non-nitrogenous compounds are dominated by prenyl quinones, linear or cyclic, and among the nitrogen containing group, nucleosides, cyclic peptides and a high variety of alkaloids can be mentioned [78]. While the majority of ascidian metabolites are amino acid derived [79], the genus *Aplidium* is noted for the propensity to biosynthesize terpene derivatives [78]. The finding of rossinones B-E in *A. fuegiense*, reflects this outcome, since meroterpenes are typically found in sponges and seaweeds [80]. Rossinones A and B were firstly isolated from an Antarctic unidentified *Aplidium* from the Ross Sea. While modest bioactivities characterized rossinone A, rossinone B exhibited antileukemic, antiviral, and antiinflammatory properties [29]. Biosynthetically, cyclic prenylated quinones, such as rossinones B-E seem to derive from linear hydroquinones, like rossinone A [81]. Interestingly, neither acyclic hydroquinones nor putative quinone-containing precursors of rossinones were detected in *A. fuegiense* [40; Annex I].

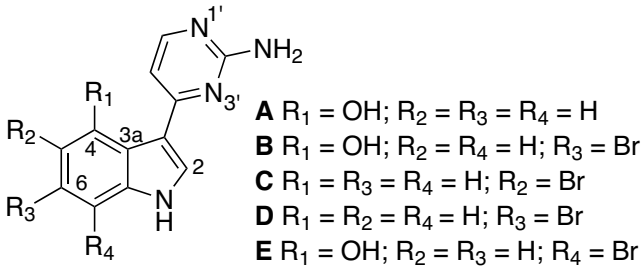
It would be interesting to find out where all these compounds are synthesized. In other colonial species special tunic cells (bladder; lacking in *Aplidium* and *Synoicum* [64], or pigment cells) concentrate defensive chemicals [36, 38, 39]. Final metabolites seem to end in storage compartments in the outer tunic, while other intermediate products remain in inner producing tissues (zooids) [38]. This could explain the distribution observed for the rossinone compounds in *A. fuegiense*. Here, Rossinone B is the majoritary and most active defensive metabolite. It was found predominantly in inner tissues, but also in the tunic in small amounts. The other minoritary rossinones (C-E) instead, are only present in internal areas of the colony, presumably

as precursors. Alternatively, these products could derive from symbiotic microbes. Among the known microorganism-derived products, terpenes are uncommon and indole alkaloids predominate [22, 82-85]. In many species, especially colonial, microsymbionts are usually sited in the tunic [33, and reviewed in 3, 34, 35]. The presence of the intermediate products exclusively in the inner tissues [40, and present study], suggests that rossinone terpenoids probably do not derive from a microbial source, or at least not from a tunical symbiont.

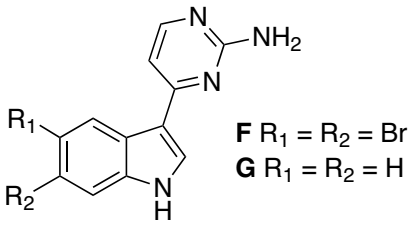
The meridianins are a family of indole alkaloids with potent cytotoxicity and kinase inhibitory activity, especially meridianins B and E, considered an important scaffold for cancer therapeutics [86, 87]. Rossinones and meridianins are indeed interesting products for pharmacological research. The new minoritary meridianins (I-U) (Online Resource 1; Annex I) and some unreported dimeric derivates indicate that the meridianins constitute a bulky group of alkaloids very rich in concentration and in diversity. Many deterrents appear taking part of a family of related metabolites, which are effective as a mixture, but often also as isolated forms, such is the case of both tambjamines and meridianins [13, 22]. Colonies of *A. falklandicum* and *A. meridianum* have external yellowish pigmentation as the fraction containing the mixture of meridianin compounds (A-G). As has been proposed for other species containing bright-colored alkaloids [30, 39, 84], the meridianins could be photoprotective. Antarctic benthic invertebrates have no apparent reason to have warning bright colorations in a system where grazing pressure by visually oriented predators, such as fish, is generally lacking. Yet many organisms are highly pigmented and the related bioactive pigments are themselves feeding deterrents and/or antifoulants. The role of coloration here may respond to an evolutionary selection for, or retention of, pigmentation driven by predation pressure. As a result, relict pigments originally selected by aposematism or UV-screening are conserved because of their defensive properties. Among these bioactive pigments are the variolins from *Kirkpatrickia variolosa*, discorhabdins from *Latrunculia apicalis*, suberitenones from *Suberites sp.* and those from *Dendrilla membranosa* [reviewed in 65, 84].

The meridianins have been obtained from geographically distinct populations and from several ascidian species: *A. meridianum*, *A. falklandicum* [13, 25, 28] and *Synoicum sp.* [88]. The two *Aplidium* species though, are being revised and might actually be synonymised in the future (M. Tatián, unpublished data). It is intriguing, however, that meridianin D, even if being a majoritary meridianin, has been repeatedly isolated from *A. meridianum* samples but not from other species, maybe representing a specific feature [13, 25, 28, 88]. But, furthermore meridianin A, B and E, have been recently reported to correspond to the so-called psammopemmins A, C and B respectively, described from the Antarctic sponge *Psammopemma sp.* [88, 89]. Thus, the broad existence of these alkaloids in Antarctic animals, along with that of the closely related aplicyanins and variolins [90] could respond, either to symbiotic associations and microbe elaboration, or to co-evolution and retention of biosynthetic pathways of metabolites with adaptive functions (Fig. 2 and 5).

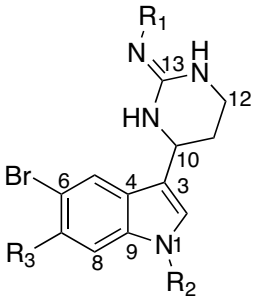
Figure 5. Chemical structures of meridianin-related indole alkaloids obtained from Antarctic marine organisms: Aplicyanins A-F from the ascidian *A. cyaneum*, Psammopemmins A-C from the sponge *Psammopemma sp.* and variolins A, B and D from the sponge *Kirkpatrickia variolosa*.



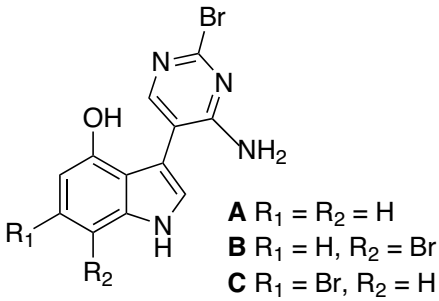
Meridianins A-E



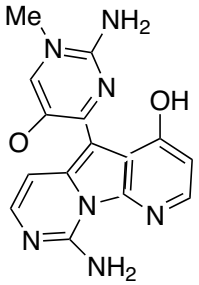
Meridianins F-G



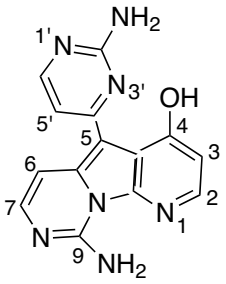
Aplicyanins A-F



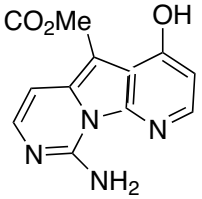
Psammopemmins A-C



Variolins A



Variolins B



Variolins D

The versatility of these alkaloids in terms of ecological functionality justifies the broad acquisition of these metabolites by a number of Antarctic species [13, 25, 28, 88, 89]. A similar situation happens with the tambjamine alkaloids, found in bryozoans and ascidians (and molluscs feeding on them) from a variety of habitats, which are moreover related to bacterial pigments [22, 83]. Meridianins as aminopyrimidine indoles might derive from a pyrimidic base by connection of a pyrimidine ring onto an indole system [87]. In fact, 2-deoxythymidine was detected in our *Synoicum* and *Aplidium* ascidian samples. Actually, nucleosid-derivates are

frequent in *Aplidium* species [78], and maybe precursors of complex secondary metabolites [21, and previous reviews of the series].

5. Conclusions

Defensive strategies of some temperate and Antarctic colonial ascidians were proposed to be highly variable, and to be poorly based on organic chemistry. In lieu, our results indicate that selective pressures for chemical defenses against predation are important in the evolution of Antarctic colonial ascidians, since all the species here analyzed had effective lipophilic deterrents. Moreover many of the samples tended to store more repellent agents into the internal regions of the colony, in particular this was observed in the species *A. fuegiense, A. millari*, and *Synoicum adareanum* orange and B&W colorations. In fact, the isolated deterrent metabolites analyzed from *Aplidium* specimens seem to have different patterns of within-colony allocation, which along with the diverse molecule-type may suggest also a distinct origin. Whereas the rossinones were characteristic of internal tissues, where their synthesis is likely to occur, the meridianins have displayed greater concentrations to the outer regions in one of our previous investigations. The meridianins, moreover, have been found in several ascidian species of the genera *Aplidium* and *Synoicum* and in sponges from Antarctic waters, driving to the suspicion that they might represent relict pigments retained for their multifunctional defensive roles. As many other bioactive alkaloid pigments, the meridianins could be hypothesized to derive from symbiotic microbes. In agreement with other Antarctic surveys with ascidians and sponges, our crude ether extracts exhibited low prevalence of antibacterial properties, even if the meridianin fraction by its own did show inbitory activity to a sympatric bacteria. This represents one of the very few studies in which deterrents were identified and localized in Antarctic ascidians. Further investigations should be undertaken to increase our knowledge in the nature and functioning of chemical defenses in the Southern Ocean.

Acknowledgments

We thank M. Paone, F. Castelluccio, C. Jiménez, S. Taboada, J. Cristobo, B. Figuerola, C. Angulo and J. Moles for their precious support and help in the lab. Thanks are due to S. Catazine for the artwork. Also we are grateful to W. Arntz and the crew of R/V Polarstern. UTM (CSIC), “Las Palmas”, and BAE “Gabriel de Castilla” crews provided logistic sustain. Funding was provided by the Ministry of Science and Innovation of Spain (CGL/2004-03356/ANT, CGL2007-65453/ANT, CGL2010-17415/ANT and CTQ2008-04024/BQU).

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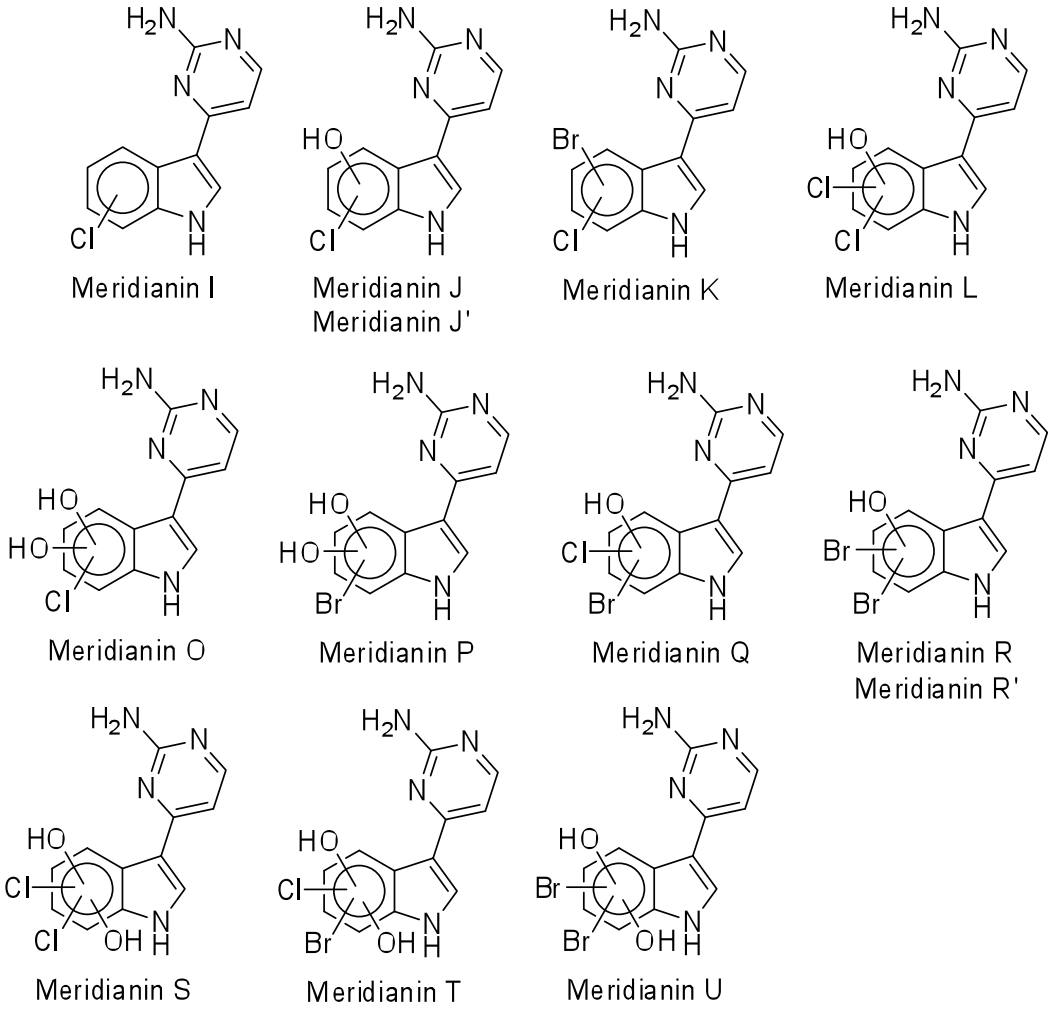
Electronic Supplementary Information



ESI_1 Proposed chemical structures of the new minoritary meridianins I-U detected by LC-HRMS/MS from *Aplidium falklandicum* 1.

Capítulo 3.6. Resumen en castellano de la Publicación VI

Productos naturales de ascidias coloniales antárticas de los géneros *Aplidium* y *Synoicum*: variabilidad y rol defensivo

LAURA NÚÑEZ-PONS, MARIANNA CARBONE, JENNIFER VÁZQUEZ, JAIME RODRÍGUEZ, ROSA Mª NIETO, Mª MERCEDES VARELA, MARGHERITA GAVAGNIN, y CONXITA AVILA. 2012. *Marine Drugs* Submitted.

Resumen

Las ascidias antárticas proliferan en un sistema donde la presión ecológia causada principalmente por invertebrados depredadores es muy intensa. En general las ascidias han desarrollado múltiples estrategias defensivas, en su mayoría relacionadas con propiedades físicas, nutritivas o químicas de la túnica. Una de ellas es la defensa química basada en metabolitos secundarios. En nuestro estudio, analizamos una serie de muestras de ascidias antárticas coloniales de los géneros *Aplidium* y *Synoicum*, para evaluar la incidencia de repelentes orgánicos y su posible variabilidad. Las fracciones etéreas de 15 muestras incluyendo especímenes de las especies *A. falklandicum, A. fuegiense, A. meridianum, A. millari* y *S. adareanum* se utilizaron en experimentos de alimentación utilizando dos relevantes depredadores simpátricos: la estrella de mar *Odontaster validus*, y el anfípodo *Cheirimedon femoratus*. Todas las muestras resultaron repelentes contra ambos depredadores; sin embargo, se observó que en algunas de las colonias existe una tendencia a concentrar las defensas en zonas internas de la colonia y no en la túnica. Cuatro meroterpenoides, rossinones B-E, y los ya conocidos alkaloides indólicos, meridianinas A-G, junto con otras meridianinas minoritarias, fueron aisladas de algunas de las muestras. Algunos de estos metabolitos aislados se utilizaron en los experimentos contra depredadores y revelaron potentes actividades de repelencia, demostrando así su papel ecológico activo frente a la depredación. Los extractos, así como los compuestos aislados, se probaron también en tests antibacterianos contra una cepa antártica marina. En este caso, únicamente las meridianinas A-G mostraron inhibición del crecimiento bacteriano, lo que sugiere un papel multifuncional para estos compuestos. Se discute el posible origen de ambos tipos de metabolitos, los rossinones y las meridianinas, atendiendo a su distribución en la naturaleza, así como a su localización en nuestros especímenes coloniales.

Capítol 3.6. Resum en català de la Publicació VI

Products naturals d'ascídis colonials antàrtiques dels gèneres *Aplidium* i *Synoicum*: variabilitat i funció defensiva

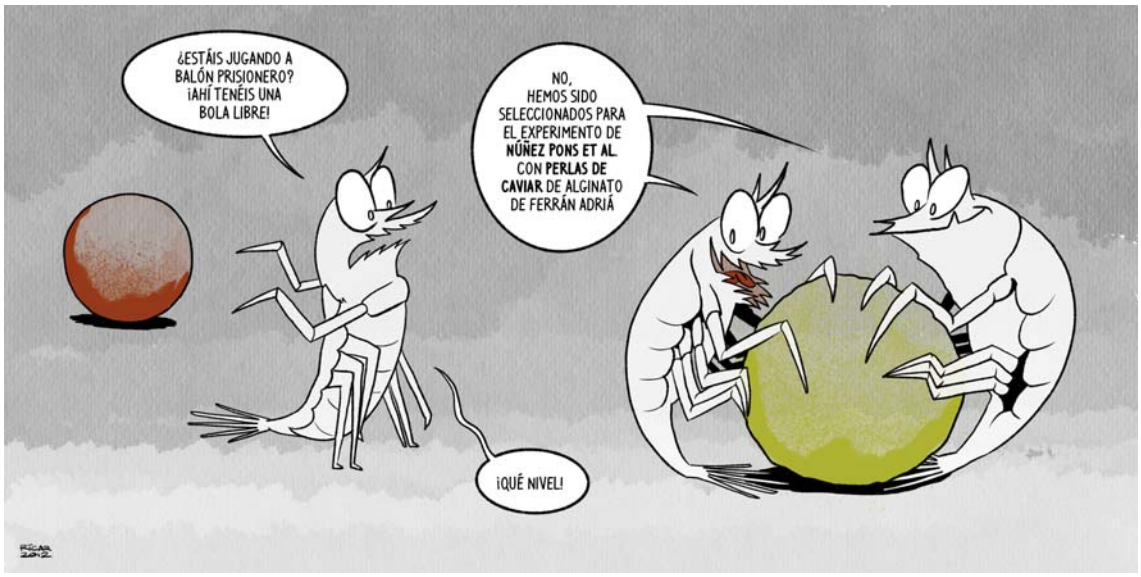
LAURA NÚÑEZ-PONS, MARIANNA CARBONE, JENNIFER VÁZQUEZ, JAIME RODRÍGUEZ, ROSA Mª NIETO, Mª MERCEDES VARELA, MARGHERITA GAVAGNIN, i CONXITA AVILA. 2012. *Marine Drugs* Submitted.

Resum

Les ascídies antàrtiques proliferen en un sistema on la pressió ecològica causada principalment per invertebrats predadors és molt intensa. En general les ascídies han desenvolupat múltiples estratègies defensives, majoritàriament relacionades amb propietats físiques, nutritives o químiques de la túnica. Una d'elles és la defensa química basada en metabòlits secundaris. Al nostre estudi, analitzem una sèrie de mostres d'ascídies antàrtiques colonials dels gèneres *Aplidium* i *Synoicum*, per tal d'avaluar la incidència de repel·lents orgànics i la seua possible variabilitat. Les fraccions etèries de 15 mostres incloent espècimens de les espècies *A. falklandicum, A. fuegiense, A. meridianum, A. millari* i *S. adareanum* es varen utilitzar en experiments d'alimentació emprant dos rellevants depredadores simpàtrics: l'estrella de mar *Odontaster validus*, i l'amfípode *Cheirimedon femoratus*. Totes les mostres varen resultar repel·lents contra ambdós depredadors, malgrat això, es va observar que en algunes de les colònies existeix una tendència a concentrar les defenses en zones internes de la colònia i no en la túnica. Quatre meroterpenoides, rossinones B-E, i els ja coneguts alcaloides indòlics, meridianines A-G, conjuntament amb altres meridianines minoritàries, varen ser aïllades d'algunes de les mostres. Alguns d'aquests metabòlits aïllats varen ser utilitzats als experiments contra depredadors i varen revelar potents activitats de repel·lència, demostrant així ell seu paper ecològic actiu front a la predació. Els extractes, així com els composts aïllats, es varen provar també en tests antibacterians contra una soca antàrtica marina. En aquest cas, únicament les meridianines A-G varen mostrar inhibició del creixement bacterià, el que suggereix un paper multifuncional per aquests composts. Es discuteix el possible origen d'ambdós tipus de metabòlits, les rossinones i les meridianines, atenent a llur distribució en la natura, així com a llur localització als nostres espècimens colonials.

CHAPTER 4.

GLOBAL DISCUSSION



CHAPTER 4. GLOBAL DISCUSSION

The results of this study on the ecology of Antarctic benthic communities cover various aspects of the chemical defense and the use of marine natural products, as mediators of ecologically relevant protective mechanisms. Throughout our study, we integrated chemical and ecological analysis of several groups of marine invertebrates, to achieve a better understanding of the potential of primary and secondary metabolites as defenses, as well as their allocation. As described in the introduction, the investigation of bioactivities within extracts or isolated compounds with realistic ecological value is more intricate in Antarctica respect to other regions, and some groups are still understudied. An important contribution of this thesis is the design of a new protocol for feeding preference bioassays, using a relevant sympatric consumer, to evaluate deterrence in potential prey items. Further accomplishments to be mentioned are the identification and distribution of metabolites participating actively in antipredatory, and occasionally antifouling processes, in hexactinellid sponges, soft corals and colonial ascidians.

Here I comment the results in the frame of the information gathered over these years of research and provide a comprehensive view of the data obtained within the current research on marine chemical ecology. The most significant achievements will be exposed over the five sections that conform this global discussion, while more details are presented in the respective publications. Further work in progress and future perspectives are also explained in the last section of this chapter.

4.1. Detection of repellent defenses through assays against two relevant predators

The evolution of protection to reduce consumption assists prey in their constant battle against predators (Harvell, 1984; Cruz-Rivera and Hay, 2003; Yamauchi and Yamamura, 2005). Generalist feeders ingest a wide assortment of species mitigating possible toxicities of defensive chemicals and compensating poor quality food items. Thus, generalists spread predation through several prey without targeting on a single one, inducing the acquisition of defenses by a wide range of co-existing species (Harvell, 1984; Cruz-Rivera and Hay, 2003; Yamauchi and Yamamura, 2005; Sotka et al., 2009). A classical benthic Antarctic community is considered stable, adapted to marked seasonalities of current regimes and nutrient supply, and composed of many defended organisms with long life-spans subjected to intense generalist predation (Dayton et al., 1974; Amsler et al., 2000a).

When studying predator-prey interactions in ecosystems with difficult accessibility, like Polar ones, the selection for a model predator becomes an intricate task if we want to obtain realistic results. In our case though, easily collectable consumers could be used along with our samples coming from shallow and mostly deep sea-bottoms, thanks to the fact that most potential prey and predator species share a predominant circumpolar and eurybathic distribution (Dayton et al., 1974; Gutt et al., 2000). The selection of the putative experimental predators for this PhD project was fundamental, since they would be used to assess the presence of defensive chemistry in our Antarctic samples, allowing comparable approaches. The lyssianasid opportunistic amphipod *Cheirimedon femoratus* was finally chosen after our search for an appropriate experimental consumer. This species fulfills the criteria of being voracious, ubiquitous and omnivorous, which also characterize the other model predator, *O. validus*. We designed a new protocol for feeding preference bioassays offering caviar-textured alginate pearls to the amphipod. The widespread incidence of deterrence found in invertebrate and algal samples coming from a broad depth range of the Weddell Sea and South Shetland Archipelago, reflected the liability of this generalist consumer as model putative predator. In addition, the new experimental protocol provided many methodological benefits, as well as a great discriminatory potential for deterrent metabolites (Núñez-Pons et al., 2012). This is probably due to the well-developed gustatory gnathopods typical of scavenging lysianassid amphipods (Kaufmann, 1994). Instead, other Antarctic omnivorous amphipods often used by other researchers, for instance *Gondogeneia antarctica*, are problematic as experimental models, for exhibiting preference for artificial foods containing organic extracts, which are phagostimulatory (Amsler et al., 2005; Amsler et al., 2009; Koplovitz et al., 2009). Another aspect to take into account when choosing a predator in Antarctica is the low metabolic rates that characterize this biota (Clarke, 1983; Dayton et al., 1994). A polar consumer may take several days to ingest a food item presented. This greatly affects experimentation, since the offered diets may degrade before consumption takes place, or the assays may last for too long, which may be a limitation for Antarctic campaigns. For instance, other abundant species, such as *Sterechinus neumayeri* and *Nacella concina*, are unsuitable for feeding assessments because their ingestion rates are too low in laboratory conditions (*i.e.* Amsler et al., 2005; Núñez-Pons et al., 2012; and pers. obs.). Hence, the voracity of an experimental predator is another requirement.

The common sea star *Odontaster validus* was selected for our experiments for being a keystone predator (Dayton et al., 1974), and it was fed on diets based on frozen shrimp. This asteroid is the most used Antarctic predator model (reviewed in Avila et al., 2008; McClintock et al., 2010). However, only seldom experiments are done using direct feeding assays measuring the actual ingestion (Avila et al., 2000; Iken et al., 2002; Núñez-Pons et al., 2010; Núñez-Pons

et al., 2012a; Taboada et al., 2012). Even if tests that measure pre-digestive reactions related to the tube-feet may certainly serve to detect the presence of repulsive metabolites (reviewed in McClintock and Baker, 1997a; Avila et al., 2008; McClintock et al., 2010), other mechanisms happening after digestion cannot be appreciable. One of such is the combination effect of nutritional content with chemical defense (Duffy and Paul, 1992; Cruz-Rivera and Hay, 2003; Sotka et al., 2009). For instance, as reported in the results, a moderate-to-poor chemical defense seems to be accompanied by a low nutritional value to reduce palatability in hexactinellid sponges, in some ascidian tunics, or in the axes of the pennatulacean *Umbellula antarctica*. This likely constitutes a strategy of metabolic thrift in hexactinellids and ascidians. Instead, in *U. antarctica* the lack of nematocysts in the stalk may be compensated with the allocation of weak activity to this otherwise undefended region, contributing to the global defense of this coral. The overall acceptance obtained in the tests with *O. validus*, coupled with the significant unpalatability exhibited towards *C. femoratus* in these samples drives us to the conclusion that these specimens must contain poor chemical defense. We base our arguments on the Optimality Theory (OT), which assumes common chemical defenses to deter a variety of co-occurring predators (Herms and Mattson, 1992). Hence, low concentrations of deterrents, and thus weak repellent activities, in some extracts might have been less (or not) evident in the sea star tests because of the higher nutritional quality of shrimp cubes respect to the alginate pearls (by Atwater factors; Atwater and Benedict, 1902). As mentioned in the introduction, more nutritious foods, like shrimp cubes, may interact with deterrents constraining the types or concentrations that could be efficacious, in particular for energy-poor organisms (Duffy and Paul, 1992; Cruz-Rivera and Hay, 2003). Indeed, performing two different assays of direct ingestion, with two different diets and two different predators allowed us to detect interactions between repellency *vs* energy content. For more ecologically realistic results, it would be optimal to perform feeding assays using artificial foods of similar energetic value as the prey organism assayed. In the case of *O.valiudus* however, this sea star does not easily feed on prepared items made with agar, carragenate or alginate (Avila et al., 2000; Iken et al., 2002; and pers. obs.), so this approach is for the moment limited for this model species. Certainly, the type of feeding experiment to be conducted, depending on the activities we want to measure, is also an important choice to consider. As we said, we largely focused in interactions with lipophilic extracts (diethyl ether fraction) rather than hydrophilic extracts (butanolic fraction or water residue) because most of the reported effective repellent secondary metabolites from macroalgae and invertebrates are lipid-soluble (Paul et al., 2007; Sotka et al., 2009). And actually, our data agreed with these postulates, since we found a broad incidence of repellent activities in most of the lipophilic fractions from all the groups assessed. Lipidic defenses appear normally sequestered inside mucous secretions, glands or vesicles (Brown and Bythell, 2005; Avila, 2006), therefore it is unlikely that a fast chemoreception reaction of the predator

prior to ingestion occurs (Sotka et al., 2009). In order to evaluate pre- and post-ingestive responses of consumers, and for dealing with lipophilic metabolites, we decided to conduct direct feeding assays with sufficiently long duration to permit that ingestion could occur. Therefore divergences in methodologies between the present study and previous surveys in Antarctic waters make direct comparisons problematic (for reviews McClintock and Baker, 1997a; McClintock and Baker, 1997b; Avila et al., 2008; McClintock et al., 2010).

Even if the asteroid *Odontaster validus* and the amphipod *Cheirimedon femoratus* have both circumpolar-eurybathic distributions, and voracious, extensive, generalist diets (scavenger, detritivore, planktivore; Bregazzi, 1972; McClintock, 1994; De Broyer et al., 2007), the distinct habits and mode of approaching food items of both predators in nature may promote variable defensive responses in potential prey. In general, sea stars are mobile macropredators that initiate extraoral digestion from the surface of the prey (Sloan, 1980). Amphipods feed on minute peripheral bites, and rarely arrive to internal tissues unless feeding is prolonged in time. However, when the prey have a body with holes, the amphipod's small size may allow them to reach inner regions. Thus, we could compare the data of both deterrent experiments using two predators, and estimate divergent responses of potential prey organisms belonging to 31 species from four major groups; algae, sponges, cnidarians and ascidians, along with a bryozoan, a holothurian and a pterobranch samples. Overall, more deterrent activities were found towards amphipods than against asteroids, principally in fractions coming from algae and sponges, mostly hexactinellids, in which amphipods may especially affect in defense distribution. Actually, no particular within-sponge arrangement of unpalatable activities was observed, in accordance with the low prevalence of defense allocation reported in previous surveys of Peters et al. (2009) with shallow species, and our group for deep-sea Antarctic demosponges (Taboada et al., 2012). This, however, contradicts the predictions of the ODT for Antarctic organisms (Rhoades and Gates, 1976), as well as other findings of sponges showing clear storage of defensive metabolites in the outer layers (Furrow et al., 2003). In the mentioned studies of Peters et al. (2009) and Taboada et al. (2012) actually, there were also a couple of species displaying this pattern of external defense allocation, supposedly as an adaptation to avoid sea star attacks. Amsler and co-workers have ruled out amphipods as a source of significant spongivory and responsible for promoting chemical defense in Antarctic shallow-water demosponges, after observing that sponge extracts stimulated rather than inhibited feeding. But the sponge-associated amphipod used to asses this activity was *Gondogeneia antarctica* (Amsler et al., 2009), which proved to exhibit skewed increased preferences to food containing extracts (Amsler et al. 2005b; Amsler et al., 2009; Koplovitz et al. 2009). This fact may indicate that such assumptions cannot be extrapolated to all amphipod species, and surely not to *C. femoratus*. Moreover, most of the results exposed above refer to demosponges, that are diverse

in essence from glass sponges, which conform the bulk of the sponges analyzed here. Hexactinellids have a particular anatomy respect to demosponges. They are characterized for possessing a volcano shape with conspicuous oscula that allows the entrance of amphipods to inner body regions, where they may reside and feed. In marine ecosystems, biosubstrata offer structural and/or chemical asylum for small crustaceans from predation, like in the case of macrophytes, sponges, and a few others (Kunzmann, 1996; Loerz, 2003; Huang et al., 2007; Amsler et al., 2009; McClintock et al., 2009; Zamzow et al., 2010). Most of the samples tested here from both, Weddell Sea and South Shetland Islands, contained chemical repellents. In fact, except for glass sponges, with a weaker chemical defense system, ascidians and corals were efficiently defended by potent deterrents against both, asteroids and amphipods. Hence, they could represent host-refuges for *C. femoratus* from larger predators, such as prospective fish (Richardson, 1975), as the OT predicts (Herms and Mattson, 1992). Moreover, host organisms represent sources of nutrition, either by direct profit of their tissues, or often by indirect (casual) ingestion when grazing on detritus or associated microbiota, such as fouling diatoms (Kunzmann, 1996; Amsler et al., 2000b; De Broyer et al., 2001; Graeve et al., 2001; Amsler et al., 2009; Zamzow et al., 2010). The sponges here studied for instance, were readily invaded internally and externally by rich diatom populations, thus providing available indirect food sources. Amphipods densely congregate and reside on their living host, constituting a potential threat to which develop defenses, sometimes worse than larger wandering echinoderms or fish (Hay et al., 1987; McClintock and Baker, 2001; Toth et al., 2007). Indeed, the interactions that result from these lax associations between amphipods and biosubstrata depend on the chemical potential of the host, and the feeding habits of the small consumer (reviewed in Sotka et al., 2009). Generalist amphipods associate with chemically defended biosubstrata (Poore et al., 2000), likely because their assorted diets allow them to reduce the consumption of recurrent host repellents (Sotka et al., 2009; Paul et al., 2011). Thus, even if in nature defended organisms maybe foraged fortuitously while profiting other resources (Graeve et al., 2001), in the laboratory, repellence for these tissues can be notable. In fact, the great incidence of unpalatabilities reported in our tests with the opportunistic *C. femoratus* could be related to this phenomenon. Finally, we must consider that hydrophilic metabolites not assessed in this study could also participate in defense, especially towards asteroids that displayed lower rejection levels, as well as the fact that amphipods are reported to be especially susceptible to lipidic deterrents (Sotka et al., 2009).

Once the new methodology using *C. femoratus* proved its validity for chemical defense detection in Antarctic waters, and it was contrasted with the sea star assays, we proceeded to study more specific features of the chemical ecology. This consisted in the elucidation and specific role of some of the responsible defensive natural products in target invertebrate groups:

hexactinellid sponges, soft corals, and colonial ascidians. Lipid-soluble deterrents normally occur in concentrations less than 2% dry mass (Sotka et al., 2009), except in a few cases. One of such exceptional cases is the higher concentrations found for meridianins in the ascidians *Aplidium falklandicum* and *A. meridianum* (Núñez-Pons et al., 2010; results section), as well as the iludalanes in the soft coral *Alcyonium grandis* (Carbone et al., 2009; results section). Here the deterrence cannot be attributed to a specific metabolite, but to the whole mixture of compounds. The production of groups of metabolites that are potentially mimetic based on their similar structures could increase the total concentration, and therefore the signal of the bioactive constituent (Paul et al., 1990; Slattery et al., 1997a; Núñez-Pons et al. 2010). In both cases, for meridianins and illudalanes, the total mixture is very rich, hence, as secondary metabolites representing a metabolic expense, they must play an important role for the animal's integrity. Instead, lipidic primary metabolites used as defenses, like wax esters (**12**-**13**) in *Alcyonium* soft corals, may normally appear in higher concentrations (results section), since they are not costly for the organism because they already possess a vital function, in this case as energy reserves (Sargent et al., 1977). As for the products from hexactinellid sponges, the keto-steroid with mild defensive properties is a primary intermediate metabolite (Núñez-Pons et al., 1012). It was found in quite high concentrations; however, it is still not well understood whether it is just a metabolic end of the degradation route of cholesterol, or if it performs other primary function (Blumenberg et al., 2002). We also found a characteristic mixture of glucoceramides that do not seem to participate in protection against predation, but instead may be useful, as many other lipids, for chemotaxonomical studies.

Secondary metabolites are usually responsible for defensive activities (Paul, 1992), but also sterols, from primary metabolism, provide antifouling and antipredation protection in soft corals, sponges and sea spiders (Bobzin and Faulkner, 1992; Tomaschko, 1994; Slattery et al., 1997a; Fleury et al., 2008; Moran and Woods, 2009; Núñez-Pons et al., 2012a). We also have described this for our Antarctic samples. Production of allelochemicals is costly (Rhoades and Gates, 1976), but this expenditure might be offset by the use of primary metabolites for ecological roles. Actually, although defensive primary metabolites are energetically cheaper, they are also less potent than those from secondary metabolism, and must be combined with other mechanisms to achieve an effective repellence to the producing organism. Thus, in relation with the metabolites found, and in correlation with the ODT, we postulate that our target invertebrate groups maybe combining several types of defensive metabolites along with other tactics, to achieve an overall energy saving strategy of protection. In hexactinellids for instance, it is presumed that metabolic saving is obtained by producing primary metabolite derivatives with weak bioactive defensive properties, coordinated with a poor nutritious value. In soft corals instead, the combination of primary and secondary metabolites provides

repellence under lower cost. Contrastingly, in colonial ascidians the compounds found to actively participate in defense are more often secondary metabolites. However, in some species, defenses are stored in internal regions, following the ODT, because gonadal tissues are apparently more valuable for the overall species survival. This is due to the great investment these clonal organisms address to produce and brood large lecitotrophic eggs and complex larvae, linked to the trend to produce defended larval stages (Lindquist et al., 1992; Lambert, 2005). Thus, the storage of lower amounts of metabolically expensive deterrents in the more exposed tunics, along with these having a poor energy content, constitutes another metabolic saving defensive mechanism, allowing larger concentrations of secondary metabolites to protect internal, more valuable reproductive regions.

4.2. Hexactinellid sponges: weak defense and poor nutritional value

Glass sponges represent an unattractive meal, with a 10% dry mass of organic material (McClintock, 1987; Barthel, 1995). In spite of this, they are readily attacked by some Antarctic benthic macroconsumers, like sea stars and nudibranchs, and foraged by associated mesofauna, including isopods, amphipods, polychaetes and others (Dayton et al., 1974; Dayton, 1979; Barthel and Tendal, 1994; Kunzmann, 1996; McClintock et al., 2005). Actually, hexactinellids constitute rich and accessible resources of sterols for crustaceans that are unable to *de novo* biosynthesize vital steroids, such as ecdysteroid hormones for molting (Goad, 1981; Blumenberg et al., 2002). Moreover, our samples revealed through SEM a rich diatom populations hosted in their tissues, representing an additional food source to be exploited.

Hexactinellids combine low nutritional value provided by the high spicule content with poor lipophilic-based chemical defenses to reduce predation (Duffy and Paul, 1992; Barthel, 1995; Chanas and Pawlik, 1995; Waddell and Pawlik, 2000; Cruz-Rivera and Hay, 2003; Jones et al., 2005). This could also allow more energy to be used for effective regeneration after tissue loss due to foraging episodes, especially by asteroids (Leys and Lauzon, 1998; Walters and Pawlik, 2005; Leong and Pawlik, 2010). But, contradicting previous convictions on glass sponges yielding inactive extracts (McClintock, 1987; McClintock et al., 2005), we obtained significant bioactivity against omnivorous amphipods, which is suspected to arise from products derived from primary metabolism. Porifera have been extensively investigated for their associations with microorganisms (Hentschel et al., 2006), and for being the most prolific marine producers of natural compounds, including: terpenoids, alkaloids, peptides and polyketides, as well as unique sterols and sphingolipids with remarkable chemodiversity (see Blunt et al., 2012 and previous reviews of the series). Nonetheless, there is a growing suspicion that many bioactive chemicals found in sponges could be symbiont-derived, mainly from bacteria and cyanobacteria

(Jayatilake et al., 1996; Taylor et al., 2007; Sabdono and Radjasa, 2008). Secondary metabolism is instead presumed to be poor in hexactinellids, along with an insignificant procariotic symbiosis (Leys et al., 2007). This is consistent with our findings, at least for sponge typical products of lipophilic nature (see Blunt et al., 2012 and previous reviews). The analysis performed with some fractions of our Antarctic glass sponges instead, led to the isolation of two types of rich primary metabolite derivatives, absent in demosponges: a steroid, 5α(H)-cholestan-3-one (**1**), present in most of the glass sponge extracts, and a peculiar glycoceramide mixture (**2**) found in all the hexactinellid samples. Both compounds were obtained from internal and external regions. It is noteworthy the characteristic composition of the ceramide mixture in the species analyzed, containing only two main glucosphingolipids (GSL) –C24 and –C22 fatty acid homologues (**2a-b**), which suggested a possible chemotaxonomical value (see below). Since all demosponges and hexactinellids revealed the same profile of diatom species typical from Austral blooms in the SEM observations, we conclude that none of the isolated lipids were diatom derived.

The steroid 5α(H)-cholestan-3-one displayed a minor antipredatory role, whereas the glycoceramides (**2a-b**), known from a superior plant (Falsone et al., 1987), and now here firstly reported in sponges, had no repellent activity. These ceramides could likely play a role within the syncytial membrane of glass sponges, as similar ceramides do in plants. They were found in all the rossellids from Antarctic and non-Antarctic waters, and could thus serve as molecular markers for the Rossellidae family of sponges. Our data also imply that, to some extent, similar kinds of GSL might be characteristic within the order Lyssacinosida. Since the taxonomic relationships within the phylum Porifera are still under discussion, some investigations on lipidic markers have been carried out to further contribute to sponge taxonomy, being complementary to the classical morphological and molecular biology approaches (Bergquist et al., 1980; Reiswig and Mackie, 1983; Lawson et al., 1984; Bergquist et al., 1986; Bergquist et al., 1991; Thiel et al., 2002; Leys, 2003; Worheide et al., 2012). Thus, since sphingolipids have been already used in chemotaxonomy in microorganisms (Takeuchi et al., 1995), we believe that GSL, such as glycoceramides (**2a-b**), could contribute in the near future to settle the taxonomy of Hexactinellida (Barthel, 1992; Göcken and Janussen, 2011; Janussen, pers. comm.). Contrastingly, the keto-steroid (**1**) is a transient functional metabolite (Smith et al., 1972; Taylor et al., 1981; Blumenberg et al., 2002), probably not useful for chemotaxonomy.

4.3. *Alcyonium* soft corals: a combination of primary and secondary metabolites

Alcyonacean soft corals are rich and accessible prey items (La Barre et al., 1992b). They lack massive carbonate skeletons, while their nematocyst system is weak for defense (Schmidt, 1974; Brusca and Brusca, 2003). Moreover, their sclerites are primarily structural, and are presumably ineffective against Antarctic keystone predators (McClintock, 1994). Indeed, soft corals are believed to majorly rely on the chemistry for protection (La Barre et al., 1986b; Wylie and Paul, 1989; Sammarco and Coll, 1992; Hines and Pawlik, 2012). Accordingly, the current study reports lipid-soluble deterrents originating from both primary and secondary metabolites, which appear to be coordinated to provide a global effective repellence to the colonies. Illudalane terpenoids (**1**-**9**) demonstrated to actively participate as chemical defenses in *Alcyonium grandis*, and wax esters (**12**-**13**) do it in all the *Alcyonium* samples at natural whole-colony concentrations. Both, illudalane terpenoids (**1**-**9**) and the wax compounds (**12**-**13**) seem to cooperate in predation avoidance in *A. grandis*. Illudalanes (**10**-**11**) from *A. roseum* 1 could not be assayed, but due to the great resemblance with the illudalanes (**1**-**9**), we expect them to possess deterrent properties too, and to collaborate in an additive way with waxes as well. Actually, *A. roseum* 1, containing illudalanes (**10**-**11**) showed unpalatability, whereas *A. roseum* 2 lacking these metabolites was palatable, even if both possessed wax esters. This also suggests that wax esters might not be as effective in whole-colony protection without other co-occurring repellents. In the rest of species tested (*A. antarcticum*, *A. haddoni* and *A. paucilobulatum*), we propose a synergistic effect of waxes along with other unreported minor metabolites to achieve effective deterrence. Moreover, three *Alcyonium* samples displayed some sort of inhibition against a marine Antarctic bacterium. Antimicrobial activities against non-associated strains of co-occurring bacteria are common in Antarctic and non-Antarctic soft corals (Ducklow and Mitchell, 1979; Rublee et al., 1980; Slattery et al., 1995; Kelman et al., 1998; Ritchie, 2006).

To the best of our knowledge, only three Antarctic soft coral species have been studied for their chemical ecology (Slattery and McClintock, 1997). *A. paessleri* (synonymized with *A. antarcticum*; Verseveldt and Van Ofwegen, 1992) has been now here again investigated. *A. antarcticum* has demonstrated to possess a broad variety of bioactive agents, working synergistically for several ecological functions (Slattery et al., 1990; Slattery and McClintock, 1995; Slattery et al., 1995; Slattery et al., 1997a; Slattery and McClintock, 1997). Indeed, this species seems to possess a variable secondary metabolite arsenal (Slattery and McClintock, 1997). In fact, in our analysis, *A. antarcticum* did not yield any of the previously reported terpenoids (Palermo et al., 2000; Rodríguez-Brasco et al., 2001; Manzo et al., 2009). Intraspecific variability in the secondary metabolite profile, observed in *A. antarcticum*, and also in *A. roseum*, could respond to different reasons: intraspecific or genetic variability (Harvell et al., 1993), chemical defense induction (Slattery et al., 2001; Hoover et al., 2008), or

symbiotic production (Kelecom, 2002). Illudalanes of the alcyopterosin series, typically found in fungi and ferns (Gribble, 1996; Suzuki et al., 2005), have been reported in the Antarctic deep-sea soft corals *A. paessleri* (*A. antarcticum*) and *A. grandis* (Palermo et al., 2000; Carbone et al., 2009), and now here also in *A. roseum*. All this may reflect a broad evolutionary retention of the metabolic pathway and/or a symbiotic origin of illudalane compounds, along with other soft coral bioactive terpenoids. The defensive terpenoid pukalide could be another example, appearing in several Pacific *Sinularia* species (Wylie and Paul, 1989; Van Alstyne et al., 1994; Slattery et al., 2001), and also in the Antarctic *A. antarcticum* (Manzo et al., 2009).

In soft corals, wax esters are the main storage energy reserves, which decrease in concentration after competitive interactions, due to the cost of the production of secondary metabolites (terpenoids) (Fleury et al., 2004). Hence, if waxes serve as defensive metabolites, this could represent a better optimization of the available metabolic energy. Wax esters might have evolved as lipidic reserves in corals, instead of most common triglycerides, for providing further advantages. Waxes are indigestible (Benson et al., 1978; Place, 1992), and as reported in our results, they can confer unpalatability to the otherwise accessible and energy-rich coral tissues and mucus. Only crown-of-thorns starfishes (*Acanthaster* spp) voraciously feed on living corals because of a unique adaptation: a wax-digesting enzyme system (Benson et al., 1975). Unexpectedly, *C. femoratus* was less sensitive to wax fractions (**12**-**13**) than *O. validus*, probably because Antarctic amphipods use wax esters as reserves, while asteroids lack such compounds (Sargent et al., 1977).

Our soft coral extracts contain a complex mixture of ether-soluble substances (primary and secondary metabolites), obtained from internal tissue but also from mucus. Even if not specifically analyzed here, mucus is essential in protective processes for the underlying coral tissues. It contains wax esters (about 60% of the mucolipid composition), sterols, and seldom mucus-borne terpenes, serving as a medium into which allelochemicals are exuded to fight against predation, fouling and competition (Ducklow and Mitchell, 1979; Coll et al., 1982; Miyamoto et al., 1994; Slattery et al., 1997a; Wang et al., 2008). Compounds **12**-**13** are common marine waxes, due to their function as energy reserves, and for being a major component of the coral mucus, their concentrations maybe very variable, as observed in our samples. The bioactive illudalane terpenoids (**1**-**11**), along with waxes (**12**-**13**), are likely secreted within the mucus in the living species studied, where they may take over their defensive role. Further studies are needed to determine the importance of mucus secretion in Antarctic corals.

4.4. Colonial ascidians: secondary metabolites and intra-colonial allocation

Ascidians are also nutritious and available prey. Regarding possible defensive mechanisms, the specimens of this study lacked mechanical protection afforded by a tough tunic (Varela, 2007; pers. obs.). Likewise, bioaccumulation of acids or heavy metals, employed to dissuade predators, especially in colonial ascidians, has not been reported within their tunic (McClintock et al., 2004; Koplovitz et al., 2009; Koplovitz and McClintock, 2011). Actually, closely related species of the family Polyclinidae report absence of bladder cells (Stoecker, 1980b; Stoecker, 1980a; Hirose, 2001; Lebar et al., 2011). Yet, defensive strategies of some temperate and Antarctic colonial ascidians were proposed to be highly variable, and to be poorly based on organic chemistry (Teo and Ryland, 1994; Tarjuelo et al., 2002; Koplovitz et al., 2009). In lieu, our results indicate that selective pressures for chemical defenses against predation are important in the evolution of Antarctic colonial ascidians, since all the species here analyzed had effective lipophilic deterrents. Moreover, the sea stars bioassays demonstrated that some of the species tend to store more repellent agents into the internal regions of the colony, such as *A. fuegiense, A. millari*, and *Synoicum adareanum* black and white (B&W) morph, as well as two samples of the orange (O) coloration. This suggested that the assumptions of the ODT to concentrate defenses into the outer tissues in Antarctic organisms are inappropriate here, as has been put forward before (Peters et al., 2009; Núñez-Pons et al., 2010; 2012a, b). But as mentioned above, the predominant presence of deterrents in inner tissues (gonads) in compound ascidians is likely related to the production of chemically defended larval stages (Young and Bingham, 1987; Lindquist and Fenical, 1991; Lindquist et al., 1992; Tarjuelo et al., 2002). A combination of low energetic value, along with a weak chemical defense in the more exposed but also less valuable tunics, is presumed to contribute to the overall protection in these colonies, along with internal storage of deterrents, following the assumptions of the ODT. The lower extract yields produced by most tunics respect to inner tissues likely reflect these facts in our samples. Other species, instead, like *Aplidium falklandicum, A. meridianum*, and *S. adareanum* (O) 2 seemed to lack within-specimen defense allocation, possessing secondary defensive metabolites all throughout the colony. In these cases the ODT is not accomplished. Some patterns of within-colony allocation of deterrent activities are correlated with the distribution of active defensive secondary metabolites. *Aplidium falklandicum* and *A. meridianum* were shown to possess protective deterrent chemicals, the meridianins (A-G). Meridianins are present both in inner and outer tissues in quite rich concentrations, even if they seem to be more concentrated in the external zones (Núñez-Pons et al., 2010). Rossinone B proved to take part in the whole-colony chemical defense of *A. fuegiense*, but it was predominant in internal regions.

Our samples were free of evident epibionts, indicating the existence of antifouling agents, like typically revealed in other colonial ascidians (Davis and Wright, 1990; Davis, 1991; Lindsay et al., 1995; Teo and Ryland, 1995; Davis and Bremner, 1999; Bryan et al., 2003). However, in agreement with previous Antarctic surveys with ascidians and sponges (Peters et al., 2010; Koplovitz et al., 2011), our crude ether extracts, as well as the rossinone B, exhibited low prevalence of antibacterial properties. Individually, isolated meridianins did not show antimicrobial activity against cosmopolitan bacteria or yeasts (Núñez-Pons et al., 2010). However, the mixture of meridianins A-G did inhibit the growth of a sympatric marine bacterium, showing to be multipurpose defenses. Like other ascidian alkaloids, the meridianins could be encapsulated in vesicles where they may perform their ecological activity in higher concentrations, avoiding auto-toxicity (López-Legentil et al., 2005; Seleghim et al., 2007). Further analyses are required to ascertain the histological location of meridianins in Antarctic ascidians.

Six species of Antarctic ascidians have been subject to chemical analysis so far, all belonging to the genera *Aplidium* and *Synoicum*. One of such is *S. adareanum*. Indeed, the variable morphologies, bioactivities, and secondary metabolite profile found in several specimens from diverse areas suggest a need for further taxonomical resolution in this species (Diyabalanage et al., 2006; Miyata et al., 2007; Varela, 2007; Koplovitz et al., 2011; our analyses). Another hypothesis is the possible symbiotic origin of some compounds (Riesenfeld et al., 2008). *Aplidium* ascidians are renowned for the variability of the metabolites provided: non-nitrogenous compounds are dominated by prenyl quinones, linear or cyclic, and among the nitrogen containing group, nucleosides, cyclic peptides and a high variety of alkaloids can be mentioned. Moreover, the genus is noted for the propensity to biosynthesize terpene derivatives (Zubía et al., 2005). We found the meroterpenes, rossinones B-E, in *A. fuegiense* (Carbone et al., 2012; and present study). Here, Rossinone B is the majoritary and most active defensive metabolite, found predominantly in inner tissues, but also in the tunic in small amounts. The other minoritary rossinones (C-E) instead, are only present in internal areas of the colony, presumably as precursors. Rossinones A and B were firstly isolated from an Antarctic unidentified *Aplidium* from the Ross Sea (Appleton et al., 2009).

Meridianins are indole alkaloids and were originally described from *A. meridianum* from South Georgia Islands. Seven main meridanins A, B, C, D, E, F and G, were reported, frequently appearing together as a mixture, yet F and G are less abundant (Hernández Franco et al., 1998; Seldes et al., 2007). We reported here their presence in *A. falklandicum* for the first time. From relative chemical quantifications, we confirmed that B/E are the most common meridianins followed by C/D and then A, while F and G are clearly minor compounds. We also assigned the carbon and proton values of meridianins F and G in DMSO (Núñez-Pons et al.,

2010), and reported the identification of new minoritary meridianins (I-U) and some unreported dimeric derivates (Rodríguez et al., in prep.). The meridianin composition in external and internal lipophilic extracts of *A. falklandicum* varied slightly among our samples and compared to *A. meridianum*. It is to note the absence of meridianin D in *A. falklandicum* samples even if being a major indolic metabolite, which could be exclusive of the species *A. meridianum* (Núñez-Pons et al., 2010). Actually, due to the high intraspecific variability of colonial ascidians, *A. meridianum* and *A. falklandicum* might be soon synonymized, and considered as two morphotypes of the same species (Varela, 2007; Tatián, pers. comm.).

The different patterns of distribution of the secondary metabolites in *Aplidium* specimens, along with their diverse molecule-type, may suggest a distinct origin of such compounds. Whereas the rossinones were characteristic of internal tissues, where their synthesis is likely to occur (Carbone et al., 2012), the meridianins have displayed greater concentrations into the external regions (Núñez-Pons et al., 2010). Among the known microorganism-derived products, terpenes are uncommon and indole alkaloids predominate (Paul et al., 1990; Kelecom, 2002; Franks et al., 2005; Bandaranayake, 2006; Ivanova et al., 2007). Furthermore, microsymbionts are usually sited in the tunic of colonial asdidians (Schmidt et al., 2005; and reviewed in Sings and Rinehart, 1996; Hildebrand et al., 2004; Hirose, 2009). The brightly colored yellow meridianins have been found in several ascidian species of the genera *Aplidium* (Hernández Franco et al., 1998; Seldes et al., 2007; Núñez-Pons et al., 2010) and *Synoicum* (Lebar and Baker, 2010), as well as in the sponge *Psammonemma sp*. (Butler et al., 1992; Lebar and Baker, 2010), driving to the suspicion that they might represent relict pigments retained for their multifunctional defensive roles (reviewed in Bandaranayake, 2006). As many other bioactive alkaloid pigments, meridianins are hypothesized to derive from symbiotic microbes (Paul et al., 1990; Franks et al., 2005). Future studies should shed some light into this topic.

4.5. Concluding remarks and future perspectives

Cheirimedon femoratus demonstrated to be a very appropriate model to perform feeding experiments for the detection of chemical defences in Antarctica. Briefly, Antarctic seaweed and sponges that commonly host amphipod populations, with hexactinellids considered energetically scant, yielded apolar extracts that were majorly unpalatable towards *C. femoratus*. As a generalist amphipod, *C. femoratus,* with reduced swimming activity, associates opportunistically with biosubstrata. This leads to a more constant pressure on host-and-prey organisms than more wandering macropredators, like *O. validus*, which focuses on ubiquitous prey with less recurrent encounters. Antarctic poriferans and macroalgae hold high diversities of amphipods, representing potential direct or indirect prey, since host tissues maybe consumed

per se, or being attacked also when feeding on detritus, diatoms or other attached organisms. Thus, in some cases amphipods could replace asteroids as main inducers of defense distribution, questioning the previous expectations of the ODT. In fact, in relation with this, there was a general lack of chemical defense allocation within our sponges. Instead, fractions from ascidians and cnidarians were fairly deterrent to both, sea stars and amphipods. Ascidians and cnidarians are prolific bioactive metabolite producers, which is indeed reflected throughout this study with the elucidation of some of such metabolites. They are considered energy-rich prey items, while they are less remarkable as hosts. Besides, some organisms likely display several anti-predation strategies, sometimes simultaneously, such as nematocysts in some hydrozoan and pennatulacean cnidarians, or nutritiously unattractive tunics in certain ascidians. Also, the benthopelagic swimming activities of the elpidiid holothurian *Peniagone vignioni* (Wigham et al., 2008), the sessile "trap-door" avicularia that can act as traps for small crustaceans and the calcified structure of the bryozoan *Isoschizoporella secunda* (Winston, 1986; Carter et al., 2010), as well as the secreted reinforced encasement of the pterobranch *Cephalodiscus nigrescens* (Ridewood, 1911), are examples of complementary defensive mechanisms presumably used by some of the samples of this study. The divergent results, the majority showing unpalatability only in the amphipod assay, correspond to samples possessing lower amounts of repellents, possibly correlated with poor energetic values. Indeed, the great percentage of coincident activities indicated that apolar deterrents were common, and operative for both consumers, according with the OT (Herms and Mattson, 1992), even if amphipods appear more sensitive.

We believe that the ecological success of our target groups in Antarctic communities is to some extent related to the presence of chemical defenses. In hexactinellid sponges these seem to be weaker yet compensated with a low energetic content, and to derive from primary metabolites. Some GSL instead, could have a chemotaxonomical value as chemical markers in rossellid sponges. In colonial ascidians defensive secondary metabolites with quite potent activities appear to predominate, and in some species these are accumulated in internal tissues, likely for the production of defended larvae. While in soft corals, chemical protection is obtained from products originating from both, primary and secondary metabolism, which seem to cooperate in an additive way. Moreover, these metabolites are likely exuded within the coral mucus in the living specimens, where they take over their defensive function. Some bioactive secondary metabolites isolated from various species, genera and even phylum, from different geographic areas, like the meridianins, suggest a broad evolutionary retention of such products, but also a possible symbiotic origin, and consequent retention of the biotic association for the profitable bioactivities provided. Regarding bacterial fouling, our colonial ascidians exhibited poor antibiotic activity, while some of the soft coral samples did display inhibition.

A latitudinal cline with a higher diversity in marine secondary metabolites in the tropics than in temperate regions was proposed in the past. Regarding polar waters, the research effort has been much lower, and therefore, it is not possible to make any final conclusion yet. Nonetheless, many Antarctic organisms are yielding a notable number of natural products with interesting bioactivities. As far as we know, our studies represent the very few (or the only) Antarctic studies in which ecologically relevant metabolites have been identified in hexactinellid sponges, Antarctic soft corals, and, in colonial ascidians, in these last ones including also intracolonial allocation. With our research, and with this PhD Thesis, we believe we are providing interesting contributions to the Antarctic ecology in the field of protective chemistry through defensive natural products, but also because for the majority of the species here analyzed, almost nothing was known about their ecology until now.

As it is mentioned throughout this Thesis, we are aware of the limitations of testing only lipophilic extracts, and we plan to analyze other fractions as well in our next Antarctic campaigns. Among our next goals the incidence of anti-diatom activity, quite extended in Antarctic invertebrates, will be included. Moreover, there is a general need to extend our studies to the field, and to experiment additional functional and allelochemical roles of compounds. Also, we plan to evaluate if defensive chemistry in Antarctic organisms is static or can be induced in response to ecological constraints, by quantifying the deterrents before and after episodes of attacks. The relationship of nutritional quality *vs* chemical defense is another issue to be addressed. Actually, *C. femoratus* provides the advantage to be fed on artificial prepared foods, making it useful to assess, in further studies, the potential of deterrent metabolites in relation with the energetic content of prepared diets. Finally, mechanisms by which animals are able to discriminate, detect and choose between chemically and non-chemically defended foods are not currently understood, and future investigations on sensory processes and feeding behavior are needed. These might start with simple preference tests, to further combine studies including the fate of deterrents within consumer's tissues (reception, absorption, distribution, metabolism, and excretion), chemical sensory factors (taste), as well as nutrient–deterrent and consumer–prey interactions with respect to generalist versus specialist predators in Antarctica.

CHAPTER 5.

FINAL CONCLUSIONS

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1) Feeding deterrency in Antarctic marine organisms: bioassays with an omnivorous lyssianasid amphipod:

- **1.1.** The lyssianasid amphipod *Cheirimedon femoratus* proved to be an excellent model for the evaluation of unpalatable chemical defenses against predators in Antarctic communities.

- **1.2.** The newly designed protocol provided many methodological benefits to perform feeding preference experiments with simultaneous food choice in Antarctic conditions, with an additional high discriminatory potential for detection of unpalatable activities.

- **1.3.** A large incidence of bioactivities were reported from invertebrates and algae against *C. femoratus*, presumably due to the opportunistic lax associations this species establishes with living substrata, using them both as habitat and potential (direct or indirect) prey.

2) Comparative study of unpalatability in Antarctic benthic organisms towards two relevant sympatric consumers: does it taste matter?:

- **2.1.** Deterrent activities were more frequent towards *C. femoratus* than against *Odontaster validus*, principally in nutritiously-poor samples, and in macroalgae and sponges, in which amphipods may especially affect the predictions of the ODT.

- **2.2.** The localized pressure exerted by sedentary populations of amphipods on their biosubstrata may be more significant in promoting defensive chemistry than the foraging activities of more wandering sea stars. Thus, amphipods could in some cases replace asteroids as main Antarctic predators, which might also explain the poor prevalence of defense allocation in most of the samples.

- **2.3.** Several species assayed could combine different defensive traits to avoid predation, such as low nutritional quality, swimming capability, or the possession of devices like stinging nematocysts, sessile “trap-door” avicularia, or external reinforcements.

3) Chemo-ecological studies on hexactinellid sponges from the Southern Ocean:

- **3.1.** Hexactinellids yielded remarkably higher unpalatable activities towards the amphipod, while no apparent allocation of lipophilic defenses was noted. A combination of low nutritional value and weak chemical defenses probably derived from primary metabolites, along with an enhanced regenerative potential seem to cooperate in glass sponges to fight against sea star predation in Antarctic waters.

- **3.2.** No secondary metabolites, typical of other sponges were detected in our glass sponges.

- **3.3.** Instead, two lipidic products (absent in demosponges) were isolated from our hexactinellids. The steroid 5α(H)-cholestan-3-one demonstrated a minor role as deterrent against *O. validus*, while the glucoceramides **2a-b**, reported previously only in plants and characteristic of all the rossellids of the study (Antarctic and non-Antarctic), are proposed to take part of syncytial structures. They could be chemical markers of the family Rossellidae, serving as chemotaxonomical tools, and along with other similar GLS contribute to the classification of the class Hexactinellida.

4) Chemical ecology of *Alcyonium* soft corals from Antarctica:

- **4.1.** Antarctic *Alcyonium* soft corals make an extended use of lipophilic chemical defenses to fight against predation and, to some extent, against bacterial fouling.

- **4.2.** Illudalanes **1-9** were present in *A. grandis*, and illudalanes **10-11** were now firstly described from *A. roseum*, while wax esters **12-13** were common to all the samples. The illudalane terpenoids, as secondary metabolites, along with the waxes, representing the main energy reserves in corals, and thus primary metabolites, seem to synergistically cooperate in predation avoidance. In other samples with no illudalanes, other minoritary deterrents may cooperate with waxes as well.

- **4.3.** The use of primary metabolites as defenses is an energy saving tactic. In part, the success of corals in marine ecosystems maybe due to the accumulation of indigestible wax in tissues and mucus. It is likely that along with waxes, the illudalanes are exuded within coral mucus to develop there their ecological roles in the living species.

5) Chemical defenses of tunicates of the genus *Aplidium* from the Weddell Sea (Antarctica):

- **5.1.** All ascidian samples possessed defenses towards *O. validus*. The meridianins A-G demonstrated to be responsible for such strong feeding deterrent activities. They were abundant in inner as well as in external body-regions of the specimens analyzed, even if they were more concentrated in the tunic. Antibiotic tests with cosmopolitan microbes instead, revealed no significant activity of the meridianins.

- **5.2.** These indole alkaloids are here firstly reported in *A. falklandicum*. It is noteworthy however, that meridianin D was exclusive of *A. meridianum*, suggesting a characteristic feature of this species. Current taxonomical studies though, may propose to synonymize both species, leaving them as two conspecific morphotypes.

- **5.3.** The carbon and proton assignments were for the first time reported in DMSO for meridianins F and G, along with the relative chemical quantification of meridianins, which showed variable relative concentrations in the different samples.

6) Natural products from Antarctic colonial ascidians of the genera *Aplidium* and *Synoicum*: variability and defensive role:

- **6.1.** All samples from both genera proved to be efficiently defended against *O. validus* and *C. femoratus*. At least in *Aplydium* ascidians this protection is majorly attributed to the presence of deterrent secondary metabolites, such as the meridianin alkaloids in *A. falklandicum* and *A. meridianum*, and the rossinone meroterpenoids in *A. fuegiense*. Such bioactivity was proved in both types of metabolites, although only the isolated meridianin mixture inhibited a sympatric marine bacterium.

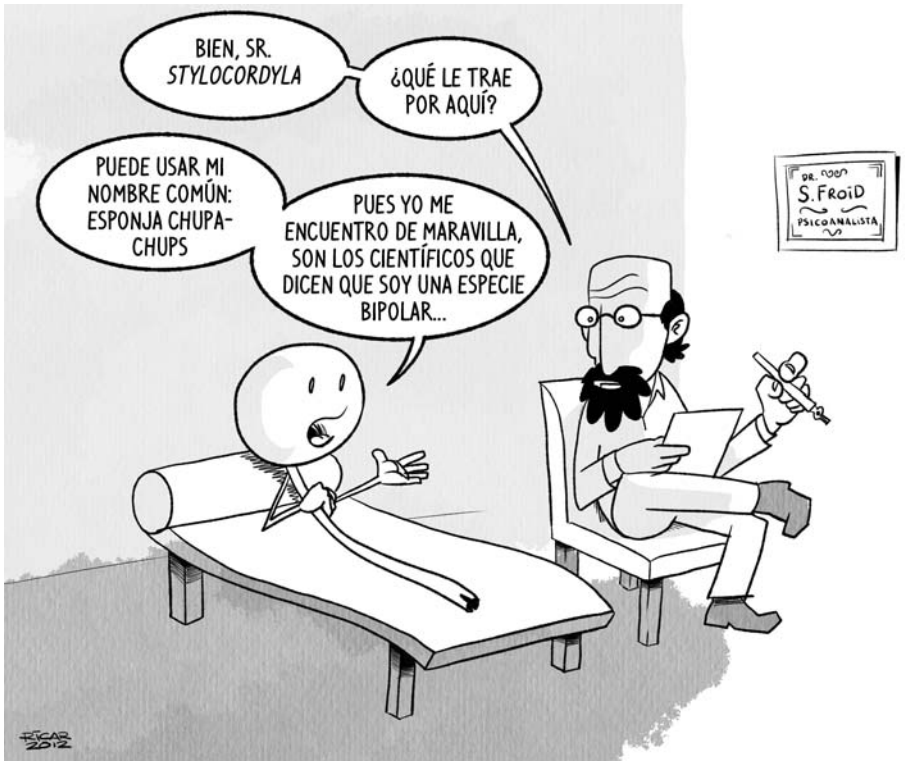
- **6.2.** Some species showed chemical defense allocation, with higher concentrations towards the internal regions, likely for the production of defended larvae. This was observed in *A. fuegiense, A. millari* and *Synoicum adareanum* B&W morph, and in 2 samples of the O coloration. This pattern was correlated with the distribution of the isolated deterrents. While the rossinones are predominant in inner tissues, being likely produced by zooids, the meridianins appear in the whole colony, still in higher quantities in the tunic.

- **6.3.** The meridianins are proposed to be relict pigments retained for their relevant multipurpose ecological activities in several Antarctic species of *Aplidium* and *Synoicum* ascidians. But, as other pigmented alkaloids, they may as well derive from symbiotic microbes.

7) The three studied groups of Antarctic invertebrates appear to rely on chemistry for defense, even if displaying diverse patterns in the usage of primary and secondary metabolites, and in the within-body defense distribution, coordinated with other existing protective mechanisms.

CHAPTER 6.

LITERATURE CITED



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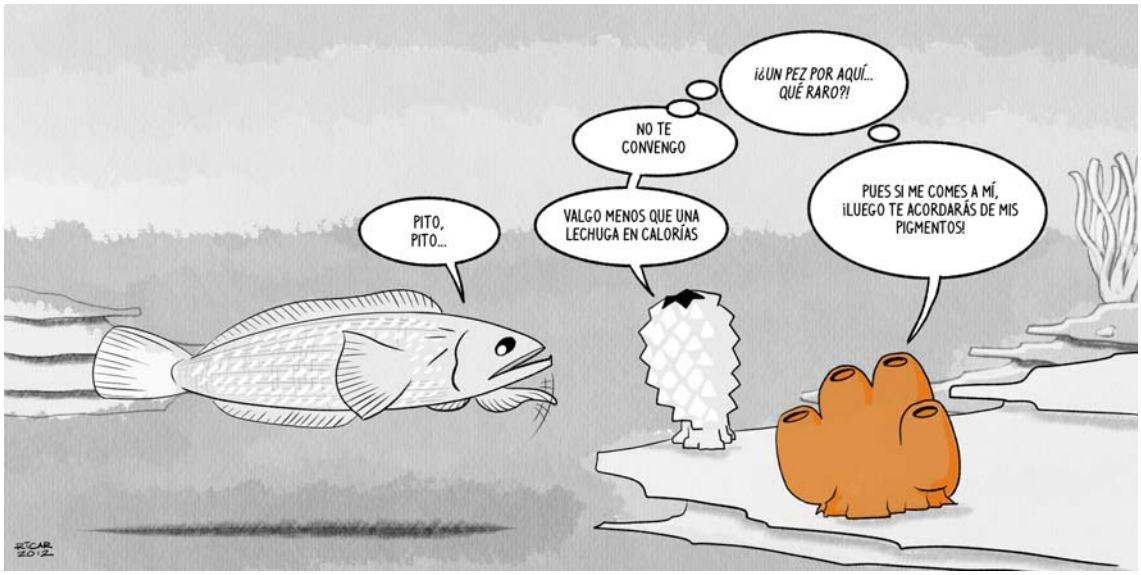
CHAPTER 7.

RESUMEN EN LENGUAS OFICIALES DE LA UB



CHAPTER 7.1. RESUMEN EN CASTELLANO

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7.1.A. Ecosistemas marinos antárticos y ecología química marina

La mayor parte de la fauna antártica evolucionó durante el Cretácico, con la división de Gondwana, que dio lugar a la formación de los continentes actuales, incluida la Antártida (Clarke y Crame, 1989; Crame, 1992). Hace 22 millones de años se estableció la corriente circumpolar, que conllevó el enfriamiento y aislamiento del continente blanco, promoviendo un importante endemismo faunístico (Crame, 1999; Gili et al., 2000). De hecho, la biota antártica está compuesta de fauna autóctona primitiva, fauna euribática originaria de aguas profundas, y especies provenientes de Sudamérica, con la que mantiene un único puente de conectividad a través de las Islas del Arco de Escocia (Brey et al., 1996; McClintock y Baker, 1997a; Brandt et al., 2007; Primo y Vazquez, 2009; Demarchi et al., 2010). Se trata por lo tanto de un bentos muy primitivo que ha convivido lo suficiente como para formar interacciones ecológicas robustas (Aronson et al., 2007; Amsler et al., 2000a).

Los ecosistemas antárticos están caracterizados por sus bajas temperaturas, por la marcada estacionalidad en la disponibilidad de recursos alimentarios y por su estabilidad. Salvada la zona más somera (por encima de los 33m) expuesta a eventos destructivos causados por el hielo (Smale, 2007), las comunidades bentónicas se consideran acomodadas biológicamente, y estructuradas por la depredación y la competencia (Gutt, 2000; Dayton et al., 1974). En la plataforma continental las comunidades antárticas gozan de mucha biodiversidad (Burton, 1932; Koltun, 1970; Dayton et al., 1974; Dayton, 1979; Dayton, 1989; Blunt et al., 1990; Arntz et al., 1997; Brandt et al., 2007), albergando ricas asociaciones de suspensívoros dominadas por esponjas, corales blandos, briozoos, hidroideos y ascidias, además de macroalgas en las zonas fóticas (Arntz et al., 1997; Gutt et al., 2000; Wiencke et al., 2007). En los niveles tróficos superiores encontramos enormes densidades de crustáceos (De Broyer y Jazdzewski, 1996; Huang et al., 2007), así como macroinvertebrados tipo nemertinos, y equinodermos variados (DeLaca y Lipps, 1976; Dearborn, 1977; Gutt et al., 2000; Obermuller et al., 2010), y también peces (Richardson, 1975; Eastman, 1993). Los principales depredadores generalistas del bentos sésil aquí son las abundantes estrellas y nemertinos, además de poblaciones de anfípodos. También hay espongívoros especialistas, como el nudibranquio *Austrodoris kerguelenensis* que se alimenta de hexactinélidas del género *Rossella*, o el asteroideo *Perknaster fuscus*, especializado en *Mycale acerata*, y que junto a *Acodontaster conspicuus* regulan la abundancia de esta esponja colonizadora de los fondos antárticos (Dayton et al., 1974). Durante mucho tiempo se sostuvo que la presión por depredación, junto con las defensas químicas eran

gradualmente menores con el aumento de la latitud. Esta teoría latitudinal consideraba básicamente la depredación causada por peces (Bakus y Green, 1974), que en la Antártida es relativamente baja, pero que es sustituida por otra muy intensa provocada por macroinvertebrados, principalmente estrellas de mar (McClintock, 1989; Baker et al., 1993; Amsler et al., 2000a; McClintock y Baker, 2001; Avila, 2006). A parte de esto, ciertos dispositivos eficaces defensivamente contra peces en los trópicos, como pueden ser los escleritos o las espículas, en la Antártida no parecen serlo, dado que los principales depredadores aquí practican otros hábitos alimenticios, como las estrellas, que realizan una pre-digestión extra oral (Hyman, 1955; Sloan, 1980). En efecto, la incidencia de defensas químicas ha demostrado ser muy elevada entre los organismos antárticos (Taboada et al., 2012; y revisado en Amsler et al., 2000a; Avila et al., 2008; McClintock et al., 2010), hecho con el cuál concuerdan nuestros resultados (Capítulos 3.1 y 3.2).

La intermitencia en la entrada de alimento en los sistemas antárticos hace que la acumulación de lípidos de reserva en forma de triglicéridos o ceras, juegue un papel importante (Sargent et al., 1977). Por esta misma razón, además, los organismos antárticos, ya sean suspensívoros sésiles, así como especies vágiles del fondo, incluyendo a los principales depredadores, han desarrollado hábitos oportunistas (Bregazzi, 1972; Dayton et al., 1974; Arnaud, 1977; McClintock, 1994; Orejas, 2001; Orejas et al., 2001; Tatian et al., 2002; Orejas et al., 2003; Tatian et al., 2004; De Broyer et al., 2007). Además, las comunidades antárticas suelen carecer de zonación faunística, siendo mayormente circumpolares y euribáticas (Dell, 1972; Arnaud, 1977; White, 1984), lo que hace que las especies dominantes compartan junto con sus depredadores hábitats tanto profundos como someros (Dayton et al., 1974; McClintock, 1994; Gutt et al., 2000). Esto facilita mucho el estudio de ecosistemas de difícil acceso como el que aquí se ha estudiado, dado que nuestras muestras son en su mayor parte de fondos profundos, con lo que este hecho faunístico nos permite probar nuestras muestras con organismos de poca profundidad sin perder rigor ecológico. Además, hemos de tener en cuenta que los científicos antárticos estamos limitados a realizar nuestros experimentos en las bases o barcos disponibles durante un tiempo limitado. En nuestro caso, la experimentación se llevó a cabo en la (BAE) Gabriel de Castilla, en la Isla Decepción, Islas Shetland del Sur (62º 59.369' S, 60º 33.424' W).

En lo referente a eventos de epibiosis, y ligado con los intensos “blooms” estivales de macroalgas, en aguas australes la invasión causada por diatomeas parece sobrepasar aquella bacteriana (Slattery et al., 1995; Amsler et al., 2000b; Bavestrello et al., 2000; Cerrano et al., 2000; Peters et al., 2010; Koplovitz et al., 2011), a diferencia de otras latitudes (Cervino et al., 2006). Pero también se han descrito asociaciones simbióticas entre esponjas y diatomeas, mucho más comunes en especies antárticas que en otras zonas (Gaino et al., 1994; Cattaneo-

Vietti et al., 1996; Hamilton et al., 1997; Cerrano et al., 2004a; Cerrano et al., 2004b; Taylor et al., 2007). En el Capítulo 3.3 abordamos este tema indirectamente con fines metabólicos, dado que las diatomeas podrían proveer a las esponjas de productos característicos (Gaino et al., 1994; Cerrano et al., 2004a; Cerrano et al., 2004b).

Los organismos marinos al estar sometidos a una constante presión ecológica, causada por la depredación, la competición por el espacio y los recursos, así como por eventos de recubrimiento por epibiontes (Barnes y Hughes, 1988), han de desarrollar una serie de mecanismos de defensa. Estos mecanismos pueden ser adaptaciones de tipo ecológico (selección del nicho), comportamental (hábitos nocturnos), o fisiológico (optimizando sus ritmos reproductivos y/o de crecimiento). Existen también formas de protección física, como esqueletos externos o internos (conchas, espinas, espículas o escleritos), o renovación constante de capas superficiales de tejido o mucus, etc. Y además existen defensas químicas, que incluyen agentes tóxicos o repelentes, que suelen derivar del metabolismo secundario (Paul, 1992; Eisner y Meinwald, 1995; McClintock y Baker, 2001). No obstante, existen casos de metabolitos primarios con propiedades defensivas (Bobzin y Faulkner, 1992; Tomaschko, 1994; Slattery et al., 1997a; Fleury et al., 2008; Moran y Woods, 2009; Núñez-Pons et al., 2012a). De hecho, en nuestras investigaciones encontramos ambos tipos de metabolitos causando repelencia (Capítulo 3.3 y 3.4).

La actividad más estudiada en ecología química es la de defensa contra la depredación, y normalmente los depredadores generalistas, que son los más abundantes, son más susceptibles a metabolitos secundarios, en su mayoría de naturaleza lipofílica (Paul, 1992; Eisner y Meinwald, 1995; McClintock y Baker, 2001; Sotka et al., 2009). En este sentido, durante este proyecto de doctorado nos hemos centrado en el estudio de las actividades y los agentes químicos contenidos en las fracciones lipofílicas más apolares de nuestros especímenes, o sea aquellos contenidos en los extractos etéreos, dejando otras fracciones para futuras investigaciones. En las comunidades bentónicas, aquellos organismos sésiles, de cuerpo blando y de tipo clonal, como esponjas, octocorales y ascidias, son los que predominantemente se defienden químicamente contra diversos tipos de depredadores (ver revisiones de Paul, 1992; Pawlik, 1993; Hay, 1996; McClintock y Baker, 2001; Paul et al., 2011). La producción de metabolitos secundarios es energéticamente costosa y los organismos han de compensar estos costes con aquellos destinados al mantenimiento, crecimiento y reproducción, lo que ha llevado a la elaboración de una serie de teorías de gestión y ahorro energético (Coley et al., 1985; Cronin, 2001). La más ampliamente aceptada es la Teoría de Defensa Optimizada (ODT; Rhoades y Gates, 1976), que contempla que la producción de defensas químicas debe ir correlacionada con el riesgo de ataque, y que debe existir una distribución anatómica diferencial de las mismas hacia estructuras más valiosas o más expuestas a depredadores. Otras teorías de ahorro energético también

plantean el uso polivalente de mecanismos defensivos, comunes contra varios depredadores (Teoría de la Optimización, OT; Herms y Mattson, 1992), o en el caso del Modelo de Defensa Inducible (IDM; Harvell, 1990), la producción de metabolitos defensivos estaría directamente correlacionada con el riesgo de ataque. En efecto, muchos organismos marinos son capaces de producir compuestos defensivos, o incrementar su concentración tras episodios de depredación sobre sus tejidos (Cronin y Hay, 1996; Toth et al., 2007; Thoms et al., 2006; Thoms y Schupp, 2008; Slattery et al., 2001; Hoover et al., 2008; Lindquist, 2002). En la Antártida sin embargo, los procesos de defensas químicas inducibles no han sido demostrados todavía (Avila et al., 2008; McClintock et al., 2010). A pesar de que las defensas químicas actuando como repelentes alimentarios han sido ampliamente reconocidas, los mecanismos que promueven el rechazo en el depredador no se conocen aún. En general las defensas contra la depredación están más relacionadas con el mal sabor que con la toxicidad (Paul, 1992; McClintock y Baker, 2001). Otro factor a tener en cuenta en conjunto con las defensas químicas es el valor nutricional, pues algunos repelentes son más (o sólo) efectivos en combinación con comidas de poca calidad energética y viceversa; las dietas nutritivas pueden enmascarar la actividad repelente (Duffy y Paul, 1992).

Como mencionamos anteriormente, otro desafío al que están sometidos los organismos marinos es al recubrimiento epibiótico y a la invasión de microbios patógenos. De hecho las defensas contra el recubrimiento están bastante extendidas en el bentos marino (Fusetani, 2004; Paul et al., 2011). Los procesos de recubrimiento son sucesiones ecológicas, y comienzan con la adsorción de macromoléculas y la colonización bacteriana. Por ello, el evitar la formación de estas películas iniciales resulta una estrategia efectiva para evitar posteriores eventos (Zobell y Allen, 1935). En nuestros modestos estudios acerca de actividades para luchar contra el recubrimiento nos basamos en tests antibióticos contra bacterias marinas del entorno.

A la hora de realizar experimentos para estudios de ecología química, es importante elegir bien el parámetro con el que vamos a calcular la concentración natural de nuestros extractos, fracciones o compuestos aislados a probar, dependiendo de la actividad que vamos a investigar, y de las especies implicadas. Los parámetros más usados son el volumen, el peso seco y el peso húmedo. Pero siempre hemos de tener en cuenta que el cálculo de la concentración natural en un espécimen, aunque sea diseccionado, será una aproximación, y que nunca podrá mimetizar lo que realmente ocurre en la naturaleza, debido a fenómenos como la distribución diferencial o encapsulamiento de metabolitos en determinadas estructuras. Teniendo en cuenta estas limitaciones y por trabajar con muestras acuáticas, hemos utilizado para nuestros cálculos el peso seco. De este modo, al eliminar la humedad evitamos desviaciones importantes que se pueden originar en organismos porosos y de tejido blando como las esponjas, los corales o las ascidias. Además, para conseguir resultados ecológicamente reales y válidos es importante

utilizar organismos experimentales simpátricos, que compartan hábitat con las muestras que queramos examinar, de lo contrario estaremos obteniendo indicios de una bioactividad, la cual carece de valor ecológico relevante (Paul et al., 2007). En este aspecto nuestros experimentos, fueron siempre realizados *in situ* (en la Antártida) y con organismos simpátricos.

7.1.B. Productos naturales marinos y defensa química en el ámbito antártico

Existe un gran número de productos naturales reconocidos, que se agrupan en clases, entre ellos los poliquétidos, terpenos, hidroquinonas, depsipéptidos y los más numerosos, los alcaloides. También, aunque menos comunes, encontramos derivados de metabolitos primarios, a saber nucleósidos, carbohidratos, esteroides y ácidos grasos (Blunt et al., 2012 y revisiones anteriores). Los metabolitos secundarios provienen de la dieta, o pueden ser biotransformados a partir de precursores, o bien pueden ser sintetizados *de novo* (Paul, 1992; McClintock y Baker, 2001). Sin embargo recientemente se ha levantado la sospecha de que muchos de los metabolitos bioactivos aislados de invertebrados marinos sean producidos por microorganismos asociados, dado que muchos poseen ricas poblaciones de microsimbiontes en sus tejidos (revisado por Kobayashi y Ishibashi, 1993; Hildebrand et al., 2004; Piel, 2009). Aunque probablemente sesgado por los intereses y las técnicas usadas por cada químico, en general a cada filo le caracterizan una serie de tipos de productos; por ejemplo, a los cnidarios los terpenoides; las esponjas, que son el grupo más estudiado, han proporcionado terpenoides y metabolitos nitrogenados, y las ascidias suelen poseer derivados de aminoácidos (Davidson, 1993; Blunt et al., 2012). En este sentido algunos metabolitos, sobretodo lípidos, son útiles para estudios quimiotaxonómicos (Bergquist et al., 1991; Thiel et al., 2002; Berge y Barnathan, 2005; Imbs y Dautova, 2008). En el Capítulo 3.3 aportamos una modesta contribución de este tipo de compuestos en esponjas hexactinélidas. Ciertamente, se han descrito muchísimos metabolitos secundarios, que no participan en procesos primarios, pero para muy pocos se conoce la función ecológica que desempeñan. De hecho, muchos compuestos se evalúan para bioactividades con fines farmacológicos, si bien su significado para el propio organismo queda de lado (Munro et al., 1987; Scheuer, 1990; Hay y Fennical, 1996; Taboada et al., 2010). Entre las funciones ecológicas que se han encontrado están la toxicidad, la repelencia alimentaria, la inhibición del recubrimiento y/o infección, y la mediación en procesos de competencia espacial (Paul, 1992; McClintock y Baker, 2001; Avila et al., 2008; Blunt et al., 2012).

El grueso de los estudios en ecología química antártica se ha llevado a cabo evaluando actividades de repelencia contra depredadores con organismos de aguas someras (accesibles mediante buceo) en las zonas de McMurdo Sound (Mar de Ross) y el oeste de la Península Antártica, y se basan mayormente en los trabajos de McClintock y colaboradores. Mientras, las

regiones de algunas islas sub-antárticas y zonas profundas del Mar de Weddell están empezando ahora a ser también investigadas. Por el contrario, las áreas del este de la Antártida, y los Mares de Amundsen y Bellingshausen son prácticamente desconocidos en estos ámbitos (McClintock y Baker, 1997a; Lebar et al., 2007; Avila et al., 2008; McClintock et al., 2010; Taboada et al., 2012). A pesar de todo, dada la distribución general circumpolar y euribática de la biota (Dell, 1972; Arnaud, 1977; White, 1984), se considera que los conocimientos adquiridos son bastante aplicables a amplias zonas de la Antártida. Por lo pronto, en lo referente a agentes químicos defensivos, se ha observado que éstos son frecuentes en organismos antárticos pertenecientes a los principales grupos taxonómicos (Avila et al., 2008; McClintock et al., 2010; Taboada et al., 2012), lo cual va en concordancia con los grupos que se han estudiado en la presente tesis (capítulos 3.1 y 3.2). No obstante, sólo en contadas ocasiones se han identificado las moléculas activas (Núñez-Pons et al., 2010; Núñez-Pons et al., 2012a; y anteriormente revisado en Avila et al., 2008). El mayor número de metabolitos con función defensiva han sido aislados de esponjas. En este sentido, cabe recalcar que muchos de estos compuestos son pigmentos repelentes y responsables de los vivos colores que caracterizan a las esponjas que los proporcionan. En principio, en la Antártida no tiene sentido la presencia de coloraciones aposemáticas (de aviso) por ser un sistema cuyos depredadores principales (las estrellas) se orientan químicamente, y por ser pocos o inexistentes los depredadores visuales, tipo peces o tortugas. Se plantea aquí que estas sustancias puedan representar pigmentos vestigiales, que en su día podrían haber tenido un valor aposemático cuando el clima era más cálido y convivían con otros depredadores, y que se han mantenido evolutivamente por sus propiedades bioactivas inherentes (revisado en Bandaranayake, 2006; Avila et al 2008; McClintock et al., 2010). Un fenómeno similar se propone en algunas de nuestras ascidias coloniales en el Capítulo 3.6. Otros metabolitos con actividad defensiva en organismos antárticos se han obtenido de algas, corales, moluscos (Avila et al 2008; McClintock et al., 2010; y referencias allí incluidas) y recientemente como parte de esta tesis en asdicias (Núñez-Pons et al., 2010; Capítulos 3.5 y 3.6).

Las predicciones de la ODT tienen el cuenta el tipo de depredador y de la presa, además de otros mecanismos de defensa alternativos. Se ha planteado que los organismos en la Antártida deberían concentrar sus defensas en las zonas externas, donde serían más efectivas contra los principales depredadores (Rhoades y Gates, 1976), entre ellos las estrellas que se alimentan evaginando el estómago sobre su presa (Sloan, 1980). Pero en organismos perforados, consumidores de pequeño tamaño como los anfípodos capaces de acceder a tejidos internos, podrían promover otro tipo de distribución. En concreto, las hexactinélidas antárticas, que constituyen una parte importante de nuestras muestras (Capítulo 3.3), y poseen forma de volcán y grandes ósculos, son claros ejemplos (Núñez-Pons et al., 2012a). Pero además, hay que tener

en cuenta los ciclos vitales en algunos grupos. Por ejemplo, en las ascidias coloniales existe una tendencia a producir larvas protegidas químicamente contra depredadores, por lo que las defensas químicas suelen aparecer en tejidos internos, en las gónadas (Lindquist et al., 1992). En la Antártida se ha descrito la localización de defensas en el manto de algunos opistobranquios (Avila et al., 2000; Iken et al., 2002), pero sobretodo en esponjas se han encontrado varias especies con repelentes concentrados en las capas superficiales (*i.e.* Furrow et al., 2003; Peters et al., 2009), aunque también hay otras especies que no exhiben una clara distribución de las defensas químicas (Peters et al., 2009). Uno de nuestros recientes trabajos también aborda el tema de la localización de las defensas en un amplio rango de grupos zoológicos (Taboada et al., 2012). En los trabajos de la presente tesis las predicciones de la ODT (Rhoades y Gates, 1976) son siempre consideradas, y la distribución de sustancias repelentes se ha estudiado en aquellas muestras que lo permitieron por tamaño, forma y tipo de organismo. Pero la presencia de productos bioactivos en zonas externas puede cumplir otras funciones, como la inhibición de recubrimiento, o la mediación de interacciones aleloquímicas (Rhoades, 1979; Paul, 1992; Slattery y McClintock, 1997; McClintock y Baker, 2001; Avila et al., 2008; McClintock et al., 2010). Por ejemplo, se ha observado que una especie de coral blando antártico del género *Alcyonium* es capaz de inducir necrosis por contacto en la esponja colonizadora *Mycale acerata*. Esto junto con los potentes agentes antirecubrimiento descritos en esta especie, que al parecer son liberados al agua circundante, sugiere la presencia de propiedades ecológicas importantes en el mucus superficial de estos corales (Slattery y McClintock, 1997), como sucede en corales de otras latitudes (Brown y Bythell, 2005). De hecho, los corales blandos y las ascidias coloniales carecen de recubrimiento evidente por epibiontes (revisado en McClintock et al., 2010; obs. pers.). En recientes trabajos se ha detectado una actividad antibacteriana escasa en ascidias y esponjas, respecto a aquella relevante descrita en corales blandos. Sin embargo los tres grupos poseen potentes inhibidores contra diatomeas, indicando que éstas pueden ser más influyentes que las bacterias en altas latitudes (Slattery y McClintock, 1997; Peters et al., 2010; Koplovitz et al., 2011). Nuestros modestos tests en este tópico evaluaban actividades inhibitorias contra bacterias marinas del entorno.

En resumen, la ecología química marina dibuja un mapa en el que gran parte del conocimiento adquirido proviene de ecosistemas someros tropicales y templados, cuya mayor accesibilidad permite establecer relaciones ecológicas fácilmente (Paul, 1992, McClintock y Baker, 2001; Avila et al., 2008; McClintock et al., 2010). Las áreas polares por su aislamiento geográfico y duras condiciones han recibido mucha menos atención (Lippert, 2003; Avila et al., 2008; McClintock et al., 2012). En las aguas del Polo Sur la mayor parte de las investigaciones se focalizan en la presencia de repelentes para evitar la depredación, y los grupos más

estudiados son las macroalgas y las esponjas. Dentro de las esponjas sin embargo, casi todas las especies investigadas son demosponjas y a pesar de que las hexactinélidas sean uno de los componentes formadores de los fondos marinos antárticos, no se sabe casi nada sobre ellas en este campo (Avila et al., 2008; McClintock et al., 2010). En efecto, a parte de nuestra reciente publicación (Núñez-Pons et al., 2012a) presentada en el Capítulo 3.3, sólo existe otro trabajo con esponjas hexactinélidas que relaciona su contenido nutricional y espicular con la defensa química (McClintock, 1987). Otros de los grupos más estudiados son los moluscos y las ascidias, estas últimas gracias a dos trabajos recientes que revelaron escasas defensas químicas contra la depredación y la colonización bacteriana, tanto en especies solitarias como clonales (Avila et al., 2008; Koplovitz et al., 2010; 2011; McClintock et al., 2010). El siguiente grupo que ha recibido más consideración son probablemente los cnidarios, seguidos por otros organismos (Avila et al., 2008; McClintock et al., 2010). Pero la mayoría de trabajos con cnidarios son puramente químicos, de aislamiento e identificación de moléculas (Slattery et al., 1994; Slattery et al., 1997b; Palermo et al., 2000; Rodríguez-Brasco et al., 2001; Gavagnin et al., 2003; Iken y Baker, 2003; Carbone et al., 2009; Manzo et al., 2009; y revisado en Avila et al., 2008). De hecho, tan sólo 3 especies de corales blandos han sido analizadas desde el punto de vista de sus defensas químicas, demostrando un gran y polivalente arsenal (Slattery y McClintock, 1997). En los últimos meses, nuestro grupo ha finalizado una extensa investigación sobre la incidencia de defensas químicas y su localización en un amplio grupo de invertebrados antárticos. Este estudio representa una contribución interesante a la ecología química antártica porque las muestras examinadas procedían de fondos profundos del Mar de Weddell e Isla de Bouvet, con lo que muchas especies no habían sido nunca investigadas (Taboada et al., 2012). Por tanto, el escenario que deja la ecología química antártica es el de una biota ampliamente protegida mediante defensas químicas, pero con muchos grupos aún claramente infraestudiados. Además, la identidad de los productos bioactivos y ecológicamente responsables, junto con aspectos en su distribución, su modo de operar en interacción con otras moléculas, y su origen se encuentran todavía en su más pronta infancia (Avila et al., 2008; McClintock et al., 2010). Por todo ello, y con el fin contribuir a la ecología antártica y a cómo funcionan los mecanismos de defensa a través de sustancias orgánicas, este proyecto de tesis se ha focalizado en estudiar organismos relevantes del bentos antártico, presumiblemente portadores de defensas químicas, y con poca investigación previa. Entre ellos, hemos seleccionado las esponjas hexactinélidas, los corales blandos y las ascidias coloniales.

7.1.C. Defensas químicas contra dos relevantes depredadores antárticos

La selección de los depredadores experimentales es fundamental para obtener resultados realistas como hemos visto, pero también porque, en nuestro caso, fueron los que, a lo largo de este proyecto de tesis, utilizamos para evaluar la presencia de actividad defensiva de repelencia alimentaria. Los mecanismos de defensa química en la Antártida se han demostrado mediante varios tipos de experimentos y con diferentes posibles depredadores. En experimentos de alimentación directa, por ejemplo, se han usado peces, anémonas y anfípodos, ofreciéndoles tejidos frescos de la presa, o bien dietas artificiales de agar incluyendo los extractos de los organismos a probar (*i.e*. McClintock et al., 1991; McClintock et al., 1992; McClintock et al., 1993; Slattery y McClintock, 1995; McClintock y Baker, 1997b; Koplovitz et al., 2009). Por el contrario, con equinodermos, que son los principales depredadores antárticos (estrellas; Dayton et al., 1974), principalmente se han explotado las capacidades quimioreceptivas de sus pies ambulacrales (Sloan, 1980; McClintock, 1994) mediante tests de corta duración que evalúan las reacciones de los mismos, sin que llegue a darse la ingestión de la propia dieta presentada conteniendo los extractos (*i.e*. McClintock, 1987; McClintock et al., 1990; McClintock et al., 1992; McClintock et al., 1993; McClintock et al., 1994a; McClintock et al., 1994b; McClintock et al., 1994c; Slattery y McClintock, 1995; McClintock y Baker, 1997; Slattery et al., 1997a; Slattery y McClintock, 1997; McClintock et al., 2000; Amsler et al., 1999; Koplovitz et al., 2009; Peters et al., 2009). Los trabajos existentes con experimentos de alimentación efectiva usando estrellas de mar antárticas, en concreto *Odontaster validus*, se limitan básicamente a aquellos desarrollados por nuestro grupo (*i.e.* Bryan et al., 1998; Avila et al., 2000; Iken et al., 2002; Núñez-Pons et al., 2010; Núñez-Pons et al., 2012a; Taboada et al., 2012). En efecto, discrepancias en las actividades de algunas especies pueden ser debidas a las distintas metodologías y/o depredadores usados. En nuestra opinión los métodos donde se valora la ingestión efectiva y no sólo las primeras reacciones, son más apropiados, pues permiten valorar respuestas que pueden darse después de la ingestión, como la interacción entre el valor nutricional y los repelentes presentes en una presa. Además, nuestro estudio se ha focalizado en la fracción más apolar (extracto etéreo) de nuestras muestras, dado que la mayoría de los repelentes marinos descritos son de naturaleza lipofílica. Éstos suelen aparecer secuestrados dentro de vesículas o tejidos, lo que dificulta una recepción de los mismos antes de la ingesta (Sotka et al., 2009). Somos conscientes de las limitaciones que tiene estudiar sólo estas fracciones, y de hecho los extractos butanólicos y residuos acuosos los conservamos para futuras investigaciones.

La abundante estrella de mar *Odontaster validus*, con distribución circumpolar y euribática (Dearborn, 1977; McClintock et al., 1988; Dearborn et al., 1983), y de hábitos omnívoros oportunistas (Dearborn, 1977; McClintock, 1994), ha sido extensamente usada como

depredador modelo experimental (para revisiones consultar Avila et al., 2008; McClintock et al., 2010), y ha sido también seleccionada para realizar parte de los experimentos de esta tesis. En la búsqueda de otro depredador experimental relevante en las comunidades bentónicas Antárticas, consideramos la influencia de las poblaciones de anfípodos, las cuáles exhiben una altísima diversidad, tanto en el número de especies, como en los estilos de vida y hábitos alimenticios (De Broyer y Jazdzewski, 1996). Además aparecen en densidades muy elevadas (300,000 individuos m^{-2}; Huang et al., 2007) asociados a sustratos vivos (con frecuencia algas y esponjas), que hacen de huéspedes y potenciales presas (directas o indirectas). Representan por tanto un grupo interesante con el que estudiar la incidencia de defensas repelentes en organismos sésiles. De hecho, ya se han probado algunas especies como consumidores modelo, pero las más usadas, *Gondogeneia antarctica* y *Paramoera walkeri,* muestran limitaciones, bien por ser herbívoras acotando su uso a algas, o bien por mostrar preferencia por comidas preparadas conteniendo extractos (Amsler et al., 2005). Entre otras especies, el anfípodo lyssianásido *Cheirimedon femoratus*, por ser abundante y voraz oportunista omnívoro, y por tener distribución circumpolar y euribática (Bregazzi, 1972; De Broyer et al., 2007), fue finalmente elegido para diseñar un nuevo protocolo de experimentos de preferencia alimentaria. En ellos, se observó una enorme incidencia de defensas químicas entre nuestras muestras de invertebrados y algas procedentes de un amplio rango de profundidades y de las zonas del Mar de Weddell y Archipiélago Shetland del Sur. Esto demostró la adaptabilidad de este anfípodo como depredador experimental para detectar agentes repelentes. Pero además el método en sí proporcionó una serie de ventajas metodológicas y un gran poder discriminatorio para detectar repelencias (Capítulo 3.1.), probablemente relacionado con el hecho de que los lyssianásidos poseen unos gnatópodos gustativos muy desarrollados (Kaufmann, 1994). De hecho, ambos consumidores elegidos, la estrella *O. validus* y el anfipodo *C. femoratus*, muestran capacidades notables para la localización de rastros de alimento (Kidawa, 2005b; Kidawa, 2005a; Smale et al., 2007; Kidawa, 2009), lo que podría favorecer la detección de repelentes. Además, ambas especies son tremendamente abundantes y fácilmente recolectables en nuestra zona de experimentación, BAE Gabriel de Castilla, en Isla Decepción, convirtiéndolas en buenos modelos experimentales.

A pesar de que ambos depredadores sean ampliamente oportunistas-generalistas, sus hábitos alimenticios y de atacar a su presa son distintos (Bregazzi, 1972; McClintock, 1994; De Broyer et al., 2007), y esto puede provocar diferentes respuestas. La estrella de mar *O. validus* muestra dificultades al ser alimentada con dietas artificiales de agar, alginato o carragenato (Avila et al., 2000; Iken et al., 2002; y obs. pers.), por lo que para los tests utilizamos dietas basadas en gambas congeladas. Para el anfípodo *C. femoratus* usamos perlas de caviar de alginato, preparadas con el kit del famoso cocinero Ferrán Adrià. Las perlas de alginato en general

poseían menos contenido energético que las gambas (según Atwater factors; Atwater y Benedict, 1902), lo que podía influir en la percepción de los posibles repelentes (Duffy y Paul, 1992; Cruz-Rivera y Hay, 2003). Cabe pues la posibilidad de que algunas defensas químicas fuesen menos evidentes en los tests con estrellas, quedando ligeramente enmascaradas por el mayor contenido nutricional de las gambas respecto al de las perlas de alginato, y también respecto al de algunas de las muestras en sí. Esto en cierto modo nos permitía comparar actividades repelentes cambiando el depredador y la dieta, lo cual viene descrito en el Capítulo 3.2. para muestras de organismos de 31 especies diferentes, pertenecientes a cuatro principales grupos: algas, esponjas, cnidarios y ascidias, además de muestras de un briozoo, una holoturia y un pterobranquio.

Las actividades de repelencia fueron más frecuentes en los tests con anfípodos que contra las estrellas, sobretodo en aquellas muestras provenientes de macroalgas y esponjas hexactinélidas, en las que los anfípodos podrían particularmente afectar a la producción y distribución de sus defensas. De hecho, los anfípodos antárticos al asociarse en especial con algas y esponjas (Kunzmann, 1996; Huang et al., 2007; Amsler et al., 2009; Zamzow et al., 2010), aunque también con otros organismos (Loerz, 2003; McClintock et al., 2009), consumen de manera directa tejidos del huésped, o indirectamente al ingerir detritus o microbiota (diatomeas) adheridas (Kunzmann, 1996; Amsler et al., 2000b; De Broyer et al., 2001; Graeve et al., 2001; Amsler et al., 2009; Zamzow et al., 2010). Así, estas congregaciones de anfípodos generalistas, ejercen una presión ecológica localizada en biosustratos sésiles, que puede ser más intensa que aquella provocada por depredadores móviles de mayor tamaño, como peces o equinodermos (Hay et al., 1987; McClintock y Baker, 2001; Toth et al., 2007). Las interacciones que se generan de estas asociaciones transitorias dependen del potencial químico del huésped, y de los hábitos del anfípodo (Sotka et al., 2009). De modo que, aunque los anfípodos ingieran tejidos de sus huéspedes accidentalmente en la naturaleza mientras aprovechan otras fuentes, en experimentos de laboratorio con dietas artificiales las repelencias por estos tejidos pueden hacerse notables, lo que podría explicar la enorme cantidad de repelencias detectada con *C. femoratus*. Las fracciones de cnidarios y asdicias demostraron contener potentes repelentes contra los dos depredadores. A parte de esto, algunas especies podrían explotar varias estrategias alternativas, como los nematocistos en algunos hidroideos y pennatuláceos, túnicas de bajo valor nutricional en algunas ascidias, o la capacidad locomotriz de las holoturias elípidas (Wigham et al., 2008), las avicularias defensivas de algunos briozoos (Winston, 1986; Carter et al., 2010), o también encapsulamientos reforzados como los de los pterobranquios (Ridewood, 1911). Por lo general, aquellas muestras repelentes sólo en los tests de *C. femoratus* correspondían a muestras o regiones corporales de bajo contenido energético, como las hexactinélidas, o como las túnicas de algunas ascidias y los haces de algunos corales, donde una

menor concentración de repelentes podría ir correlacionada con el bajo valor nutricional. A parte de esto, el gran porcentaje de actividades repelentes en ambos experimentos indica la existencia de defensas químicas efectivas contra ambos tipos de consumidores, en concordancia con la OT.

7.1.D. Aspectos quimio-ecológicos en esponjas hexactinélidas antárticas

Los Poríferos han despertado mucho interés por sus asociaciones con microorganismos, y por ser fuentes de un enorme repertorio de metabolitos bioactivos, de los que muchos se piensa tengan un origen simbiótico bacteriano (Taylor et al., 2007; Blunt et al., 2012). Por el contrario existen muy pocos estudios de química o ecología química en hexactinélidas (Guella et al., 1988; McClintock et al., 2000; Blumenberg et al., 2002; Thiel et al., 2002). Pero se cree que por tener una organización sincitial, diferente al resto de esponjas, y con un mesohilo casi ausente, las relaciones simbióticas con bacterias son prácticamente inexistentes, al igual que la producción de metabolitos secundarios (Leys et al., 2007). En cambio en la Antártida se han descrito asociaciones de hexactinélidas con diatomeas (Cattaneo-Vietti et al., 1996). Las hexactinélidas suelen habitar en los fondos profundos de todos los océanos, a los cuales es difícil acceder y recolectar, lo que ha dificultado enormemente su estudio (Leys et al., 2007). En el Mar de Weddell, por contra estas esponjas dominan la plataforma continental, entre los 100 y los 600m, creando espectaculares formaciones tridimensionales que dan cobijo a un gran número de organismos (Barthel, 1992; Barthel y Gutt, 1992; Gutt, 2007; Janussen y Tendal, 2007).

Las hexactinélidas contienen un bajo valor nutricional (10% tejido orgánico en peso seco) (McClintock, 1987; Barthel, 1995). Pero a pesar de eso, en los fondos antárticos son objeto de una intensa depredación por parte de estrellas de mar del género *Odontaster*, y nudibranquios, además de por mesofauna asociada: isópodos, anfípodos, poliquetos y otros (Dayton et al., 1974; Dayton, 1979; Barthel y Tendal, 1994; Kunzmann, 1996; McClintock et al., 2005). En nuestros tests, las hexactinélidas mostraron una actividad repelente muy baja contra estrellas, pero muy considerable contra *C. femoratus*, esta última presumiblemente derivada de asociaciones transitorias y/o por el mayor potencial discriminatorio del test con anfípodos (Núñez-Pons et al., 2012; Capítulos 3.1. y 3.2.). Sin embargo, cabe recordar que han de considerarse también los metabolitos polares no probados y presentes en otras fracciones, que podrían afectar a las estrellas. Es probable que las hexactinélidas combinen un bajo valor nutricional debido a su alto contenido en espículas (Barthel, 1995), con poca producción de productos repelentes (Duffy y Paul, 1992; Chanas y Pawlik, 1995; Waddell y Pawlik, 2000; Cruz-Rivera y Hay, 2003; Jones et al., 2005), para reducir la depredación, en especial de

estrellas. Además, esto les podría permitir dirigir más energía para la regeneración de tejidos dañados después de episodios de ataque (Leys y Lauzon, 1998; Walters y Pawlik, 2005; Leong y Pawlik, 2010).

Los agentes causantes de la elevada incidencia de actividades repelentes contra los anfípodos parecen derivar del metabolismo primario. De hecho, en nuestro análisis no detectamos metabolitos secundarios evidentes, al menos de aquellos característicos de otras esponjas (Blunt et al., 2012 y revisiones precedentes). En su lugar, aislamos dos tipos de productos lipídicos característicamente abundantes y además ausentes en demosponjas antárticas: un keto-esteroide 5α(H)-cholestan-3-one (**1**), presente en la mayoría de las hexactinélidas, y una mezcla particular de ceramidas (**2**) característica de todas las muestras de hexactinélidas. La composición de la mezcla de ceramidas en nuestras muestras es muy peculiar, pues se trata de una mezcla muy simple que sólo contiene dos glucoesfingolípidos (GSL) principales, que son dos homólogos con ácidos grasos de –C24 y –C22 (**2a-b**), lo que nos sugirió un posible valor quimiotaxonómico (ver abajo). Las observaciones en SEM nos indicaron que ambos compuestos no parecen derivar directamente de una fuente de diatomeas, pues todas las esponjas, demosponjas y hexactinélidas del estudio mostraron el mismo patrón de especies de diatomeas en sus tejidos, las cuales eran típicas de los "blooms" estivales australes.

El esteroide 5α(H)-cholestan-3-one produjo repelencia en los tests con *O. validus* demostrando al menos un papel minoritario como defensa ante la depredación. En cambio las glicoceramidas (**2a-b**), conocidas previamente de una planta, *Euphorbia biglandulosa* (Falsone et al., 1987), y ahora descritas por vez primera en una esponja, carecían de actividad alguna. Sospechamos que estas ceramidas podrían jugar un papel importante y formar parte de la membrana sincitial de las hexactinélidas, de la misma manera que similares ceramidas lo hacen en las plantas. Las glicoceramidas (**2a-b**) se encontraron en todas las muestras de hexactinélidas rosséllidas, antárticas y del hemisferio norte, con lo que podrían representar marcadores químicos de esponjas pertenecientes a la familia Rossellidae. Nuestros datos además nos sugieren que en cierta medida otros tipos similares de GSL podrían ser característicos del orden Lyssacinosida (ver Figura 7 del capítulo 3.3). Las relaciones taxonómicas dentro del filo Porifera actualmente están sujetas a un gran debate (Reiswig y Mackie, 1983; Leys, 2003; Worheide et al., 2012), y se han ido desarrollando, en paralelo con estudios morfológicos y de biología molecular clásicos, investigaciones con biomarcadores lipídicos para clarificar este asunto (Bergquist et al., 1980; Lawson et al., 1984; Bergquist et al., 1986; Bergquist et al., 1991; Thiel et al., 2002). Dado que los esfingolípidos han sido ya empleados con fines quimiotaxonómicos en microorganismos (Takeuchi et al., 1995), a nuestro parecer sería interesante saber cómo se distribuyen este tipo de GSL, similares a las ceramidas (**2a-b**), dentro de la clase Hexactinellida. Creemos que quizás estas moléculas podrían contribuir a la

clasificación de esta clase por el momento tan confusa (Barthel, 1992; Göcken y Janussen, 2011; Janussen, com. pers.). Los resultados detallados de esta sección se pueden consultar en el Capítulo 3.3.

7.1.E. Ecología química de corales blandos antárticos del género *Alcyonium*

Los antozoos son el tercer taxón en dominancia en el bentos del Mar de Weddell (Arnaud, 1977; Orejas, 2001). El género de corales blandos *Alcyonium* es particularmente común y está representado por 8 especies antárticas, algunas muy abundantes. Los corales blandos carecen de la protección ofrecida por esqueletos masivos de carbonato cálcico. En su lugar están formados por un tejido blando (coenénquima) que presenta incrustaciones de escleritos diminutos y espinosos que dan soporte (Brusca y Brusca, 2003), los cuáles se ha sugerido que son ineficaces contra los principales depredadores antárticos, las estrellas (McClintock, 1994). Además, su sistema de nematocistos es débil (Mariscal y Bigger, 1977; Brusca y Brusca, 2003) respecto al de otros cnidarios (Stachowicz y Lindquist, 2000; Bullard y Hay, 2002; Hines y Pawlik, 2012), y por tanto inefectivo como defensa (Schmidt, 1974; Sammarco y Coll, 1992). A pesar de todo esto, y de su rico valor nutricional, los corales *Alcyonium* son evitados por depredadores antárticos en fondos someros, y en efecto solamente una especie de picnogónido ha sido observada alimentándose de ellos (Slattery y McClintock, 1995; obs. pers.). Los corales blandos de hecho se encuentran altamente protegidos químicamente contra la depredación y la epibiosis, principalmente mediante terpenoides y esteroides (La Barre et al., 1986a, 1986b; Coll et al., 1987; Mackie, 1987; Wylie y Paul, 1989; Sammarco y Coll, 1992; Kelman et al., 1999; Wang et al., 2008; Hines y Pawlik, 2012). De acuerdo con estos datos, nuestro estudio con seis muestras de corales antárticos del género *Alcyonum* mostraron repelencia contra *O. validus* para las cinco especies representadas, y sólo una de las muestras carecía de actividad aparente. Igualmente las tres muestras probadas contra *C. femoratus* fueron significativamente repelentes. Tanto los terpenos iludalanos (**1**-**9**) de *Alcyonium grandis*, como los ésteres de ceras (**12**-**13**) obtenidos de todas las muestras de *Alcyonium* poseían propiedades repelentes, con la salvedad de que por falta de cantidad suficiente los iludalanos (**1**-**9**) no pudieron ser probados contra el anfípodo. Esto nos revelaba la identidad de algunos de los metabolitos involucrados en la defensa. Al parecer los illudalanos (**1**-**9**) junto con las ceras (**12**-**13**) cooperan para evitar la depredación en la especie en *A. grandis*. Los otros iludalanos (**10**-**11**) aislados de *A. roseum* 1 no pudieron ser probados en los experimentos, pero dada su gran proximidad molecular con los iludalanos (**1**-**9**), seguramente también posean características repelentes, y colaboren aditivamente junto con las ceras. De hecho, la muestra *A. roseum* 1 que contenía los iludalanos **10**-**11** mostró repelencia, mientras que la muestra conespecífica *A. roseum* 2, que carecía de estos compuestos era

inactiva, a pesar de que ambas poseían las ceras. Esto también sugiere que las ceras, a pesar de ser ellas mismas activas como sub-fracciones aisladas, parecen no ser tan efectivas en la defensa a nivel de toda la colonia sin la presencia de otros repelentes. En el resto de especies del estudio (*A. antarcticum*, *A. haddoni* y *A. paucilobulatum*) es probable que la defensa contra la depredación se consiga de forma similar gracias al efecto sinérgico de las ceras junto a otros compuestos repelentes minoritarios no identificados. A parte de esto, tres de las muestras exhibieron algún tipo de propiedad contra el recubrimiento al inhibir el crecimiento de una bacteria marina antártica. Las actividades contra cepas bacterianas del entorno no asociadas con el coral son en efecto comunes en corales blandos, tanto antárticos como no antárticos (Ducklow y Mitchell, 1979; Rublee et al., 1980; Slattery et al., 1995; Kelman et al., 1998; Ritchie, 2006).

Hasta la fecha la única especie de coral antártico del género *Alcyonium* que se ha investigado en ecología química ha sido *A. paessleri* (sinonimizado con *A. antarcticum*; Verseveldt y Van Ofwegen, 1992), que aquí hemos estudiado de nuevo. Esta especie ha demostrado una extensa variedad de actividades ecológicas basadas en compuestos orgánicos (Slattery et al., 1990; Slattery y McClintock, 1995; Slattery et al., 1995; Slattery et al., 1997a; Slattery y McClintock, 1997), además de poseer un rico pero variable arsenal de metabolitos secundarios (Slattery y McClintock, 1997). En efecto, nuestro *A. antarcticum* no proporcionó ninguno de los terpenos previamente descritos en varios trabajos (Palermo et al., 2000; Rodríguez-Brasco et al., 2001; Manzo et al., 2009). La variabilidad en el patrón de metabolitos secundarios observado en *A. antarcticum*, y ahora también en *A. roseum*, podría responder a cuestiones de variabilidad genética intraespecífica (Harvell et al., 1993), a tratarse de defensas inducibles (Slattery et al., 2001; Hoover et al., 2008), o a un origen simbiótico (Kelecom, 2002). Los iludalanos de la serie de las alcyopterosinas son típicos de hongos y helechos (Gribble, 1996; Suzuki et al., 2005), y además han sido obtenidos de los corales antárticos profundos *A. paessleri* (*A. antarcticum*) y *A. grandis* (Palermo et al., 2000; Carbone et al., 2009), y ahora también de *A. roseum*. La presencia de los iludalanos puede deberse a una retención evolutiva y/o a un origen simbiótico, como se hipotetiza para otros terpenos bioactivos de corales blandos. Un ejemplo es el pukalide, que aparece en especies pacíficas de *Sinularia* (Wylie y Paul, 1989; Van Alstyne et al., 1994; Slattery et al., 2001), y también en *A. antarcticum* (Manzo et al., 2009).

Los metabolitos secundarios son normalmente considerados los responsables de las actividades defensivas (Paul, 1992), pero hay también algunos esteroides, derivados del metabolismo primario, que proporcionan protección en corales blandos, esponjas y arañas de mar (Bobzin y Faulkner, 1992; Tomaschko, 1994; Slattery et al., 1997a; Fleury et al., 2008; Moran y Woods, 2009; Núñez-Pons et al., 2012a). El coste de la producción de compuestos bioactivos (Rhoades y Gates, 1976) podría reducirse mediante el uso de metabolitos primarios

para fines ecológicos. En los corales blandos las ceras son las principales reservas de energía, y su concentración disminuye tras interacciones competitivas como inversión para la producción de metabolitos secundarios (terpenos) defensivos (Fleury et al., 2004). Por lo tanto, si las ceras tienen propiedades defensivas en sí, esto significaría una táctica de ahorro de energía. En efecto las ceras podrían haber evolucionado como reservas lipídicas en los corales, en lugar de los más comunes triglicéridos porque proporcionan ventajas adicionales. Las ceras son indigestas (Benson et al., 1978; Place, 1992), y como demuestran nuestros resultados, pueden conferir repelencia a los tejidos y al mucus de los corales. Solamente las estrellas corona de espinas (*Acanthaster spp*) son capaces de alimentarse vorazmente de los corales, debido a una adaptación única: un sistema enzimático para digerir ceras (Benson et al., 1975). Inesperadamente, *C. femoratus* parece ser menos susceptible a los ésteres de ceras (**12**-**13**) que *O. validus*, quizás porque, así como los anfípodos antárticos hacen uso de las ceras como reservas también, las estrellas de mar carecen de estos compuestos (Sargent et al., 1977).

Nuestros extractos de corales consistían en complejas mezclas de metabolitos primarios y secundarios, obtenidos del tejido interno y del mucus. Aunque no se analizó específicamente, el muscus es esencial en procesos protectores, y es rico en ceras (60% de la composición mucolípida), esteroles y terpenos, siendo un medio donde los compuestos de defensa son exudados (Ducklow y Mitchell, 1979; Coll et al., 1982; Miyamoto et al., 1994; Slattery et al., 1997a; Wang et al., 2008). Los iludalanos (**1**-**11**), junto con las ceras (**12**-**13**), son probablemente secretados dentro del mucus en las especies estudiadas donde desempeñan un papel defensivo (los resultados de esta sección están detallados en el Capítulo 3.4.).

7.1.F. Distribución de las defensas químicas y metabolitos secundarios en ascidias coloniales antárticas

Dentro de la clase Ascidiacea, la familia Polyclinidae es una de las más abundantes en la plataforma antártica, y dentro de ésta los géneros *Aplidium* y *Synoicum* están muy bien representados (Ramos-Esplá et al., 2005). En general las ascidias coloniales presentan mucha variabilidad morfológica intraespecífica y de coloración (Varela, 2007). Las ascidias han desarrollado diversos mecanismos para prevenir la depredación, muchos relacionados con propiedades físicas y químicas de la túnica (Lambert, 2005), como pueden ser la posesión de túnicas gruesas, características de especies solitarias (Koplovitz y McClintock, 2011), túnicas con inclusiones de escleritos (Lambert, 1979; Lambert y Lambert, 1997; López-Legentil et al., 2006), o con poco valor nutricional (Tarjuelo et al., 2002). No obstante la principal línea de protección parece ser la química defensiva, que puede consistir en la acumulación de metales pesados o ácidos inorgánicos (Stoecker, 1980b; Stoecker, 1980a; Pisut y Pawlik, 2002;

McClintock et al., 2004), o en la producción de metabolitos repelentes (McClintock et al., 2004; López-Legentil et al., 2006; Núñez-Pons et al., 2010). Las especies estudiadas aquí carecían de túnicas gruesas o con escleritos (Varela, 2007; obs. pers.) y en ninguna, ni en especies relacionadas de la familia se ha descrito acumulación significativa de metales o ácidos (Stoecker, 1980b; Stoecker, 1980a; Hirose, 2001; Lebar et al., 2011). Por ello, deducimos que los metabolitos secundarios son las principales defensas utilizadas en las especies de nuestro estudio. No obstante, en estudios anteriores se observó poca prevalencia de defensas químicas (Koplovitz et al., 2009). Nuestros resultados en cambio muestran que el uso de las defensas químicas se extendía por todas las especies y muestras examinadas. Los experimentos con estrellas, además, demostraron que algunas especies tendían a concentrar los agentes repelentes hacia el interior de las colonias, como *A. fuegiense, A. millari*, y el morfotipo blanco y negro (B&W), así como dos muestras del morfotipo naranja (O) de la especie *Synoicum adareanum*. Esta distribución, en principio, parece contradecir las predicciones de la ODT. No obstante, en asdicias compuestas es frecuente producir estados larvarios protegidos contra depredadores, dada la gran inversión que se hace en la reproducción, con lo que las defensas químicas tienden a estar situadas en los tejidos internos (gónadas) (Young y Bingham, 1987; Lindquist y Fenical, 1991; Lindquist et al., 1992; Tarjuelo et al., 2002). En estos casos, las túnicas podrían combinar una producción de repelentes relativamente pobre, junto a un valor nutritivo bajo, que contribuiría a la defensa total de la colonia, complementando otros mecanismos coexistentes. De hecho, las bajas concentraciones de extracto obtenidas de estas túnicas, respecto a aquellas de las respectivas zonas internas reflejan estos hechos. En cambio otras especies analizadas, como *Aplidium falklandicum, A. meridianum*, y *S. adareanum* (O) 2, no mostraron localización de defensas intracolonial. Algunos de los patrones de distribución diferencial de actividades repelentes parecen estar ligados a la distribución de algunos de los metabolitos secundarios defensivos encontrados. *Aplidium falklandicum* y *A. meridianum* revelaron la identidad de sus repelentes, las meridianinas (A-G), que eran efectivas contra ambos *O. validus* y *C. femoratus*. Estos alcaloides están presentes tanto en la parte interna como en la externa, aunque son más abundantes en la externa (Núñez-Pons et al., 2010). Rossinone B demostró participar en la defensa de toda la colonia en *A. fuegiense*, contra anfípodos y estrellas, pero es predominante de regiones internas (Carbone et al., 2012; presente estudio).

En cuanto a procesos epibióticos, todas las muestras estaban libres de recubrimiento evidente, pero en concordancia con el estudio previo de Koplovitz et al. (2011), los extractos crudos etéreos de nuestras ascidias, así como el rossinone B carecían de actividades notables antibacterianas. En cambio las meridianinas, que aisladamente no habían reflejado propiedades antibióticas contra cepas cosmopolitas de bacterias y levaduras (Núñez-Pons et al., 2010), como

mezcla produjeron inhibición contra una bacteria simpátrica, demostrando así su polivalencia como defensas.

Por el momento seis especies de los géneros *Aplidium* y *Synoicum* han sido analizadas químicamente. En el caso de *S. adareanum* su variabilidad morfológica, bioactiva, y su diverso patrón de metabolitos secundarios entre especímenes de distintas áreas, sugieren una revisión taxonómica en esta especie (Diyabalanage et al., 2006; Miyata et al., 2007; Varela, 2007; Koplovitz et al., 2011; presente estudio), además de un posible origen simbiótico de algunos compuestos (Riesenfeld et al., 2008). El género *Aplidium* en cambio es renombrado por la cantidad y variedad de productos naturales proporcionados, sobretodo prenil quinonas, productos nitrogenados, ciclopéptidos, y una enorme variedad de alcaloides, pero lo que caracteriza al género es la propensión a producir derivados terpénicos (Zubía et al., 2005). Nosotros hemos encontrado los meroterpenoides rossinones B-E en *A. fuegiense* (Carbone et al., 2012; presente estudio). Aquí el producto mayoritario y el más bioactivo del grupo es sin duda el rossinone B, presente predominantemente en los tejidos internos, pero, aunque en cantidades muy pequeñas, también en la túnica. Los otros rossinones (C-E) en cambio se encuentran exclusivamente en el interior de la colonia, presumiblemente como precursores. Los rossinones A y B fueron descubiertos en una especie de *Aplidium* del Mar de Ross (Appleton et al., 2009). Las meridianinas en cambio son alcaloides indólicos originalmente descritos en *A. meridianum* de las Islas Georgia del Sur. Se describieron siete meridianias principales A, B, C, D, E, F y G, formando una mezcla, aunque las meridianinas F y G eran menos abundantes (Hernández Franco et al., 1998; Seldes et al., 2007). Nosotros las aislamos por vez primera en *A. falklandicum*, y a partir de cuantificaciones relativas confirmamos que B/E son las meridianinas más comunes seguidas por C/D y luego A, mientras F y G eran claramente minoritarias. También aportamos las asignaciones de carbono y protones de las meridianinas F y G en DMSO (Núñez-Pons et al., 2010), e identificamos nuevas meridianinas minoritarias (I-U), además de unos dímeros no descritos derivados de meridianinas mayoritarias en una muestra de *A. falklandicum*. Cabe destacar la ausencia de meridianina D en todas las muestras de *A. falklandicum*, a pesar de ser una meridianina mayoritaria, la cuál podría ser específica de *A. meridianum* (Núñez-Pons et al., 2010). En relación con esto, ambas especies están bajo estudio taxonómico, y se plantea sinonimizarlas como morfotipos distintos de la misma especie (Varela, 2007; Tatián com. pers.).

Los patrones de distribución de metabolitos secundarios en especímenes de *Aplidium*, junto con los tipos de moléculas encontrados, sugieren distintos orígenes para meridianinas y rossinones. Mientras los rossinones son característicos de tejidos internos, donde es probable que tenga lugar su biosíntesis (Carbone et al., 2012), las meridianinas se encuentran más concentradas en las regiones externas de las colonias (Núñez-Pons et al., 2010). Entre los

productos conocidos derivados de microorganismos, los terpenos escasean, mientras que los alcaloides predominan (Paul et al., 1990; Kelecom, 2002; Franks et al., 2005; Bandaranayake, 2006; Ivanova et al., 2007). Por otro lado los microsimbiontes suelen habitar en las túnicas de las ascidias coloniales (Schmidt et al., 2005; y revisado en Sings y Rinehart, 1996; Hildebrand et al., 2004; Hirose, 2009). Las meridianinas poseen una pigmentación amarilla viva, y se han obtenido de varias especies de ascidias coloniales antárticas de los géneros *Aplidium* (Hernández Franco et al., 1998; Seldes et al., 2007; Núñez-Pons et al., 2010) y *Synoicum* (Lebar y Baker, 2010), además de en la esponja *Psammonemma sp*. (Butler et al., 1992; Lebar y Baker, 2010). Esto nos lleva a plantear que podría tratarse de pigmentos vestigiales mantenidos en diversos organismos antárticos por su papel ecológico multifuncional (revisado en Bandaranayake, 2006). Y como otros muchos pigmentos alcaloides bioactivos, se sugiere también que deriven de microbios simbiontes (Paul et al., 1990; Franks et al., 2005). Para consultar más detalladamente los resultados de esta sección véase Capítulo 3.5. y 3.6.

7.1.G. Conclusiones

El anfípodo *Cheirimedon femoratus* demostró ser un modelo de depredador experimental muy apropiado para llevar a cabo experimentos de detección de defensas químicas en aguas antárticas. Especialmente en los grupos de las macroalgas y las hexactinélidas las defensas químicas eran más frecuentes contra el anfípodo, que hacia la estrella *O. validus*. *Cheirimedon femoratus* se asocia de manera oportunista con biosustratos sésiles del fondo provocando una presión constante en estos organismos, que puede resultar más intensa que la ejercida por macrodepredadores móviles menos recurrentes como *O. validus*. Las esponjas y algas antárticas representan potenciales huéspedes-presa para los anfípodos, que los prefieren como sustratos. Por ello, en algunos casos los anfípodos podrían sustituir a las estrellas como principales inductores de la distribución de las defensas químicas, poniendo en duda previas predicciones del funcionamiento de la ODT en el bentos antártico. De hecho, observamos una ausencia general de concentración diferencial de defensas en nuestras esponjas.

Considerando que los recursos internos de energía son limitados, el uso de metabolitos primarios para la defensa representa una estrategia efectiva de ahorro de energía. Creemos que el éxito evolutivo de nuestro grupo de estudio en las comunidades antárticas está relacionado con la presencia de defensas químicas. En esponjas hexactinélidas éstas parecen ser más débiles y derivadas del metabolismo primario, pero compensadas con un bajo valor nutricional. Algunos GSL en cambio, podrían poseer un valor quimiotaxonómico como marcadores químicos de la familia de hexactinélidas Rossellidae. En los corales blandos la protección química se obtiene tanto de metabolitos primarios como secundarios, todos ellos al parecer

operando de forma sinérgica. Sugerimos además la secreción de estos metabolitos como parte del mucus. Mientras que en las ascidias coloniales, los metabolitos defensivos parecen ser predominantemente secundarios y muy potentes, y además en algunas especies éstos tienden a acumularse en los tejidos internos de las colonias, presumiblemente para producir larvas protegidas. Las propiedades de compuestos como las meridianinas o los iludalanos no pueden ser atribuidas a un compuesto en particular, sino a la mezcla entera, que suele aparecer en cantidades importantes. La producción de grupos de compuestos potencialmente miméticos en base a su parecido estructural podría aumentar su concentración como mezcla, y con ello la señal bioactiva. Algunos metabolitos aislados de varias especies, géneros, e incluso filos, y de diferentes áreas geográficas, como sucede con las meridianinas, sugieren, o bien una extensa retención evolutiva, o un posible origen simbiótico y retención de esa asociación por las beneficiosas bioactividades conferidas. En lo referente al recubrimiento bacteriano, nuestras ascidias mostraron poca actividad, pero algunas especies de corales sí que exhibieron respuestas inhibitorias. Entre nuestros estudios futuros cabe incluir experimentos para evaluar la inhibición de invasión por diatomeas.

Se ha propuesto un gradiente descendente en la diversidad de metabolitos secundarios marinos a medida que aumentamos de latitud. En las zonas polares al haberse realizado una menor investigación, no es posible establecer una conclusión por el momento. No obstante, muchos organismos marinos antárticos están proporcionando enormes cantidades de productos naturales. Hasta donde llega nuestro conocimiento, nuestros estudios son de los únicos que revelan la identidad de metabolitos ecológicamente relevantes en la defensa de hexactinélidas, corales blandos y ascidias coloniales de la Antártida. Con la investigación de esta tesis doctoral esperamos haber contribuido a la ecología antártica, especialmente en el campo de la química defensiva a través de productos naturales, y adicionalmente por tratarse de especies en su mayoría nunca antes estudiadas.

En general, existe la necesidad de extender los experimentos ecológicos al campo. Aún hay mucho por saber sobre cómo funcionan los aleloquímicos, y en determinar si existe la defensa química inducible en la Antártida, mediante monitorización de las concentraciones de defensas antes y después de episodios de ataque. También la relación entre el valor nutricional y defensa química es algo a tener en cuenta para el futuro. De hecho *C. femoratus*, como otros anfípodos, permite por su fácil adaptabilidad a dietas artificiales, hacer este tipo de estudios variando la cantidad de repelentes y la fuente alimenticia dentro de las dietas preparadas. Finalmente, los mecanismos por los que los animales discriminan, detectan y eligen entre presas defendidas químicamente o no, son en la actualidad desconocidos. Por ello, se precisan estudios de los procesos sensoriales en depredadores y presas, así como de los efectos que provocan los repelentes en los depredadores, y las posibles diferencias respecto a generalistas y especialistas.

CHAPTER 7.2. RESUM EN CATALÀ

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7.2.A. Ecosistemes marins antàrtics i ecologia química marina

La major part de la fauna antàrtica va evolucionar durant el Cretàcic, amb la divisió de Gondwana, que va donar lloc a la formació dels continents actuals, inclosa l' Antàrtida (Clarke i Crame, 1989; Crame, 1992). Fa 22 milions d'anys es va establir el corrent circumpolar, fet que va comportar el refredament i aïllament del continent blanc, promovent un important endemisme faunístic (Crame, 1999; Gili et al., 2000). De fet la biota antàrtica es composa de fauna autòctona primitiva, fauna euribàtica originària d'aigües profundes, i espècies provinents d'Amèrica del Sud, amb la que manté un únic pont de connectivitat a través de les Illes de l'Arc d'Escòcia (Brey et al., 1996; McClintock i Baker, 1997a; Brandt et al., 2007; Primo i Vazquez, 2009; Demarchi et al., 2010). Es tracta, per tant, d'un bentos molt primitiu que ha conviscut suficientment com per formar interaccions ecològiques robustes (Aronson et al., 2007; Amsler et al., 2000).

Els ecosistemes antàrtics estan caracteritzats per les baixes temperatures, per la marcada estacionalitat en la disponibilitat de recursos alimentaris i per la seua estabilitat. Salvada la zona més somera (per sobre dels 33 m) exposada a esdeveniments destructius causats pel gel (Smale, 2007), les comunitats bentòniques es consideren acomodades biològicament, i estructurades per la predació i la competència (Gutt, 2000; Dayton et al., 1974). A la plataforma continental, les comunitats antàrtiques gaudeixen de molta biodiversitat (Burton, 1932; Koltun, 1970; Dayton et al., 1974; Dayton, 1979; Dayton, 1989; Blunt et al., 1990; Arntz et al., 1997; Brandt et al., 2007), albergant riques associacions de suspensívors dominades per esponges, coralls tous, briozous, hidroideus i ascidis, a més de macroalgues a les zones fòtiques (Arntz et al., 1997; Gutt et al., 2000; Wiencke et al., 2007). Als nivells tròfics superiors trobem enormes densitats de crustacis (De Broyer i Jazdzewski, 1996; Huang et al., 2007), així com macroinvertebrats tipus nemertins, i equinoderms variats (DeLaca i Lipps, 1976; Dearborn, 1977; Gutt et al., 2000; Obermuller et al., 2010), i també peixos (Richardson, 1975; Eastman, 1993). Els principals predadors generalistes del bentos sèssil aquí són les abundants estrelles i nemertins, a més de poblacions d'amfípodes. També hi ha espongívors especialistes, com el nudibranqui *Austrodoris kerguelenensis* que s'alimenta d'hexactinèl·lides del gènere *Rossella*, o l'asteroideu *Perknaster fuscus*, especialitzat en *Mycale acerata*, i que conjuntament a *Acodontaster conspicuus* regulen l'abundància d'aquesta esponja colonitzadora dels fons antàrtics (Dayton et al., 1974). Durant molt de temps es va sostindre que la pressió per predació, conjuntament amb les defenses químiques eren gradualment menors amb l'augment de la latitud. Aquesta teoria

latitudinal considerava bàsicament la predació causada per peixos (Bakus i Green, 1974), que a l'Antàrtida és relativament baixa, però que està substituïda per una altra molt intensa provocada per macroinvertebrats, principalment estrelles (McClintock, 1989; Baker et al., 1993; Amsler et al., 2000a; McClintock i Baker, 2001; Avila, 2006). Apart d'açò, dispositius eficaços contra peixos als tròpics, com poden ser els esclerits o les espícules, a l'Antàrtida no semblen ser-ho, donat que els principals predadors ací practiquen altres hàbits alimentaris, com les estrelles, que realitzen una pre-digestió extra oral (Hyman, 1955; Sloan, 1980). En efecte, la incidència de defenses químiques ha demostrat ser molt elevada entre els organismes antàrtics (Taboada et al., 2012; i revisat en Amsler et al., 2000a; Avila et al., 2008; McClintock et al., 2010), i els nostres resultats coincideixen amb açò (Capítols 3.1 i 3.2).

La intermitència en l'entrada d'aliment als sistemes antàrtics fa que l'acumulació de lípids de reserva en forma de triglicèrids o ceres, jugue un paper important (Sargent et al., 1977). Per aquesta mateixa raó, a més, els organismes antàrtics, ja siguen suspensívors sèssils així com espècies vàgils del fons, incloent als principals predadors, han desenvolupat hàbits oportunistes (Bregazzi, 1972; Dayton et al., 1974; Arnaud, 1977; McClintock, 1994; Orejas, 2001; Orejas et al., 2001; Tatian et al., 2002; Orejas et al., 2003; Tatian et al., 2004; De Broyer et al., 2007). A més, les comunitats antàrtiques solen mancar de zonació faunística, sent majorment circumpolars i euribàtiques (Dell, 1972; Arnaud, 1977; White, 1984), el que fa que les espècies dominants comparteixen conjuntament amb llurs predadors hàbitats tant profunds com somers (Dayton et al., 1974; McClintock, 1994; Gutt et al., 2000). Açò facilita molt l'estudi d'ecosistemes de difícil accés com el que ens disposem a estudiar, donat que les nostres mostres són en la seua major part de fons profunds, amb el que aquest fet faunístic ens permet provar les nostres mostres amb organismes de poca profunditat sense perdre rigor ecològic. A més hem de tindre en compte que els científics antàrtics estem limitats a realitzar els nostres experiments a les bases o vaixells disponibles. Al nostre cas, l'experimentació es va realitzar a la (BAE) Gabriel de Castilla, a la Illa Decepció, Illes Shetland del Sud (62º 59.369' S, 60º 33.424' W). En allò referent a esdeveniments d'epibiosi, i lligat amb els intensos blooms estivals de macroalgues, en aigües australs, la invasió causada per diatomees sembla sobrepassar aquella bacteriana (Slattery et al., 1995; Amsler et al., 2000b; Bavestrello et al., 2000; Cerrano et al., 2000; Peters et al., 2010; Koplovitz et al., 2011), la qual cosa difereix d'altres latituds (Cervino et al., 2006). Però també s'han descrit associacions simbiòtiques entre esponges i diatomees, molt més comunes en espècies antàrtiques que en altres zones (Gaino et al., 1994; Cattaneo-Vietti et al., 1996; Hamilton et al., 1997; Cerrano et al., 2004a; Cerrano et al., 2004b; Taylor et al., 2007). Al Capítol 3.3 abordem aquest tema indirectament amb fins metabòlics, donat que les diatomees poden proveïr a les esponges de productes característics (Gaino et al., 1994; Cerrano et al., 2004a; Cerrano et al., 2004b).

Els organismes marins en estar sotmesos a una constant pressió ecològica, causada per la predació, la competició per l'espai i els recursos, així com per esdeveniments de recobriment per epibionts (Barnes i Hughes, 1988), han de desenvolupar una sèrie de mecanismes de defensa. Aquests mecanismes poden ser adaptacions de tipus ecològic (selecció del nínxol), comportamental (hàbits nocturns), o fisiològic (optimitzant els ritmes reproductius i/o de creixement). Existeixen també formes de protecció física, com esquelets externs o interns (closques, espines, espícules o esclerits), o renovació constant de capes superficials de teixit o mucus, etc. i a més estan les defenses químiques, que inclouen agents tòxics o repel·lents, que solen derivar del metabolisme secundari (Paul, 1992; Eisner i Meinwald, 1995; McClintock i Baker, 2001). Però existeixen casos de metabòlits primaris amb propietats defensives (Bobzin i Faulkner, 1992; Tomaschko, 1994; Slattery et al., 1997a; Fleury et al., 2008; Moran i Woods, 2009; Núñez-Pons et al., 2012a). De fet a les nostres investigacions trobem ambdós tipus de metabòlits causant repel·lència (Capítol 3.3 i 3.4).

L'activitat més estudiada en ecologia química és la de defensa contra la predació, i normalment els predadors generalistes, que són els més abundants, són més susceptibles a metabòlits secundaris, en la seua majoria de naturalesa lipofílica (Paul, 1992; Eisner i Meinwald, 1995; McClintock i Baker, 2001; Sotka et al., 2009). En aquest sentit, durant aquest projecte de doctorat ens hem centrat en l'estudi de les activitats i els agents químics continguts en les fraccions lipofíliques més apolars dels nostres espècimens, és a dir aquells continguts als extractes eteris, deixant altres fraccions per a futures investigacions. A les comunitats bentòniques, aquells organismes sèssils, de cos tou i de tipus clonal, como esponges, octocoralls i ascidis, són els que predominantment es defenen químicament contra diversos tipus de predadors (veure revisions de Paul, 1992; Pawlik, 1993; Hay, 1996; McClintock i Baker, 2001; Paul et al., 2011). La producció de metabòlits secundaris és energèticament costosa i els organismes han de compensar aquestes despeses amb aquells destinats al manteniment, creixement i reproducció, fet que ha portat a l'elaboració de una sèrie de teories de gestió i estalvi energètic (Coley et al., 1985; Cronin, 2001). La més amplament acceptada és la Teoria de Defensa Optimitzada (ODT; Rhoades i Gates, 1976), que contempla que la producció de defenses químiques ha d'anar correlacionada amb el risc d'atac, i que ha d'existir una distribució anatòmica diferencial de les mateixes cap a estructures més valuoses o més exposades a predadors. Els nostres estudis, com molts altres, integren els postulats de la ODT. Altres teories d'estalvi energètic també plantegen l'ús polivalent de mecanismes defensius, comuns contra varis predadors (Teoria de l'Optimització, OT; Herms i Mattson, 1992), o en el cas del Model de Defensa Induible (IDM; Harvell, 1990), la producció de metabòlits defensius directament correlacionada amb el risc d'atac. En efecte, molts organismes marins són capaços de produïr composts defensius, o incrementar la seua concentració després d'episodis de

predació sobre els seus teixits (Cronin i Hay, 1996; Toth et al., 2007; Thoms et al., 2006; Thoms i Schupp, 2008; Slattery et al., 2001; Hoover et al., 2008; Lindquist, 2002). Malgrat això, a l'Antàrtida, els processos de defenses químiques induïbles no han estat demostrats encara (Avila et al., 2008; McClintock et al., 2010). Malgrat que les defenses químiques actuant com a repel·lents alimentaris han estat amplament reconegudes, els mecanismes que promouen el rebuig en el predador no es coneixen encara. En general, les defenses contra la predació estan més relacionades amb el mal sabor que no pas amb la toxicitat (Paul, 1992; McClintock i Baker, 2001). Un altre factor a tindre en compte conjuntament amb les defenses químiques és el valor nutritiu, doncs alguns repel·lents són més (o només) efectius en combinació amb menjars de poca qualitat energètica i al contrari, les dietes nutritives poden emmascarar l'activitat repel·lent (Duffy i Paul, 1992). Malgrat que no en profunditat, aquest concepte es pot contemplar al capítol 3.2.

Com mencionem anteriorment, un altre desafiament al que estan sotmesos els organismes marins és al recobriment epibiòtic i a la invasió de microbis patògens. De fet, les defenses contra el recobriment estan prou esteses al bentos marí (Fusetani, 2004; Paul et al., 2011). Els processos de recobriment són successionals, i comencen amb l'adsorció de macromolècules i la colonització bacteriana. Per això, evitar la formació d'aquestes pel·lícules inicials resulta una estratègia efectiva per evitar posteriors esdeveniments (Zobell i Allen, 1935). Als nostres modestos estudis sobre les activitats per lluitar contra el recobriment ens basem en tests antibiòtics contra bacteris marins de l'entorn.

A l'hora de realitzar experiments per a estudis d'ecologia química, és important triar bé el paràmetre amb que anem a calcular la concentració natural dels nostres extractes, fraccions o composts aïllats a provar, depenent de l'activitat que anem a investigar, i de les espècies implicades. Els paràmetres més emprats són el volum, el pes sec i el pes humit. Però sempre hem de tindre en compte que el càlcul de la concentració natural en un espècimen, malgrat que siga disseccionat, serà una aproximació, i que mai no podrà mimetitzar allò que realment ocorre en la natura, degut a fenòmens com la distribució diferencial o encapsulament de metabòlits en determinades estructures. Nosaltres, tenint en compte aquestes limitacions insalvables i per treballar amb mostres aquàtiques, hem utilitzat per als nostres càlculs el pes sec. D'aquesta manera, en eliminar la humitat evitem desviacions importants que es poden originar en organismes porosos i de teixit tou com les esponges, els coralls o els ascidis. A més, per aconseguir resultats ecològicament reals i vàlids és important utilitzar organismes experimentals simpàtrics, que comparteixen hàbitat amb les mostres que volem examinar, del contrari estarem obtenint indicis d'una bioactivitat, la qual manca de valor ecològic rellevant (Paul et al., 2007). En aquest aspecte els nostres experiments, varen ser sempre realitzats *in situ* (a la Antartida) i amb organismes simpàtrics.

7.2.B. Productes naturals marins i defensa química a l'àmbit antàrtic

Existeix un gran nombre de productes naturals reconeguts per classes, entre ells els poliquètids, terpens, hidroquinones, depsipèptids i els més nombrosos, els alcaloides. També malgrat que menys comuns, trobem derivats de metabòlits primaris, és a dir nucleòsids, carbohidrats, esteroides i àcids grassos (Blunt et al., 2012 i revisions anteriors de la sèrie). Els metabòlits secundaris provenen de la dieta, o poden ser biotransformats a partir de precursors, o bé poden ser sintetitzats *de novo* (Paul, 1992; McClintock i Baker, 2001). No obstant això, recentment s'ha alçat la sospita de que molts de els metabòlits bioactius aïllats d'invertebrats marins siguen produïts per microorganismes associats, donat que molts posseeixen riques poblacions de microsimbionts als seus teixits (revisat per Kobayashi i Ishibashi, 1993; Hildebrand et al., 2004; Piel, 2009). Malgrat que probablement esbiaixat pels interessos i les tècniques emprades per a cada químic, en general cada fílum es caracteritza per una sèrie de tipus de productes; per exemple als cnidaris els terpenoides; les esponges, que són el grup més estudiat han proporcionat terpenoides i metabòlits nitrogenats, i lels ascidis solen especialitzar-se en derivats d'aminoàcids (Davidson, 1993; Blunt et al., 2012). En aquest sentit alguns metabòlits, sobretot lípids, són útils per a estudis quimiotaxonòmics (Bergquist et al., 1991; Thiel et al., 2002; Berge i Barnathan, 2005; Imbs i Dautova, 2008). Nosaltres, al Capítol 3.3 aportem una modesta contribució d'aquest tipus en esponges hexactinèl·lides. Certament s'han descrit moltíssims metabòlits secundaris, els quals no participen en processos primaris, però per a molt pocs es coneix la funció ecològica que desenvolupen. De fet, molts composts s'avaluen per a bioactivitats amb finalitats farmacèutiques, si bé el camp de la seua significació en el propi organisme es deixa de costat (Munro et al., 1987; Scheuer, 1990; Hay i Fennical, 1996; Taboada et al., 2010). Entre les funcions ecològiques que s'han trobat estan la toxicitat, la repel·lència alimentària, la inhibició del recobriment i/o infecció, i la mediació en processos de compètencia espacial (Paul, 1992; McClintock i Baker, 2001; Avila et al., 2008; Blunt et al., 2012).

El gruix dels estudis en ecologia química s'han fet avaluant activitats de repel·lència contra predadors amb organismes d'aigües someres (accessibles mitjançant submarinisme) a les zones de McMurdo Sound (Mar de Ross) i l'oest de la Península Antàrtica, i es basen majorment en els treballs de McClintock i col·laboradors. Mentre que, les regions d'algunes Illes subantàrtiques i zones profundes del Mar de Weddell comencen a ser també investigades. Pel contrari les àrees de l'est de l'Antàrtida, i les Mars d'Amundsen i Bellingshausen són pràcticament desconegudes en aquests àmbits (McClintock i Baker, 1997a; Lebar et al., 2007; Avila et al., 2008; McClintock et al., 2010; Taboada et al., 2012). Malgrat tot, donada la distribució general circumpolar i euribàtica de la biota (Dell, 1972; Arnaud, 1977; White, 1984), es considera que els coneixements adquirits siguen prou aplicables a amples zones de l'Antàrtida. De moment, en allò referent a agents químics defensius, s'ha observat que aquests

són freqüents en organismes antàrtics pertanyents als principals grups taxonòmics (Avila et al., 2008; McClintock et al., 2010; Taboada et al., 2012), lo qual cosa va en concordància amb els grups que s'han estudiat a la present tesi (capítols 3.1 i 3.2). Malgrat tot, en comptades ocasions s'han identificat les molècules actives (Núñez-Pons et al., 2010; Núñez-Pons et al., 2012a; i anteriorment revisat en Avila et al., 2008). El major nombre de metabòlits amb funció defensiva han estat aïllats d'esponges. En aquest aspecte cal recalcar que molts d'aquests composts són pigments repel·lents i responsables dels vius colors que caracteritzen les esponges que els proporcionen. En principi a l'Antàrtida no té sentit la presència de coloracions aposemàtiques (d'avís) per ser un sistema els predadors principals de les quals (les estrelles) s'orienten químicament, i per mancar de predadors visuals tipus peixos o tortugues. Però es planteja que aquestes substàncies poden representar pigments vestigials, que al seu dia podria haver tingut un valor aposemàtic quan el clima era més càlid i hi habitaven altres predadors, i que s'han mantingut evolutivament per les seues propietats bioactives inherents (revisat en Bandaranayake, 2006; Avila et al 2008; McClintock et al., 2010). Un fenomen semblant es proposa en alguns dels nostres ascidis colonials al Capítol 3.6. Altres metabòlits amb activitat defensiva en organismes antàrtics s'han obtingut d'algues, coralls, mol·luscs (Avila et al 2008; McClintock et al., 2010; i referenias allí incluídas) i recentment com a part d'aquesta tesi en ascidis (Núñez-Pons et al., 2010; Capítols 3.5 i 3.6).

Les prediccions de l'ODT tenen en compte el tipus de predador i de presa, a més d'altres mecanismes de defensa alternatius. S'ha plantejat que els organismes de l'Antartida hauríen de concentrar les seues defenses a les zones externes, on serien més efectives contra els principals predadors (Rhoades i Gates, 1976), entre ells les estrelles que s'alimenten evaginant l'estòmac sobre la seua presa (Sloan, 1980). Però en organismes perforats, consumidors de petita mida com els amfípodes capaços d'accedir a teixits interns, podrien promoure un altre tipus de distribució. En concret les hexactinèl·lides antàrtiques, que constitueixen una part important de les nostres mostres (Capítol 3.3), i posseeixen forma de volcà i grans òsculs, són clars exemples (Núñez-Pons et al., 2012a). Però, a més, cal tindre en compte els cicles vitals en alguns grups, per exemple en els ascidis colonials existeix una tendència a produir larves protegides químicament contra predadors, pel que les defenses químiques solen aparèixer en teixits interns, a les gònades (Lindquist et al., 1992). A l'Antàrtida s'ha descrit la localització de defenses al mantell d'alguns opistobranquis (Avila et al., 2000; Iken et al., 2002), però sobretot en esponges s'han trobat vàries espècies amb repel·lents concentrats a les capes superficials (*p.e.,* Furrow et al., 2003; Peters et al., 2009), malgrat que també hi ha altres espècies que no exhibeixen una clara distribució de les defenses químiques (Peters et al., 2009). Un dels nostres recents treballs també aborda el tema de la localització de les defenses en un ampli rang de grups zoològics (Taboada et al., 2012). Als treballs de la present tesi les prediccions de l'ODT (Rhoades i Gates,

1976) són sempre considerades, i la distribució de repel·lents s'ha estudiat en aquelles mostres que ho varen permetre per mida, forma i tipus d'organisme. Però, la presència de productes bioactius a zones externes pot acomplir d'altres funcions, com la inhibició del recobriment, o la mediació d'interaccions al·leloquímiques (Rhoades, 1979; Paul, 1992; Slattery i McClintock, 1997; McClintock i Baker, 2001; Avila et al., 2008; McClintock et al., 2010). Per exemple, s'ha observat que una espècie de corall tou antàrtic del gènere *Alcyonium* és capaç d'induir necrosi per contacte a l'esponja colonitzadora *Mycale acerata*. Aquest fet conjuntament amb els potents agents d'anti-recobriment descrits en aquesta espècie, que sembla que són alliberats a l'aigua circumdant, suggereix la presència de propietats ecològiques importants al mucus superficial d'aquests coralls (Slattery i McClintock, 1997), com succeeix en coralls d'altres latituds (Brown i Bythell, 2005). De fet, els coralls tous i els ascidis colonials manquen de recobriment evident per epibionts (revisat en McClintock et al., 2010; obs. pers.). En recents treballs s'ha detectat una activitat antibacteriana escassa en ascidis i esponges, respecte a aquella rellevant descrita en coralls tous. Però, els tres grups posseeixen potents inhibidors contra diatomees, indicant que aquestes poden ser més influents que els bacteris en altes latituds (Slattery i McClintock, 1997; Peters et al., 2010; Koplovitz et al., 2011). Els nostres modestos tests en aquest tòpic avaluaven activitats inhibitòries contra bacteris marins de l'entorn.

En resum, l'ecologia química marina dibuixa un mapa en el que gran part del coneixement adquirit prové d'ecosistemes somers tropicals i temperats, l'accessibilitat dels quals permet establir relacions ecològiques fàcilment (Paul, 1992, McClintock i Baker, 2001; Avila et al., 2008; McClintock et al., 2010). Les àrees polars pel seu aïllament geogràfic i dures condicions han rebut menys atenció (Lippert, 2003; Avila et al., 2008; McClintock et al., 2012). A les aigües del Pol Sud la major part de les investigacions es focalitzen en la presencia de repel·lents per evitar la predació, i els grups més estudiats són les macroalgues i les esponges. Dins de les esponges però, quasi totes les espècies investigades són demosponges i malgrat que les hexactinèl·lides siguen un dels components formadors dels sols marins antàrtics, no es sap quasi res sobre elles en aquest camp (Avila et al., 2008; McClintock et al., 2010). En efecte, apart de la nostra recent publicació (Núñez-Pons et al., 2012a) presentada al Capítol 3.3, només existeix un altre treball amb esponges hexactinèl·lides que relaciona el seu contingut nutritiu i espicular amb la defensa química (McClintock, 1987). Altres dels grups més estudiats són els mol·luscs i els ascidis, aquests darrers gràcies a dos treballs recents que varen revelar escasses defenses químiques contra la predació i la colonització bacteriana, tant en espècies solitàries com clonals (Avila et al., 2008; Koplovitz et al., 2010; 2011; McClintock et al., 2010). El següent grup que ha rebut més consideració són probablement els cnidaris, seguits per d'altres organismes (Avila et al., 2008; McClintock et al., 2010). Però la majoria de treballs amb cnidaris són purament químics d'aïllament de molècules (Slattery et al., 1994; Slattery et al., 1997b; Palermo et al.,

2000; Rodríguez-Brasco et al., 2001; Gavagnin et al., 2003; Iken i Baker, 2003; Carbone et al., 2009; Manzo et al., 2009; i revisat en Avila et al., 2008), i només tres espècies de coralls tous han estat analitzades des del punt de vista de llurs defenses químiques, demostrant un gran i polivalent arsenal (Slattery i McClintock, 1997). Durant els darrers mesos, el nostre grup ha finalitzat una extensa recerca sobre la incidència de defenses químiques i llur localització en un ampli grup d'invertebrats antàrtics. Aquest estudi representa una contribució interessant a l'ecologia química antàrtica perquè les mostres examinades provenien de fons profunds del Mar de Weddell i Illa de Bouvet, amb el que moltes espècies no havien estat mai investigades (Taboada et al., 2012). Per la qual cosa l'escenari que deixa l'ecologia química antàrtica és el d'una biota amplament protegida mitjançant defenses químiques, però amb molts grups encara clarament infraestudiats. A més, la identitat dels productes bioactius i ecològicament responsables, conjuntament amb aspectes en la seua distribució, mode d'operar en interacció amb altres molècules, i el seu origen es troben encara a la seua infantesa (Avila et al., 2008; McClintock et al., 2010). Per tot açò, i amb la fi de contribuir a l'ecologia antàrtica i a esbrinar cóm funcionen els mecanismes de defensa a través de substàncies orgàniques, aquest projecte de tesi s'ha focalitzat en estudiar organismes rellevants del bentos antàrtic, presumiblement portadors de defenses químiques, i amb poca investigació al seu darrere. Entre ells hem seleccionat les esponges hexactinèl·lides, els coralls tous i els ascidis colonials.

7.2.C. Defenses químiques contra dos rellevants predadors antàrtics

La selecció dels predadors experimentals és fonamental, per obtindre resultats realistes com hem vist, però també perquè, en el nostre cas varen ser amb els que, al llarg d'aquest projecte de tesi, avaluem la presència d'activitat defensiva de repel·lència alimentària. Els mecanismes de defensa química a l'Antàrtida s'han demostrat mitjançant varis tipus d'experiments i amb diferents possibles predadors. En experiments d'alimentació directa, per exemple, s'han emprat peixos, anemones i amfípodes, oferint-los teixits frescos de la presa, o bé dietes artificials d'agar incloent els extractes dels organismes a provar (*p.e*. McClintock et al., 1991; McClintock et al., 1992; McClintock et al., 1993; Slattery i McClintock, 1995; McClintock i Baker, 1997b; Koplovitz et al., 2009). Pel contrari amb equinoderms, que són els principals predadors antàrtics (estrelles; Dayton et al., 1974), principalment s'han explotat les capacitats quimioreceptives dels peus ambulacrals (Sloan, 1980; McClintock, 1994) mitjançant tests de curta durada que avaluen les reaccions dels mateixos, sense que arribe a donar-se ingestió de la pròpia dieta presentada contenint els extractes (*p.e*. McClintock, 1987; McClintock et al., 1990; McClintock et al., 1992; McClintock et al., 1993; McClintock et al., 1994a; McClintock et al., 1994b; McClintock et al., 1994c; Slattery i McClintock, 1995; McClintock i Baker, 1997; Slattery et al., 1997a;

Slattery i McClintock, 1997; McClintock et al., 2000; Amsler et al., 1999; Koplovitz et al., 2009; Peters et al., 2009). Els treballs existents amb experiments d'alimentació efectiva emprant estrelles de mar antàrtiques, en concret *Odontaster validus*, es limiten bàsicament a aquells desenvolupats pel nostre grup (*p.e.* Bryan et al., 1998; Avila et al., 2000; Iken et al., 2002; Núñez-Pons et al., 2010; Núñez-Pons et al., 2012a; Taboada et al., 2012). En efecte, discrepàncies en les activitats d'algunes espècies poden ser degudes a les distintes metodologies i/o predadors utilitzats. En la nostra opinió els mètodes on es valora la ingestió efectiva i no només les primeres reaccions, són més escaients, doncs permeten valorar respostes que poden donar-se després de la ingestió, com la interacció entre el valor nutritiu i els repel·lents presents en una presa. A més el nostre estudi s'ha focalitzat en la fracció més apolar (extracte eteri) de les nostres mostres, donat que la majoria dels repel·lents marins descrits són de naturalesa lipofílica. Aquests solen aparèixer segrestats dins de vesícules o teixits, fet que dificulta una recepció dels mateixos abans de la ingesta (Sotka et al., 2009). Som conscients de les limitacions que té estudiar només aquestes fraccions, i de fet els extractes butanòlics i residus aquosos els conservem per a futures investigacions.

L'abundant estrella de mar *Odontaster validus*, amb distribució circumpolar i euribàtica (Dearborn, 1977; McClintock et al., 1988; Dearborn et al., 1983), i d'hàbits omnívors oportunistes (Dearborn, 1977; McClintock, 1994), ha estat extensament emprada com a predador model experimental (per revisions consultar Avila et al., 2008; McClintock et al., 2010), i ha estat també seleccionada per realitzar part dels experiments d'aquesta tesi. En la cerca d'un altre predador experimental rellevant en les comunitats bentòniques antàrtiques, considerem la influència de les poblacions d'amfípodes, les quals exhibeixen una altíssima diversitat, tant en el nombre d'espècies, como en els estils de vida i hàbits alimentaris (De Broyer i Jazdzewski, 1996). A més apareixen en densitats molt elevades (300,000 individus m^{-2}; Huang et al., 2007) associats a substrats vius (amb freqüència algues i esponges), que fan d'hostes i potencials presses (directes o indirectes). Representen per tant un grup interessant amb el que estudiar la incidència de defenses repel·lents en organismes sèssils. De fet, ja s'han provat algunes espècies com a consumidores model, però les més utilitzades, *Gondogeneia antarctica* i *Paramoera walkeri,* mostren limitacions, bé per ser herbívores limitant el seu ús a algues, o bé per mostrar preferència per menjars preparats contenint extractes (Amsler et al., 2005). Entre d'altres espècies, l'amfípode lissianàsid *Cheirimedon femoratus*, per ser abundant i voraç oportunista omnívor, i per tindre distribució circumpolar i euribàtica (Bregazzi, 1972; De Broyer et al., 2007), va ser triat finalment per dissenyar un nou protocol d'experiments de preferència alimentaria. En ell, es va observar una enorme incidència de defenses químiques entre les nostres mostres d'invertebrats i algues provinents d'un ampli rang de profunditats i de les zones del Mar de Weddell i l'Arxipèlag Shetland del Sud. Aquest fet va demostrar

l'adaptabilitat d'aquest amfípode com a predador experimental per detectar agents repel·lents. Però a més el mètode en sí va proporcionar una sèrie d'avantatges metodològiques i un gran poder discriminatori per detectar repel·lències (Capítol 3.1.), probablement relacionat amb que els lissianàsids posseeixen uns gnatòpodes gustatius molt desenvolupats (Kaufmann, 1994). De fet, ambdós consumidors escollits, l'estrella *O. validus* i l'amfípode *C. femoratus*, mostren capacitats notables per a la localització de rastres d'aliment (Kidawa, 2005b; Kidawa, 2005a; Smale et al., 2007; Kidawa, 2009), fet que podria afavorir la detecció de repel·lents. A més, ambdues espècies són tremendament abundants i fàcilment recol·lectables en la nostra zona d'experimentació, BAE Gabriel de Castilla, en l'Illa Decepció, convertint-les en bons models experimentals.

Malgrat que ambdós predadors siguen àmpliament oportunistes-generalistes, els seus hàbits alimentaris i d'atacar la seua presa són distints (Bregazzi, 1972; McClintock, 1994; De Broyer et al., 2007), i açò pot provocar diferents respostes als distints organismes. L'estrella de mar *O. validus* mostra dificultats en ser alimentada amb dietes artificials d'agar, alginat o carragenat (Avila et al., 2000; Iken et al., 2002; i obs. pers.), pel que per als tests utilitzem dietes basades en gambes congelades. Per a l'amfípode *C. femoratus* utilitzem perles de caviar d'alginat, preparades amb el kit del famós cuiner Ferran Adrià. Les perles d'alginat en general posseïen menys contingut energètic que les gambes (segons Atwater factors; Atwater i Benedict, 1902), el que podia influir en la percepció dels possibles repel·lents (Duffy i Paul, 1992; Cruz-Rivera i Hay, 2003). Cap doncs la possibilitat que algunes defenses químiques foren menys evidents als tests amb estrelles, quedant lleugerament emmascarades pel major contingut nutritiu de les gambes respecte al de les perles d'alginat, i també respecte al d'algunes de les mostres en sí. Açò en certa mesura ens permetia comparar activitats repel·lents canviant el predador i la dieta, la qual cosa ve descrita al Capítol 3.2. per a mostres d'organismes de 31 espècies diferents, pertanyents a quatre principals grups: algues, esponges, cnidaris i ascidis, a més de mostres d'un briozou, una holotúria i un pterobranqui.

Les activitats de repel·lència varen ser més freqüents als tests amb amfípodes que contra les estrelles, sobretot en aquelles mostres provinents de macroalgues i esponges hexactinèl·lides, en les que els amfípodes podrien particularment afectar a la producció i distribució de llurs defenses. De fet, els amfípodes antàrtics en associar-se en especial amb algues i esponges (Kunzmann, 1996; Huang et al., 2007; Amsler et al., 2009; Zamzow et al., 2010), malgrat que també amb altres (Loerz, 2003; McClintock et al., 2009), consumeixen de manera directa teixits de l'hoste, o indirectamente en ingerir detrits o microbiota (diatomees) adherides (Kunzmann, 1996; Amsler et al., 2000b; De Broyer et al., 2001; Graeve et al., 2001; Amsler et al., 2009; Zamzow et al., 2010). Així, aquestes congregacions d'amfípodes generalistes, exerceixen una pressió ecològica localitzada als biosubstrats sèssils, que pot ser més intensa que aquella

provocada per predadors mòbils de major mida, como peixos o equinoderms (Hay et al., 1987; McClintock i Baker, 2001; Toth et al., 2007). Les interaccions que es generen d'aquestes associacions transitòries depenen del potencial químic de l'hoste, i dels hàbits de l'amfípode (Sotka et al., 2009). De manera que malgrat que els amfípodes ingereixen teixits dels seus hostes accidentalment a la natura mentre que aprofiten altres fonts, en experiments de laboratori amb dietes artificials les repel·lències per aquests teixits poden fer-se notables, el que podria explicar l'enorme quantitat de repel·lències detectada amb *C. femoratus*. Les fraccions de cnidaris i ascidis varen demostrar contindre repel·lents potents contra els dos predadors. A part d'açò, algunes espècies podrien explotar varies estratègies alternatives, com els nematocists en alguns hidroïdeus i penatulàcis, túniques de baix valor nutritiu en alguns ascidis, o la capacitat locomotriu de les holotúries el·lípides (Wigham et al., 2008), les aviculàries defensives d'alguns briozous (Winston, 1986; Carter et al., 2010), o també encapsulaments reforçats com els dels pterobranquis (Ridewood, 1911). Pel general, aquelles mostres repel·lents només en els tests de *C. femoratus* corresponien a mostres o regions corporals de baix contingut energètic, com les hexactinèl·lides, o com les túniques d'alguns ascidis i els feixos d'alguns coralls, on una menor concentració de repel·lents podria anar correl·lacionada amb el baix valor nutritiu. Apart d'açò, el gran percentatge d'activitats repel·lents en ambdós experiments indica l'existència de defenses químiques efectives contra ambdós tipus de consumidors, en concordància amb la OT.

7.2.D. Aspectes quimio-ecològics en esponges hexactinèl·lides antàrtiques

Els porífers han focalitzat molt d'interès per les seues associacions amb microorganismes, i per ser fonts d'un enorme repertori de metabòlits bioactius, dels que molts es pensa tinguen un origen simbiòtic bacterià (Taylor et al., 2007; Blunt et al., 2012). Pel contrari, existeixen molts pocs estudis de química o ecologia química en hexactinèl·lides (Guella et al., 1988; McClintock et al., 2000; Blumenberg et al., 2002; Thiel et al., 2002). Però es creu que per tindre una organització sincitial, diferent a la resta d'esponges, i amb un mesohil quasi absent, les relacions simbiòtiques amb bacteris són practiment inexistents, d'igual manera que la producció de metabòlits secundaris (Leys et al., 2007). En canvi han estat demostrades a l'Antartida associacions d'hexactinèl·lides amb diatomees (Cattaneo-Vietti et al., 1996). Les hexactinèl·lides solen habitar als fons profunds de tots els oceans, i són difícils d'accedir i recol·lectar, el que ha dificultat enormement el seu estudi (Leys et al., 2007). Al Mar de Weddell per contra, aquestes esponges dominen la plataforma continental, entre els 100 i els 600 metres, creant espectaculars formacions tridimensionals que alberguen un gran nombre d'organismes (Barthel, 1992; Barthel i Gutt, 1992; Gutt, 2007; Janussen i Tendal, 2007).

Les hexactinèl·lides contenen un baix valor nutritiu (10% teixit orgànic en pes sec) (McClintock, 1987; Barthel, 1995). Però malgrat açò, als fons antàrtics són objecte d'una intensa predació per part d'estrelles de mar del gènere *Odontaster*, i nudibranquis, a més de per mesofauna associada; isòpodes, amfípodes, poliquets i d'altres (Dayton et al., 1974; Dayton, 1979; Barthel i Tendal, 1994; Kunzmann, 1996; McClintock et al., 2005). Als nostres tests les hexactinèl·lides varen demostrar una activitat repel·lent molt baixa contra estrelles, però molt considerable contra *C. femoratus*, aquesta darrera presumiblement derivada d'associacions transitòries i/o pel major potencial discriminatori del test amb amfípodes (Núñez-Pons et al., 2012; Capítols 3.1. i 3.2.). Malgrat que han de considerar-se també els metabòlits polars no provats i presents en altres fraccions que podrien afectar a les estrelles. És probable que les hexactinèl·lides combinen un baix valor nutritiu degut al seu alt contingut en espícules (Barthel, 1995), amb poca producció de productes repel·lents (Duffy i Paul, 1992; Chanas i Pawlik, 1995; Waddell i Pawlik, 2000; Cruz-Rivera i Hay, 2003; Jones et al., 2005), per reduïr la predació, en especial d'estrelles. A més açò les podria permetre dirigir més energía cap a la regeneració de teixits danyats després d'episodis d'atac (Leys i Lauzon, 1998; Walters i Pawlik, 2005; Leong i Pawlik, 2010).

Els agents causants de l'elevada incidència d'activitats repel·lents contra els amfípodes semblen derivar del metabolisme primari. De fet al nostre anàlisi no detectem metabòlits secundaris evidents, al menys d'aquells característics d'altres esponges (Blunt et al., 2012 i revisions precedents de la sèrie). Al seu lloc aïllem dos tipus de productes lipídics característicament abundants i a més mancants en demosponges antàrtiques: un quetoesteroide 5α(H)-cholaquestan-3-one (**1**), present a la majoria de les hexactinèl·lides, i una barreja particular de ceramides (**2**) característica de totes les mostres d'hexactinèl·lides. La composició de la barreja de ceramides a les nostres mostres és molt peculiar, doncs es tracta d'una barreja molt simple que només conté dos glucoesfingolípids (GSL) principals, que són dos homòlegs amb àcids grassos de –C24 i –C22 (**2a-b**), el que ens va suggerir un possible valor quimiotaxonòmic (veure sota). Les observacions en SEM ens varen indicar que ambdós composts no semblen derivar directament d'una font de diatomees, doncs totes les esponges, demosponges i hexactinèl·lides de l'estudi varen mostrar el mateix patró d'espècies de diatomees als seus teixits, les quals eren típiques dels blooms estivals australs.

L'esteroide 5α(H)-cholaquestan-3-one va produir repel·lència als tests amb *O. validus* demostrant un paper minoritari com a defensa envers la predació. En canvi les glicoceramides (**2a-b**), conegudes prèviament d'una planta, *Euphorbia biglanduelsa* (Falsone et al., 1987), i ara descrites per primer cop en una esponja, mancaven d'activitat alguna. Sospitem que aquestes ceramides podrien jugar un paper important i formar part de la membrana sincitial de les hexactinèl·lides, de la mateixa manera que ceramides semblants ho fan a les plantes. Les

glicoceramides (**2a-b**) es varen trobar en totes les mostres d'hexactinèl·lides rossellides, antàrtiques i de l'hemisferi nord, per la qual cosa podrien representar marcadors químics d'esponges pertanyents a la família Rossellidae. Les nostres dades a més ens suggereixen que en certa mesura altres tipus similars de GSL podrien ser característics de l'ordre Lyssacinosida (veure Figura 7 del capítol 3.3). Les relacions taxonòmiques dins del fílum Porifera estan subjectes a debat (Reiswig i Mackie, 1983; Leys, 2003; Worheide et al., 2012), i s'han anat desenvolupant, en paral·lel amb estudis morfològics i de biologia molecular clàssics, investigacions amb biomarcadors lipídics per clarificar aquest assumpte (Bergquist et al., 1980; Lawson et al., 1984; Bergquist et al., 1986; Bergquist et al., 1991; Thiel et al., 2002). Per tant, donat que els esfingolípids han estat emprats amb fins quimiotaxonòmics en microorganismes (Takeuchi et al., 1995), al nostre parèixer seria interessant saber com es distribueixen tipus de GSL, similars a les ceramides (**2a-b**), dins de la classe Hexactinellida. Creiem que potser aquestes molècules podrien contribuir a la classificació d'aquesta classe pel moment tan confusa (Barthel, 1992; Göcken i Janussen, 2011; Janussen, com. pers.). Els resultats detallats d'aquesta secció es poden consultar al Capítol 3.3.

7.2.E. Ecologia química de coralls tous antàrtics del gènere *Alcyonium*

Els antozous són el tercer taxó en dominància al bentos del Mar de Weddell (Arnaud, 1977; Orejas, 2001). El gènere de coralls tous *Alcyonium* és particularment comú i està representat per vuit espècies antàrtiques, algunes molt abundants. Els coralls tous manquen de la protecció oferta per esquelets massius de carbonat càlcic. En el seu lloc estan formats per un teixit tou (coenènquima) que presenta incrustacions d'esclerits diminuts i espinosos que donen suport (Brusca i Brusca, 2003), els quals s'ha proposat siguen ineficaços contra els principals predadors antàrtics, les estrelles (McClintock, 1994). A més, el seu sistema de nematocists és dèbil (Mariscal i Bigger, 1977; Brusca i Brusca, 2003) respecte al d'altres cnidaris (Stachowicz i Lindquist, 2000; Bullard i Hay, 2002; Hines i Pawlik, 2012), i per tant inefectiu com a defensa (Schmidt, 1974; Sammarco i Coll, 1992). Malgrat tot i el seu ric valor nutritiu, els coralls *Alcyonium* són evitats pels predadors antàrtics en fons somers, i en efecte només una espècie de picnogònid ha estat observada alimentant-se d'ells (Slattery i McClintock, 1995; obs. pers.). Els coralls tous de fet es troben altament protegits químicament contra la predació i l'epibiosi, principalment mitjançant productes terpenoides i esteroides (La Barre et al., 1986a, 1986b; Coll et al., 1987; Mackie, 1987; Wylie i Paul, 1989; Sammarco i Coll, 1992; Kelman et al., 1999; Wang et al., 2008; Hines i Pawlik, 2012). D'acord amb aquestes dades, el nostre estudi amb sis mostres de coralls antàrtics del gènere *Alcyonum* varen demostrar repel·lència contra *O. validus* per a les cinc espècies representades, i només una de les mostres mancava d'activitat aparent. Igualment les tres mostres provades contra *C. femoratus* varen ser significativament repel·lents. Tant els terpens iludalans (**1**-**9**) d'*Alcyonium grandis*, com els esters de ceres (**12**-**13**) obtinguts

de totes les mostres d'*Alcyonium* posseïen propietats repel·lents, amb l'excepció de que per falta de quantitat suficient els iludalans (**1-9**) no varen poder ser provats contra l'amfípode. Açò ens revelava la identitat d'alguns dels metabòlits involucrats en la defensa. Sembla que els iludalans (**1-9**) conjuntament amb les ceres (**12-13**) cooperen per evitar la predació en l'espècie *A. grandis*. Els altres iludalans (**10-11**) aïllats d'*A. roseum* 1 no varen poder ser provats als experiments, però donada la seua gran proximitat molecular amb els iludalans (**1-9**), segurament també posseeixen característiques repel·lents, i col·laboren additivament i conjunta amb les ceres. De fet, la mostra *A. roseum* 1 que contenia els iludalans **10-11** va mostrar repel·lència, Mentre que la mostra conespecífica *A. roseum* 2, que mancava d'aquests composts, era inactiva, malgrat que ambdues posseïen les ceres. Açò també suggereix que les ceres, malgrat ser elles mateixes actives com a sub-fraccions aïllades, semblen no ser tan efectives en la defensa a nivell de tota la colònia sense la presència d'altres repel·lents. En la resta d'espècies de l'estudi (*A. antarcticum*, *A. haddoni* i *A. paucilobulatum*) és provable que la defensa contra la predació s'aconsegueixi similarment gràcies a l'efecte sinèrgic de les ceres conjuntament a altres composts repel·lents minoritaris no identificats. Apart d'açò, tres de les mostres varen exhibir algun tipus de propietat contra el recobriment en inhibir el creixement d'un bacteri marí antàrtic. Les activitats contra soques bacterianes de l'entorn no associades amb el corall són en efecte comuns entre els coralls tous, tant antàrtics como no antàrtics (Ducklow i Mitchell, 1979; Rublee et al., 1980; Slattery et al., 1995; Kelman et al., 1998; Ritchie, 2006).

Fins la data la única espècie de corall antàrtic del gènere *Alcyonium* que s'ha investigat en ecologia química ha estat *A. paessleri* (sinonimitzat amb *A. antarcticum*; Verseveldt i Van Ofwegen, 1992), que aquí és novament estudiada. Aquesta espècie ha demostrat un extens llistat d'activitats ecològiques basades en composts orgànics (Slattery et al., 1990; Slattery i McClintock, 1995; Slattery et al., 1995; Slattery et al., 1997a; Slattery i McClintock, 1997), a més de posseir un ric però variable arsenal de metabòlits secundaris (Slattery i McClintock, 1997). En efecte, nostre *A. antarcticum* no va proporcionar cap dels terpens prèviament descrits en varis treballs (Palermo et al., 2000; Rodríguez-Brasco et al., 2001; Manzo et al., 2009). La variabilitat en el patró de metabòlits secundaris observat en *A. antarcticum*, i ara també en *A. roseum*, podria respondre a qüestions de variabilitat genètica intraespecífica (Harvell et al., 1993), a tractar-se de defenses induïbles (Slattery et al., 2001; Hoover et al., 2008), o a un origen simbiòtic (Kelecom, 2002). Els iludalans de la sèrie de les alciopterosines són típics de fongs i falgueres (Gribble, 1996; Suzuki et al., 2005), i a més han estat obtinguts dels coralls antàrtics profunds *A. paessleri* (*A. antarcticum*) i *A. grandis* (Palermo et al., 2000; Carbone et al., 2009), i ara també d'*A. roseum*. La presència dels iludalans es pot deure a una retenció evolutiva i/o a un origen simbiòtic, como s'hipotetitza per altres terpens bioactius de coralls tous. Un exemple és el pukalide, que apareix en espècies pacífiques de *Sinularia* (Wylie i Paul,

1989; Van Alstyne et al., 1994; Slattery et al., 2001), i també en *A. antarcticum* (Manzo et al., 2009).

Els metabòlits secundaris són normalment considerats els responsables de les activitats defensives (Paul, 1992), però hi ha també alguns esteroides, derivats del metabolisme primari, que proporcionen protecció en coralls tous, esponges i aranyes de mar (Bobzin i Faulkner, 1992; Tomaschko, 1994; Slattery et al., 1997a; Fleury et al., 2008; Moran i Woods, 2009; Núñez-Pons et al., 2012). El cost de la producció de composts bioactius (Rhoades i Gates, 1976) podria reduir-se mitjançant l'ús de metabòlits primaris per a finalitats ecològiques. Als coralls tous les ceres són les principals reserves d'energia, la concentració de la qual minva després d'interaccions competitives com a inversió per a la producció de metabòlits secundaris (terpens) defensius (Fleury et al., 2004). Per la qual cosa, si les ceres tingueren propietats defensives en sí, significaria un tàctica d'estalvi d'energia. En efecte, les ceres pot ser que haguen evolucionat com a reserves lipídiques als coralls, enlloc dels més comuns triglicèrids perquè proporcionen avantatges addicionals. Les ceres són indigestes (Benson et al., 1978; Place, 1992), i com demostren els nostres resultats, poden conferir repel·lència als teixits i al mucus dels coralls. Solament les estrelles corona d'espines (*Acanthaster spp*) són capaces d'alimentar-se voraçment dels coralls, degut a una adaptació única: un sistema enzimàtic per digerir ceres (Benson et al., 1975). Inesperadament, *C. femoratus* sembla ser menys susceptible als esters de ceres (**12-13**) que *O. validus*, potser perquè, així com els amfípodes antàrtics fan ús de les ceres com a reserves també, les estrelles de mar manquen d'aquests composts (Sargent et al., 1977).

Els nostres extractes de coralls consistien en complexes barreges de metabòlits primaris i secundaris, obtinguts del teixit intern i del mucus. Malgrat que no es va analitzar específicament, el mucus és essencial en processos protectors, i és ric en ceres (60% de la composició mucolípida), esterols i terpens, essent un medi on els composts de defensa són exsudats (Ducklow i Mitchell, 1979; Coll et al., 1982; Miyamoto et al., 1994; Slattery et al., 1997a; Wang et al., 2008). Els iludalans (**1-11**), conjuntament amb les ceres (**12-13**), són probablement secretats dins del mucus en les espècies estudiades on acompleixen el seu rol defensiu (els resultats d'aquesta secció estan detallats als Capítols 3.4.)

7.2.F. Distribució de les defenses químiques i metabòlits secundaris en ascidis colonials antàrtics

Dins de la classe Ascidiacea la família Polyclinidae és una de les més abundants a la plataforma antàrtica, i dins d'aquesta els gèneres *Aplidium* i *Synoicum* estan molt ben representats (Ramos-Esplá et al., 2005). En general els ascidis colonials presenten molta variabilitat morfològica intraespecífica i de coloració (Varela, 2007). Els ascidis han desenvolupat molts mecanismes per previndre la predació, molts relacionats amb propietats físiques i químiques de la túnica (Lambert, 2005), com poden ser la possessió de túniques gruixudes, característiques d'espècies solitàries (Koplovitz i McClintock, 2011), túniques amb inclussions d'esclerits (Lambert, 1979; Lambert i Lambert, 1997; López-Legentil et al., 2006), o amb escàs valor nutritiu (Tarjuelo et al., 2002). Però, la principal línia de protecció sembla ser la química defensiva, que pot consistir en l'acumulació de metalls pesants o àcids inorgànics (Stoecker, 1980b; Stoecker, 1980a; Pisut i Pawlik, 2002; McClintock et al., 2004), o en la producció de metabòlits repel·lents (McClintock et al., 2004; López-Legentil et al., 2006; Núñez-Pons et al., 2010). Les espècies estudiades mancaven de túniques gruixudes o amb esclerits (Varela, 2007; obs. pers.) i en cap d'elles, ni en espècies relacionades de la família s'ha descrit l'acumulació significant de metalls o àcids (Stoecker, 1980b; Stoecker, 1980a; Hirose, 2001; Lebar et al., 2011). Amb el que prediem que els metabòlits secundaris haurien de ser segurament emprats en les espècies del nostre estudi per a la defensa. Malgrat açò, en estudis anteriors es va observar una escassa prevalència de defensa química basada en química orgànica (Koplovitz et al., 2009). Els nostres resultats en canvi, mostren que l'ús de les defenses químiques s'estenia per totes les espècies i mostres examinades. Els experiments amb estrelles, a més varen demostrar que algunes espècies tendien a concentrar els agents repel·lents cap a l'interior de les colònies, com *A. fuegiense, A. millari*, i el morfotipus blanc i negre (B&W), així com dues mostres del morfotipus taronja (O) de l'espècie *Synoicum adareanum*. Aquesta distribució en principi contradeia les prediccions de l'ODT. Però, en ascidis composts és freqüent produir estats larvaris defensats contra predadors, donada la gran inversió que es fa en la reproducció, i les defenses químiques tendeixen a estar situades als teixits interns (gònades) (Young i Bingham, 1987; Lindquist i Fenical, 1991; Lindquist et al., 1992; Tarjuelo et al., 2002). En aquests casos les túniques podrien combinar una producció de repel·lents relativament pobre, conjuntament a un valor nutritiu baix, que contribuiria a la defensa total de la colònia, complementant altres mecanismes coexistents. De fet, les baixes concentracions d'extracte obtingudes d'aquestes túniques, respecte a aquelles de les respectives zones internes reflecteixen aquests fets. En canvi, altres espècies analitzades, com *Aplidium falklandicum, A. meridianum*, i *S. adareanum* (O) 2, no varen mostrar localització de defenses intracolonial. Alguns dels patrons de distribució diferencial d'activitats repel·lents semblen estar lligats amb la distribució d'alguns dels metabòlits secundaris defensius

trobats. *Aplidium falklandicum* i *A. meridianum* varen revelar la identitat dels seus repel·lents, les meridianines (A-G), que eren efectives contra ambdós *O. validus* i *C. femoratus*. Aquests alcaloides estan presents tant a la part interna com a l'externa, malgrat que són més abundants a l'externa (Núñez-Pons et al., 2010). El rossinone B va demostrar participar en la defensa de tota la colònia en *A. fuegiense*, envers amfípodes i estrelles, però es predominant en regions internes (Carbone et al., 2012; present estudi).

Pel que fa a processos epibiòtics, totes les mostres estaven lliures de recobriment evident, però en concordança amb l'estudi previ de Koplovitz et al. (2011), els extractes crus eteris dels nostres ascidis, així com el rossinone B mancaven d'activitats notables antibacterianes. En canvi les meridianines, que aïlladament no havien reflectit propietats antibiòtiques contra soques cosmopolites de bactèries i llevats (Núñez-Pons et al., 2010), com a barreja varen produir inhibició contra una bactèria simpàtrica, demostrant així la seua polivalència com a defenses.

De moment sis espècies dels gèneres *Aplidium* i *Synoicum* han estat analitzades químicament. En el cas de *S. adareanum* la seua variabilitat morfològica, bioactiva, i el seu divers patró de metabòlits secundaris entre espècimens de distintes àrees, suggereixen una revisió taxonòmica en aquesta espècie (Diyabalanage et al., 2006; Miyata et al., 2007; Varela, 2007; Koplovitz et al., 2011; present estudi), a més d'un possible origen simbiòtic d'alguns composts (Riesenfeld et al., 2008). El gènere *Aplidium* en canvi, és conegut per la quantitat i varietat de productes naturals proporcionats, sobretot prenil quinones, productes nitrogenats, ciclopèptids, i una enorme varietat d'alcaloides, però el que caracteritza al gènere és la propensió a produir derivats terpènics (Zubía et al., 2005). Nosaltres trobem els meroterpenoides rossinones B-E en *A. fuegiense* (Carbone et al., 2012; present estudi). Ací el producte majoritari i més bioactiu del grup és sens dubte el rossinone B, present predominantment als teixits interns, però, malgrat que en quantitats molt petites, també a la túnica. Els altres rossinones (C-E) en canvi, es troben exclusivament a l'interior de la colònia, presumiblement com a precursors. Els rossinones A i B varen ser descoberts en una espècie d'*Aplidium* del Mar de Ross (Appleton et al., 2009). Les meridianines en canvi, són alcaloides indòlics originalment descrits en *A. meridianum* de les Illes Georgia del Sud. Es varen descriure set meridianines principals A, B, C, D, E, F i G, presents formant una barreja, malgrat que les meridianines F i G eren menys abundants (Hernández Franco et al., 1998; Seldes et al., 2007). Nosaltres les varem aïllar per primer cop en *A. falklandicum*, i a partir de quantificacions relatives varem confirmar que B/E són les meridianines més comunes seguides per C/D i després A, mentre que F i G eren clarament minoritàries. També varem aportar les assignacions de carbó i el protònic de les meridianines F i G en DMSO (Núñez-Pons et al., 2010), i varem identificar noves meridianines minoritaries (I-U), a més d'uns dímers no descrits derivats de meridianines majoritàries en una mostra d'*A. falklandicum*. Cal destacar l'absència de

meridianina D en totes les mostres d'*A. falklandicum*, malgrat ser una meridianina majoritària, la qual podria ser específica d'*A. meridianum* (Núñez-Pons et al., 2010). En relació amb açò, ambdues espècies estan sota estudi taxonòmic, i se planteja sinonimitzar-les com a morfotipus distints de la mateixa espècie (Varela, 2007; Tatián com. pers.).

Els patrons de distribució de metabòlits secundaris en espècimens d'*Aplidium*, conjuntament amb els tipus de molècula trobats, suggereixen distints orígens per a meridianines i rossinones. Mentre que els rossinones són característics de teixits interns, on es probable que tinga lloc la seua biosíntesi (Carbone et al., 2012), les meridianines es trobaven més concentrades a les regions externes de les colònies (Núñez-Pons et al., 2010). Entre els productes coneguts derivats de microorganismes, els terpens escassegen, mentre que els alcaloides predominen (Paul et al., 1990; Kelecom, 2002; Franks et al., 2005; Bandaranayake, 2006; Ivanova et al., 2007), d'altra banda els microsimbionts solen habitar a les túniques dels ascidis colonials (Schmidt et al., 2005; i revisat en Sings i Rinehart, 1996; Hildebrand et al., 2004; Hirose, 2009). Les meridianines posseeixen una pigmentació groc viu, i s'han obtingut a l'Antartida de vàries espècies d'ascidis colonials dels gèneres *Aplidium* (Hernández Franco et al., 1998; Seldes et al., 2007; Núñez-Pons et al., 2010) i *Synoicum* (Lebar i Baker, 2010) a més de en l'esponja *Psammonemma sp*. (Butler et al., 1992; Lebar i Baker, 2010). Açò ens porta a plantejar que podria tractar-se de pigments vestigials mantinguts en diversos organismes antàrtics pel seu paper ecològic multifuncional (revisat en Bandaranayake, 2006). I com molts altres pigments alcaloides bioactius, es suggereix també que deriven de microbis simbionts (Paul et al., 1990; Franks et al., 2005). Per consultar més detalladament els resultats d'aquesta secció, mireu el Capítol 3.5. i 3.6.

7.2.G. Conclusions

L'amfípode *Cheirimedon femoratus* va demostrar ser un predador experimental model molt apropiat per realitzar experiments de detecció de defenses químiques en aigües antàrtiques. Especialment als grups de les macroalgues i les hexactinèl·lides les defenses químiques eren més freqüents contra l'amfípode, que envers l'estrella *O. validus*. *Cheirimedon femoratus* s'associa de manera oportunista amb biosustrats sèssils del fons provocant una pressió constant en aquests organismes, que pot resultar més intensa que l'exercida per macropredadors mòbils menys recurrents com *O. validus*. Les esponges i algues antàrtiques representen potencials hostes-presa per als amfípodes, que els prefereixen com a substrats. Per açò en alguns casos els amfípodes podrien substituir a les estrelles com a principals inductors de la distribució de les defenses químiques, posant en dubte prediccions prèvies del funcionament de l'ODT al bentos

antàrtic. De fet varem observar una absència general de concentració diferencial de defenses a les nostres esponges.

Considerant que els recursos interns d'energia són limitats, l'ús de metabòlits primaris per a la defensa representa una estratègia efectiva d'estalvi d'energia. Creiem que l'èxit evolutiu del nostre grup d'estudi en les comunitats antàrtiques està relacionat amb la presència de defenses químiques. En esponges hexactinèl·lides aquestes semblen ser més febles i derivades del metabolisme primari, però compensades amb un baix valor nutritiu. Alguns GSL en canvi, podrien posseir un valor quimiotaxonòmic com a marcadors químics de la família d'hexactinèl·lides Rossellidae. En els coralls tous la protecció química s'obté tant de metabòlits primaris como secundaris, sembla ser que tots ells operant de forma sinèrgica. Suggerim a més la secreció d'aquests metabòlits com a part del mucus. Mentre què en els ascidis colonials els metabòlits defensius semblen ser predominantment secundaris i molt potents, i a més en algunes espècies aquests tendeixen a acumular-se als teixits interns de les colònies, presumiblement per produir larves protegides. Les propietats de composts com les meridianines o els iludalans no poden ser atribuïdes a un compost en particular, sinó a la barreja sencera, que sol aparèixer en quantitats importants. La producció de grups de composts potencialment mimètics en base al seu paregut estructural podria augmentar la seua concentració com a barreja, i amb açò el senyal bioactiu. Alguns metabòlits aïllats de varies espècies, gèneres en inclús fílums, i de diferents àrees geogràfiques, com succeeix amb les meridianines, suggereixen, o bé una extensa retenció evolutiva, o un possible origen simbiòtic i retenció d'aquesta associació per les beneficioses bioactivitats conferides. En el referent al recobriment bacterià, els nostres ascidis varen mostrar poca activitat, però algunes espècies de coralls sí que varen exhibir respostes inhibitòries. Entre els nostres estudis futurs preveiem incloure experiments per avaluar la inhibició d'invasió per diatomees.

S'ha proposat un gradient descendent en la diversitat de metabòlits secundaris marins a mesura que augmentem de latitud. A les zones polars en haver hagut menor recerca, no és possible establir una conclusió pel moment, no obstant això molts organismes marins antàrtics estan proporcionant enormes quantitats de productes naturals. Fins on arriba el nostre coneixement, els nostres estudis són dels únics que revelen la identitat de metabòlits ecològicament rellevants en la defensa d'hexactinèl·lides, coralls tous i ascidis colonials de l'Antàrtida. Amb la recerca d'aquesta tesi doctoral esperem contribuir a l'ecologia antàrtica, especialment en el camp de la química defensiva a través de productes naturals, i per tractar amb espècies en la seua majoria mai abans estudiades.

En general, existeix la necessitat d'estendre els experiments ecològics al camp. Encara falta molt per saber com funcionen els al·leloquímics, i en determinar si existeix la defensa química

induïble a l'Antartida, mitjançant monitorització de les concentracions de defenses abans i després d'episodis d'atac. També la relació entre valor nutritiu i defensa química és digne de tindre en compte de cara al futur. De fet, *C. femoratus*, com altres amfípodes, permet per la seua fàcil adaptabilitat a dietes artificials, fer aquest tipus d'estudis variant la quantitat de repel·lents i la font alimentària dins de les dietes preparades. Finalment, els mecanismes pels que els animals discriminen, detecten i trien entre preses defensades químicament o no, són en l'actualitat desconeguts. Per aquest motiu es precisa d'estudis dels processos sensorials en predadors i preses, així com dels efectes que provoquen els repel·lents en els predadors, i respecte a generalistes i especialistes.

INFORMES DE LA DIRECTORA DE LA TESIS

INFORME I

Informe de la Directora de la Tesis sobre el factor del impacto de los artículos publicados y/o enviados a revistas científicas.

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Informe de la Directora de la Tesis sobre el factor de impacto de los artículos publicados y/o enviados a revistas científicas

Como directora de la tesis doctoral de Laura Núñez Pons, emito el siguiente informe sobre el factor de impacto de las publicaciones presentadas en la tesis:

- Publicación I. NÚÑEZ-PONS L, RODRÍGUEZ-ARIAS M, GÓMEZ-GARRETA A, RIBERA-SIGUÁN A and AVILA C. 2012. Feeding deterrency in Antarctic marine organisms: bioassays with an omnivorous lyssianasid amphipod. ***Marine Ecology Progress Series****, in press*.

Este artículo ha sido aceptado recientemente (mayo 2012) en la revista Marine Ecology-Progress Series (ISSN 0171-8630), que tiene en la última edición disponible del Journal Citation Reports (2011) un índice de impacto de **2.711**. Actualmente esperamos las pruebas de imprenta. *Marine Ecology-Progress Series* es una de las revistas que forma el "núcleo duro" del área de ciencias marinas y es una referencia obligada para cualquier estudio en esta disciplina, con diseños experimentales muy exigentes en el campo de la ecología marina más aplicada. Esta revista figura en el segundo cuartil (49 de 131) del área de "Ecology", en el primero (12 de 97) de "Marine and Freshwater Biology", y en el primer cuartil de "Oceanography" (8 de 59).

- Publicación II. NÚÑEZ-PONS L and AVILA C. 2012. Comparative study of unpalatability in Antarctic benthic organisms towards two relevant sympatric consumers: does it taste matter? ***Polar Biology****,* submitted.

Polar Biology (ISSN 0722-4060) tiene un índice de impacto de **1.659** (JCR Reports, 2011). *Polar Biology* es una de las revistas de más prestigio para este tipo de contribuciones a la biología polar, se encuentra clasificada en el área de "Ecology" en el tercer cuartil (75 de 131), y en el segundo cuartil en el área de "Biodiversity and conservation" (16 de 35).

- Publicación III. NÚÑEZ-PONS L, CARBONE M, PARIS D, MELCK D, RÍOS P, CRISTOBO J, CASTELLUCCIO F, GAVAGNIN M and AVILA C. 2012. Chemo-ecological studies on hexactinellid sponges from the Southern Ocean. ***Naturwissenschaften*** 99(5):353-368.

Naturwissenschaften (ISSN 0028-1042) tiene un índice de impacto de **2.278** (JCR Reports, 2011). Se trata de una revista que tiene un carácter multidisciplinar, con una visión amplia tanto en sistemas acuáticos como terrestres, por lo que se espera una gran difusión del trabajo. Se encuentra en el primer cuartil del area "Multidisciplinary Sciences" (11 de 55).

- Publicación IV. NÚÑEZ-PONS L, CARBONE M, VÁZQUEZ J, GAVAGNIN M and AVILA C. 2012. Chemical ecology of *Alcyonium* soft corals from Antarctica. ***Journal of Chemical Ecology****,* submitted.

Esta publicación está enviada a la revista *Journal of Chemical Ecology* (ISSN 0098-0331), que tiene un índice de impacto de **2.657** (JCR Reports, 2011). Es una revista de gran prestigio y difusión, y de más proyección para estudios de ecología que involucran la química y los productos naturales como mediadores de funciones ecológicas relevantes. Dada la temática de esta Tesis Doctoral es una revista que se ajusta mucho a los manuscritos elaborados. Dentro del área "Biochemistry and molecular Biology" se encuentra en el tercer cuartil (162 de 289), y en el segundo del área "Ecology" (51 de 131).

- Publicación V. NÚÑEZ-PONS L, FORESTIERI R, NIETO RM, VARELA M, NAPPO M, RODRÍGUEZ J, JIMÉNEZ C, CASTELLUCCIO F, CARBONE M, RAMOS-ESPLÁ A, GAVAGNIN M, and AVILA C. 2010. Chemical defenses of tunicates of the genus *Aplidium* from the Weddell Sea (Antarctica). ***Polar Biology*** 33(10):1319-1329.

Este trabajo se publicó en la revista *Polar Biology* (ver publicación II). Es de destacar que el índice de impacto del año 2010, **1.445** (JCR 2010) se ha incrementado en el año 2011 (ver

arriba). En el 2010 se encontraba clasificada en el área de "Ecology" en el tercer cuartil (80 de 130), y en el segundo cuartil en el área de "Biodiversity and conservation" (15 de 34).

- Publicación VI. NÚÑEZ-PONS L, CARBONE M, VÁZQUEZ J, RODRÍGUEZ J, NIETO RM, VARELA M, GAVAGNIN M and AVILA C. 2012. Natural products from Antarctic colonial ascidians of the genera *Aplidium* and *Synoicum*: variability and defensive role. ***Marine Drugs***, submitted.

Marine Drugs, con un factor de impacto de **3.854** (JCR 2011), y dentro del primer cuartil del área de "Chemistry, Medicinal" (7 de 59), es una revista de gran prestigio dentro del ámbito de los productos naturales marinos con bioactividad. Dado que sus publicaciones son en "Open access" (de libre acceso), se espera una importante difusión y consulta vía web. Es por tanto una revista muy adaptada al tema de este proyecto.

En resumen, todas las publicaciones de esta Tesis están publicadas o enviadas a revistas con elevado índice de impacto, en algunos casos incluso en ámbitos generales y multidisciplinarios, y no exclusivamente marinos.

Y para que conste a los efectos oportunos, firmo la presente en Barcelona, a 12 de julio de 2012.

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INFORME II

Informe de la Directora de la Tesis sobre la participación de la doctoranda en cada uno de los artículos de esta Tesis

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Informe de la Directora de la Tesis sobre la participación de la doctoranda en cada uno de los artículos presentados

Como Directora de la Tesis Doctoral de Laura Núñez Pons, emito el siguiente informe sobre la contribución de la doctoranda en las publicaciones presentadas en esta tesis:

- Publicación I:

NÚÑEZ-PONS L, RODRÍGUEZ-ARIAS M, GÓMEZ-GARRETA A, RIBERA-SIGUÁN A and AVILA C. 2012. Feeding deterrency in Antarctic marine organisms: bioassays with an omnivorous lyssianasid amphipod. ***Marine Ecology Progress Series***, *in press*.

. Contribución de la doctoranda: Diseño del trabajo, recogida, procesado e identificación de las muestras y organismos experimentales, trabajo de laboratorio en las extracciones químicas, realización de los experimentos y análisis estadístico de los mismos, redacción de la primera versión del manuscrito y revisiones posteriores.

. Contribución de los co-autores: MR-A participación en los experimentos. AG-G y AR-S identificación taxonómica de las algas y participación en la primera revisión. CA dirección y supervisión del trabajo de laboratorio, recogida, identificación y procesado de las muestras y organismos experimentales, y participación en las revisiones posteriores.

- Publicación II:

NÚÑEZ-PONS L and AVILA C. 2012. Comparative study of unpalatability in Antarctic benthic organisms towards two relevant sympatric consumers: does it taste matter? ***Polar Biology****,* submitted.

. Contribución de la doctoranda: Diseño del trabajo, recogida, procesado e identificación de las muestras y organismos experimentales, trabajo de laboratorio en las extracciones químicas, realización de los experimentos y análisis estadístico de los mismos, redacción de la primera versión del manuscrito y revisiones posteriores.

. Contribución de los co-autores: CA diseño, dirección y supervisión del trabajo, recogida, identificación y procesado de las muestras y organismos experimentales, realización de los experimentos, y participación en las revisiones posteriores.

- Publicación III:

NÚÑEZ-PONS L, CARBONE M, PARIS D, MELCK D, RÍOS P, CRISTOBO J, CASTELLUCCIO F, GAVAGNIN M and AVILA C. 2012. Chemo-ecological studies on hexactinellid sponges from the Southern Ocean. ***Naturwissenschaften*** 99(5):353-368.

. Contribución de la doctoranda: Diseño del trabajo, recogida, procesado e identificación de las muestras y organismos experimentales, trabajo de laboratorio en las extracciones químicas y purificaciones de las fracciones y compuestos aislados, realización de los experimentos y análisis estadístico de los mismos, redacción de la primera versión del manuscrito y revisiones posteriores.

. Contribución de los co-autores: MC participación en los análisis químicos, determinación de la estructura de las moléculas y participación en las revisiones posteriores. DP y DM análisis NMR de los compuestos. PR y JC identificación taxonómica de esponjas. FC participación en las extracciones y purificaciones químicas. MG supervisión del trabajo químico. CA diseño, dirección y supervisión del trabajo, recogida y procesado de las muestras y organismos experimentales, realización de experimentos, y participación en las revisiones posteriores.

- Publicación IV:

NÚÑEZ-PONS L, CARBONE M, VÁZQUEZ J, GAVAGNIN M and AVILA C. 2012. Chemical ecology of *Alcyonium* soft corals from Antarctica. ***Journal of Chemical Ecology,*** submitted.

. Contribución de la doctoranda: Diseño del trabajo, recogida, procesado de las muestras y organismos experimentales, identificación taxonómica de los corales, trabajo de laboratorio en las extracciones químicas y purificaciones de las fracciones y compuestos aislados, realización de los experimentos y análisis estadístico de los mismos, redacción de la primera versión del manuscrito y revisiones posteriores.

. Contribución de los co-autores: MC participación en los análisis químicos, determinación de la estructura de las moléculas y participación en las revisiones posteriores. JV realización de los tests antibacterianos. MG supervisión del trabajo químico. CA diseño, dirección y supervisión del trabajo, recogida y procesado de las muestras y organismos experimentales, realización de experimentos, y participación en las revisiones posteriores.

- Publicación V:

NÚÑEZ-PONS L, FORESTIERI R, NIETO RM, VARELA M, NAPPO M, RODRÍGUEZ J, JIMÉNEZ C, CASTELLUCCIO F, CARBONE M, RAMOS-ESPLÁ A, GAVAGNIN M, and AVILA C. 2010. Chemical defenses of tunicates of the genus *Aplidium* from the Weddell Sea (Antarctica). ***Polar Biology*** 33(10):1319-1329.

. Contribución de la doctoranda: Diseño del trabajo, recogida, procesado de las muestras y organismos experimentales, identificación taxonómica de los tunicados, trabajo de laboratorio en las extracciones químicas, purificaciones de las fracciones y compuestos aislados, realización de los experimentos y análisis estadístico de los mismos, redacción de la primera versión del manuscrito y revisiones posteriores.

. Contribución de los co-autores: RF, MN y FC participación en las purificaciones químicas. RMN, JR y CJ cuantificación química de las meridianinas. MV y AR-E identificación taxonómica de los tunicados. MC participación en los análisis químicos y determinación de la estructura de las moléculas. MG supervisión del trabajo químico. CA diseño, dirección y supervisión del trabajo, recogida, identificación y procesado de las muestras y organismos experimentales, realización de experimentos, y participación en las revisiones posteriores.

- Publicación VI:

NÚÑEZ-PONS L, CARBONE M, VÁZQUEZ J, RODRÍGUEZ J, NIETO RM, VARELA M, GAVAGNIN M and AVILA C. 2012. Natural products from Antarctic colonial ascidians of the genera *Aplidium* and *Synoicum*: variability and defensive role. ***Marine Drugs****,* submitted.

. Contribución de la doctoranda: Diseño del trabajo, recogida, procesado de las muestras y organismos experimentales, identificación taxonómica de los tunicados, trabajo de laboratorio en las extracciones químicas, purificaciones de las fracciones y compuestos aislados, realización de los experimentos y análisis estadístico de los mismos, redacción de la primera versión del manuscrito y revisiones posteriores.

. Contribución de los otros autores: MC participación en los análisis químicos, determinación de la estructura de las moléculas y participación en las revisiones posteriores. JV realización de los tests antibacterianos. JR y RMN detección e identificación de meridianinas minoritarias por LC-MS. MV identificación taxonómica los tunicados. MG supervisión del trabajo químico. CA diseño, dirección y supervisión del trabajo, recogida y procesado de las muestras y organismos experimentales, realización de experimentos, y participación en las revisiones posteriores.

Los co-autores de dichas publicaciones no utilizarán en ningún caso estos datos para otras Tesis Doctorales.

Y para que conste a los efectos oportunos firmo la presente en Barcelona, a 12 de julio de 2012

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ANEXO: Participación de la doctoranda en otros artículos publicados y/o en preparación relacionados directa o indirectamente con esta Tesis

- AVILA C, TABOADA S and NÚÑEZ-PONS L. 2008. Marine Antarctic chemical ecology: what is next? ***Marine Ecology*** 29:1-70. Impact factor **1.234** (JCR 2008).

- CARBONE M, NÚÑEZ-PONS L, CASTELLUCCIO F, AVILA C and GAVAGNIN M. 2009. Illudalane sesquiterpenoids of the alcyopterosin series from the Antarctic marine soft coral *Alcyonium grandis*. ***Journal of Natural Products*** 72(7):1357-1360. Impact factor **3.159** (JCR 2009). ANNEX I

- BALLESTEROS M, NÚÑEZ-PONS L, VÁZQUEZ J, CRISTOBO FJ, TABOADA S, FIGUEROLA B and AVILA C. 2011. Ecología química en el bentos antártico. ***Ecosistemas*** 20(1):54-68.

- FIGUEROLA B, NÚÑEZ-PONS L, VÁZQUEZ J, TABOADA S, CRISTOBO FJ, BALLESTEROS M and AVILA C. 2012. Chemical interactions in Antarctic marine benthic ecosystems. In: Cruzado A., (ed.). **Marine Ecosystems**. In Tech Open Access Publisher of Scientific Books and Journals. On line:http://www.intechopen.com/articles/show/title/chemical-interactions-in-antarctic-marine-benthic-ecosystems

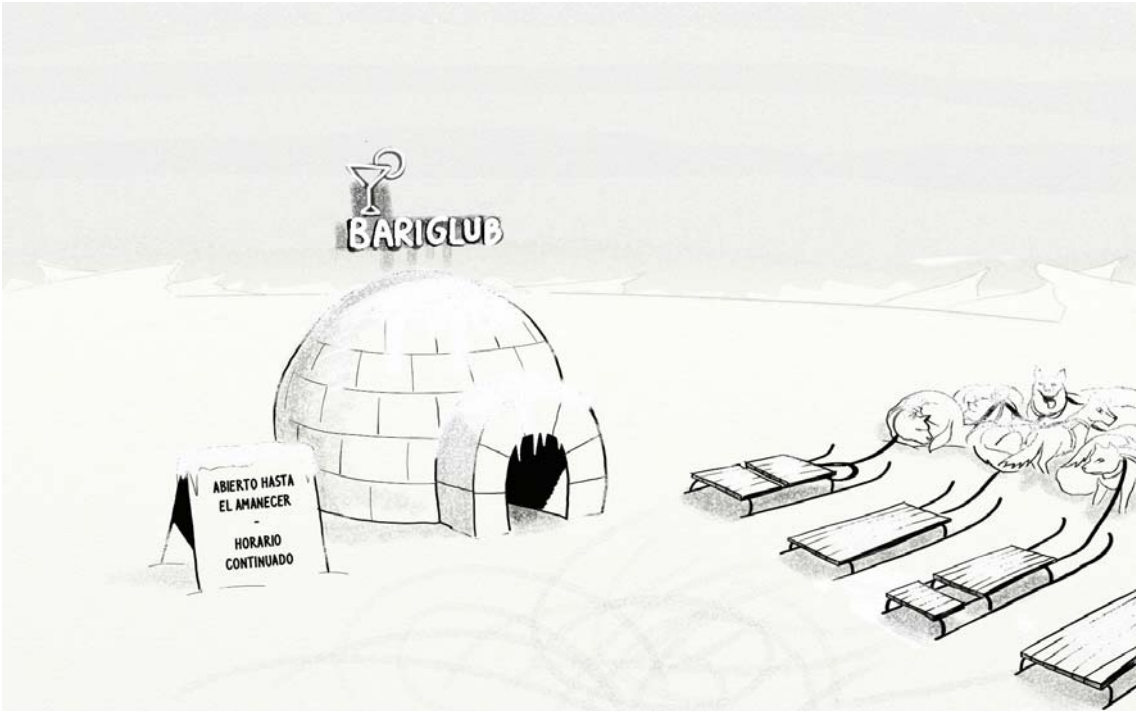
- CARBONE M, NÚÑEZ-PONS L, CASTELLUCCIO F, AVILA C and GAVAGNIN M. 2012. Rossinone-related meroterpenes from the Antarctic ascidian *Aplidium fuegiense*. ***Tetrahedron*** 68:3541-3544. Impact factor **3.025** (JCR 2011). ANNEX II

- TABOADA S, NÚÑEZ-PONS L and AVILA C. 2012. Feeding repellence of Antarctic and sub-Antarctic benthic invertebrates against the omnivorous sea star *Odontaster validus* Koehler, 1906. ***Polar Biology***, *in press*. Impact factor **1.659** (JCR 2011).

- RODRÍGUEZ J, NÚÑEZ-PONS L, NIETO RM, JIMÉNEZ C and AVILA C. *in prep*. Identification of a new group of minoritary indole alkaloids of the meridianin series from the crude extract of the Antarctic ascidian *Aplidium falklandicum* by mass spectometry. ANNEX III

ANNEX I.

OTHER PUBLICATIONS AND CHEMICAL DATA



ANNEX I

CARBONE M, NÚÑEZ-PONS L, CASTELLUCCIO F, AVILA C and GAVAGNIN M. 2009. Illudalane sesquiterpenoids of the alcyopterosin series from the Antarctic marine soft coral *Alcyonium grandis*. ***Journal of Natural Products*** 72(7):1357-1360.

Illudalane Sesquiterpenoids of the Alcyopterosin Series from the Antarctic Marine Soft Coral *Alcyonium grandis*

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Received March 10, 2009

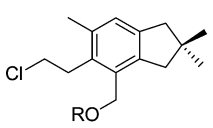
Chemical investigation of the lipophilic extract of the Antarctic soft coral *Alcyonium grandis* led us to the finding of nine unreported sesquiterpenoids, **2**−**10**. These molecules are members of the illudalane class and in particular belong to the group of alcyopterosins, illudalanes isolated from marine organisms. The structures of **2**−**10** were determined by interpretation of spectroscopic data. Repellency experiments conducted using the omnivorous Antarctic sea star *Odontaster validus* revealed a strong activity in the lipophilic extract of *A. grandis* against predation.

Illudalane sesquiterpenes[1] are a group of compounds modestly distributed in nature, being typical metabolites of both fungi of the Basidiomycotina subdivision[2] and ferns of the Pteridaceae family.[3] Among these, alcyopterosins (e.g., alcyopterosin D, **1**) represent a unique set of marine illudalanes isolated from the sub-Antarctic deep sea soft coral *Alcyonium paessleri*.[4] In the alcyopterosins, the six-membered ring of the illudalane skeleton is aromatic and either a chlorine atom or a nitrate ester function is present on the side chain of almost all members of the group.[4] Cytotoxic[4,5] and antispasmodic[6] activities have been reported for illudalane sesquiterpenes. In addition, interesting DNA-binding properties have been described for alcyopterosins and their synthetic analogues.[7,8]

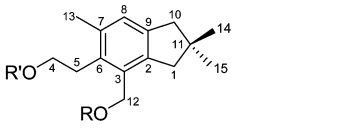
In this paper we report the structure elucidation of nine additional alcyopterosins, compounds **2**−**10**, isolated from an ether extract of the Antarctic soft coral *Alcyonium grandis* Casas, Ramil and Van Ofwegen 1997. Soft corals (order Alcyonacea) are conspicuous members of Antarctic benthic communities and possess a variety of bioactive chemicals.[9] The extract analyzed in this work exhibited feeding-deterrent activity against the generalist Antarctic predator *Odontaster validus.*

The soft coral *A. grandis* was collected in the Weddell Sea (Antarctica), during the Austral Summer of 2003−2004. The biological material was frozen at −20 °C and transferred to the laboratory in Spain, where it was later extracted with acetone. In a further Antarctic campaign in January 2006, the Et_2O-soluble portion of the acetone extract was tested in a repellency assay against *O. validus*, and it displayed significant activity. Subsequently, a portion of the extract (365 mg) was transferred to our laboratory in Italy and submitted to chemical investigation. TLC analysis of the extract showed the presence of a series of spots at R_f 0.35−0.75 (light petroleum ether/Et_2O, 8:2). The extract was then submitted to purification steps including molecular exclusion, silica gel, and reversed-phase chromatography (see Experimental Section) to give pure compounds **2** (1.3 mg), **3** (0.5 mg), **4** (0.6 mg), **5** (1.2 mg), **6** (1.3 mg), **7** (2.8 mg), **8** (0.7 mg), **9** (0.5 mg), and **10** (1.0 mg).

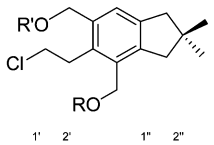
Preliminary ^{1}H NMR analysis of the new compounds showed their close structural relationship, in particular indicating that they exhibited the same illudalane aromatic carbon skeleton as that reported for the alcyopterosins (i.e., alcyopterosin D,[4] **1**). Four groups of molecules could be recognized: compounds **2**, **4**, and **6**, exhibiting oxygen functional groups at both C-4 and C-12; compounds **3**, **5**, and **9**, bearing chlorine at C-4 and oxygen functions at both C-13 and C-12; compounds **7** and **8**, with a chlorine at C-4 and an oxygenated group at C-12; and compound **10**, displaying an unusual tricyclic arrangement. The structure elucidations are reported starting from the main metabolite **7**. Other alcyopterosins are described subsequently according to the above functionalization groupings.



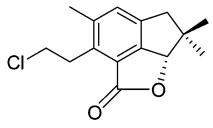
1[4]: R= H
7: R= $COCH_3$
8: R= $COCH_2CH_2CH_3$



2: R= $COCH_2CH_2CH_3$; R'= $COCH_2CH_2CH_3$
4: R= $COCH_3$; R'= $COCH_3$
6: R= $COCH_3$; R'= $COCH_2CH_2CH_3$
11[4]: R= H; R'= H



3: R= $COCH_3$; R'= $COCH_3$
5: R= $COCH_3$; R'= $COCH_2CH_2CH_3$
9: R= H; R'= H



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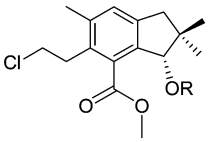

12: R= H
12a: R= *S*-MTPA
12b: R= *R*-MTPA

Compound **7** exhibited the molecular formula $C_{17}H_{23}O_2Cl$ as deduced by HRESIMS on the sodiated molecular peak at 317.1286 (M + Na). The ^{1}H NMR spectrum appeared to be very simple and displayed three singlet signals at δ_H 1.14 (6H), 2.08 (3H), and 2.33 (3H), which were attributed to two tertiary methyls (H_3-14 and H_3-15), an acetyl group, and an aromatic methyl (H_3-13), respectively. Five methylene signals at δ_H 2.69 (2H, s, H_2-10), 2.74 (2H, s, H_2-1), 3.15 (2H, t, J = 9 Hz, H_2-5), 3.57 (2H, t, J = 9 Hz, H_2-4), and 5.12 (2H, s, H_2-12) and a single aromatic methine at δ_H 7.01 (1H, s, H-8) completed the spectrum. These data were consistent with the alcyopterosin carbon skeleton containing chlorine and acetyl functional groups. The ^{13}C NMR spectrum displayed signals attributable to six sp^2 aromatic carbons (one CH and five quaternary

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10.1021/np900162t CCC: $40.75
Published on Web 05/11/2009

Table 1. ^{1}H NMR Data[a] (400 and 600 MHz, $CDCl_3$) of Compounds **7**, **8**, and **10**

	7	**8**	**10**
position	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1	2.74, s	2.74, s	5.28, s
4	3.57, t (9)	3.57, t (9)	3.80, m
5	3.15, t (9)	3.15, t (9)	3.54, m
			3.28, m
8	7.01, s	7.26, s	7.15, s
10	2.69, s	2.69, s	2.48, d (15)
			3.30, m
12	5.12, s	5.13, s	
13	2.33, s	2.42, s	2.42, s
14	1.14, s	1.14, s	1.45, s
15	1.14, s	1.14, s	0.44, s
2′	2.08, s	2.30, m	
3′		1.62, m	
4′		0.94, t (7)	

[a] Assignments made by ^{1}H−^{1}H COSY, HSQC, and HMBC (J = 10 Hz) experiments.

Table 2. ^{13}C NMR Data[a] (300 MHz, $CDCl_3$) of Compounds **2**−**10**

	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
position	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
1	46.5	46.6	46.4	47.1	46.5	46.4	46.4	47.7	87.9
2	142.3	142.4	142.4	143.4	142.4	141.9	142.5	143.4	139.9
3	135.5	133.5	135.5	134.6	135.6	134.3	134.6	137.1	n.d.
4	63.7	44.0	63.9	44.0	63.7	43.2	44.5	44.8	44.7
5	28.6	32.6	29.7	32.6	28.6	33.2	31.6	32.1	30.4
6	132.8	134.3	132.8	135.2	132.9	133.1	133.3	n.d.	133.7
7	130.4	130.9	130.3	131.1	130.3	130.3	130.5	134.2	n.d.
8	127.9	127.9	127.7	127.8	127.7	127.7	127.7	125.9	132.0
9	142.3	145.6	142.4	145.6	142.4	142.5	142.5	142.9	139.4
10	47.7	47.6	47.6	46.7	47.7	47.6	47.7	46.5	49.1
11	39.7	39.8	39.7	40.4	39.7	40.3	39.7	39.6	54.9
12	61.9	65.1	62.1	65.1	62.1	62.1	61.9	64.0	161.7
13	20.0	61.7	20.0	61.5	21.0	20.9	21.4	60.2	19.3
14	28.9	28.9	29.0	28.9	29.0	28.9	28.9	29.0	26.4
15	28.9	28.9	29.0	28.9	29.0	28.9	28.9	29.0	18.7
1′	173.3	170.6	170.6	171.1	170.2	170.8	173.6		
2′	36.2	21.0	21.0	21.1	22.3	19.8	36.2		
3′	18.4						18.1		
4′	13.6						13.4		
1″	173.1	171.1	170.9	173.5	172.7				
2″	36.2	21.0	21.0	36.1	36.2				
3″	18.4			18.4	18.4				
4″	13.6			13.7	13.7				

[a] Assignments made by HSQC and HMBC (J = 10 Hz) experiments.

C) and nine sp^3 carbons (three CH_3, two of which resonated at the same value, five CH_2, one CH, and one quaternary C) along with the signals at δ_C 170.8 (CO) and 19.8 (CH_3) due to the carbons of the acetyl function. Comparison of ^{1}H and ^{13}C NMR spectra of compound **7** with literature data[4] clearly indicated that **7** was the acetyl derivative of alcyopterosin D (**1**). Analysis of 2D-NMR experiments (^{1}H−^{1}H COSY, HSQC, and HMBC) of 12-acetylalcyopterosin D (**7**) allowed complete proton and carbon assignments as reported in Tables 1 and 2.

The molecular formula of compound **8** ($C_{19}H_{27}O_2Cl$) exhibited 28 additional mass units (C_2H_4) with respect to compound **7**. NMR data of **8** were substantially similar to those of **7** (Tables 1 and 2) and suggested that the unique difference between the two metabolites was in the nature of the acyl residue at C-12. Analysis of the ^{1}H−^{1}H COSY spectrum of compound **8** indicated that an *n*-butanoyl group was present rather than the acetyl group present in **7**. NMR analysis of 12-*n*-butanoylalcyopterosin D (**8**) led to the proton and carbon assignments listed in Tables 1 and 2.

Analysis of the ^{1}H and ^{13}C NMR spectra of **2**, molecular formula $C_{23}H_{34}O_4$, revealed the absence of chlorine and the presence of four additional carbon and two oxygen atoms with respect to compound **8**. Compound **2** contained an acyloxy group linked to C-4 [δ_H 4.15 (t, J = 8 Hz); δ_C 63.7] in the place of the chlorine substituent. The ^{1}H−^{1}H COSY spectrum of **2** clearly indicated the presence of two equivalent spin systems consistent with two *n*-butanoyl moieties esterified to 4-OH and 12-OH. This structural hypothesis was confirmed by analysis of HSQC and HMBC experiments, which also led us to assign all carbon and proton resonances (Tables 2 and 3). Compound **2** was the 4,12-bis-*n*-butanoyl derivative of the previously reported alcyopterosin O (**11**).[4]

Table 3. ^{1}H NMR Data[a] (400 and 600 MHz, $CDCl_3$) of Compounds **2**, **4**, and **6**

	2	**4**	**6**
position	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1	2.73, s	2.74, s	2.74, s
4	4.15, t (8)	4.15, t (8)	4.15, m
5	3.03, t (8)	3.03, t (8)	3.02, m
8	7.01, s	7.01, s	7.01, s
10	2.69, s	2.69, s	2.69, s
12	5.15, s	5.15, s	5.15, s
13	2.35, s	2.35, s	2.36, s
14	1.13, s	1.14, s	1.14, s
15	1.13, s	1.14, s	1.14, s
2′	2.29, m	2.06,[c] s	2.06, s
3′	1.64, m	2.07,[c] s	
4′	0.93,[b] t (7)		
2″	2.29, m		2.35, m
3″	1.64, m		1.67, m
4″	0.94,[b] t (7)		0.94, t (7)

[a] Assignments made by ^{1}H−^{1}H COSY, HSQC, and HMBC (J = 10 Hz) experiments. [b,c] Values with the same superscript may be interchanged.

Compound **4** had the molecular formula $C_{19}H_{26}O_4$, and spectroscopic data were similar to those of compound **2**. Two acetyl signals (δ_H 2.06 and 2.07) in the proton spectrum of **4** replaced signals due to the *n*-butanoyl moieties in the ^{1}H NMR spectrum of **2**, clearly indicating that the difference between **4** and **2** was in the nature of the acids esterified to the hydroxy groups at C-4 and C-12. In particular, compound **4** was the 4,12-bis-acetyl derivative of alcyopterosin O.[4] The ^{1}H and ^{13}C NMR data of **4** were in agreement with the proposed structure. All resonances were assigned as reported in Tables 2 and 3 by 2D NMR experiments.

Analysis of the spectroscopic data of compound **6**, $C_{21}H_{30}O_4$ by the HRESIMS, revealed structural features similar to those of compounds **2** and **4**. But, in this case, both an acetyl group and an *n*-butanoyl group were present in the molecule, as indicated by a singlet at δ_H 5.15 and a multiplet at δ_H 4.15 in the ^{1}H NMR spectrum. Thus the hydroxy groups at C-4 and C-12 were esterified by these acids. The positions of the acid residues were evident from analysis of the HMBC spectrum of **6**. Diagnostic long-range correlations were observed between C-1′ (δ_C 170.2) and both H_3-2′ (δ_H 2.06) and H_2-12 (δ_H 5.15) as well as between C-1″ (δ_C 172.7) and both H_2-3″ (δ_H 1.67) and H_2-4 (δ_H 4.15), inferring the indicated substitution pattern. Thus, compound **6** was 12-acetyl-4-*n*-butanoylalcyopterosin O. NMR assignments are reported in Tables 2 and 3.

The ^{1}H NMR spectrum of **3**, which had the molecular formula $C_{19}H_{25}O_4Cl$, showed some similarities to that of acetylalcyopterosin D (**7**), only differing in the presence of two signals at δ_H 5.14 (2H, s, H_2-13) and δ_H 2.10 (3H, s, −OAc) rather than the aromatic methyl singlet at δ_H 2.33 of compound **7**. Analysis of the HMBC spectrum confirmed this suggestion, as significant long-range correlations were observed between the two oxymethylenes at δ_H 5.13 and 5.14 and carbonyl carbons at δ_C 170.6 and 171.1, respectively. All proton and carbon assignments of 13-acetoxy-12-acetylalcyopterosin D (**3**) were made by 2D NMR experiments (Tables 2 and 4).

Compound **5** had the molecular formula $C_{21}H_{29}O_4Cl$ and was structurally related to compound **3**. Analysis of the NMR spectra (Tables 2 and 4) revealed that **5** differed from **3** only in the nature of the ester attached to C-13. An *n*-butanoyl moiety was present in

Table 4. ^{1}H NMR Data[a] (400 and 600 MHz, $CDCl_3$) of Compounds **3**, **5**, and **9**

	3	**5**	**9**
position	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1	2.73, s	2.73, s	2.73, s
4	3.63, t (9)	3.62, t (8)	3.76, t (8)
5	3.20, t (9)	3.20, t (8)	3.30, t (8)
8	7.20, s	7.19, s	7.19, s
10	2.77, s	2.77, s	2.82, s
12	5.13, s	5.13, s	4.69,[c] s
13	5.14, s	5.14, s	4.71,[c] s
14	1.15, s	1.14, s	1.16, s
15	1.15, s	1.14, s	1.16, s
2′	2.08,[b] s	2.10, s	
2″	2.10,[b] s	2.31, t (7)	
3″		1.66, app. sext (7)	
4″		0.94, t (7)	

[a] Assignments made by ^{1}H−^{1}H COSY, HSQC, and HMBC (J = 10 Hz) experiments. [b,c] Values with the same superscript may be interchanged.

the molecule, as indicated by the typical multiplets at δ_H 2.31 (2H, t, J = 7 Hz, H_2-2″), 1.66 (2H, app. sext, J = 7 Hz, H_2-3″), and 0.94 (3H, t, J = 7 Hz, H_3-4″) in the ^{1}H NMR spectrum. Compound **5** was thus 12-acetyl-13-*n*-butanoxyalcyopterosin D.

The spectroscopic data of compound **9** ($C_{15}H_{21}O_2Cl$) indicated that it was a diol related to both **3** and **6**. The ^{1}H NMR spectrum of **9** lacked the two acetyl signals present in the spectrum of **3** and displayed two singlets due to the isolated methylenes H_2-12 and H_2-13 at high field shifted values (δ_H 4.69 and 4.71) with respect to the corresponding signals in **3** (δ_H 5.13 and 5.14). The proposed structure was confirmed by comparing a synthetic sample obtained by acetylation of **9** with compound **3**. The proton and carbon assignments of 13-hydroxyalcyopterosin D (**9**) are reported in Tables 2 and 4.

Compound **10** had the molecular formula $C_{15}H_{17}O_2Cl$, implying seven unsaturation degrees. The ^{1}H and ^{13}C NMR spectra of **10**, named alcyopterosin P, revealed a structural arrangement different from those of the other co-occurring alcyopterosins. According to the molecular formula, the presence of a lactone moiety fused to the bicyclic alcyopterosin framework was strongly suggested by both a carboxyl signal at δ_C 161.7 in the ^{13}C NMR spectrum and the strong IR band at 1765 cm^{-1}. The ^{1}H NMR spectrum lacked two methylene signals attributed to H_2-1 and H_2-12 of the alcyopterosin skeleton, displaying in their place a methine singlet at δ_H 5.28 (s, H-1), which was correlated in the HMBC spectrum to the carboxyl carbon at δ_C 161.7. These data suggested the location of the carboxyl at C-12, and subsequently the lactone moiety had to involve C-1, C-2, and C-3. The remaining part of the molecule was the same as alcyopterosins **7** and **8** (see Tables 1 and 2 for NMR assignments).

The absolute configuration at C-1 was determined by applying the modified Mosher method[10,11] on the methyl ester derivative **12**, obtained from **10** by methanolysis and subsequent opening of the lactone ring. Treatment of compound **12** with (*R*)- and (*S*)-MTPA chlorides in dry CH_2Cl_2 and DMAP afforded the corresponding (*S*)-MTPA (**12a**) and (*R*)-MTPA (**12b**) esters, respectively. The two Mosher derivatives were characterized by 2D-NMR experiments (^{1}H−^{1}H COSY, HSQC, HMBC), and some selected ^{1}H NMR data and $\Delta\delta$ ($\delta_S - \delta_R$) are reported in the Experimental Section. The $\Delta\delta$ values observed for the signals of protons close to the hydroxyl group at C-1 indicated the *S* configuration as depicted in formula **12**, and the same configuration was assigned to C-1 of the corresponding lactone, alcyopterosin P (**10**).

The occurrence of sesquiterpenes of the alcyopterosin series in the Antarctic soft coral *A. grandis* is in agreement with the chemical data reported for the sub-Antarctic species *A. paessleri*.[4] This secondary metabolite pattern seems to be a distinctive character for both species, suggesting a close taxonomic relationship. Alcyopterosins have not been reported so far from other soft corals or from any other marine organisms. The extract containing alcyopterosins was active in a repellency assay on *O. validus*.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP 370 digital polarimeter. The UV spectra and CD curves were recorded on a Agilent 8453 spectrophotometer and a JASCO 710 spectropolarimeter, respectively. The IR spectra were taken on a Bio-Rad FTS 155 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on DRX 600, Avance 400, and DPX 300 MHz Bruker spectrometers in $CDCl_3$, with chemical shifts reported in ppm referred to $CHCl_3$ as internal standard (δ 7.26 for proton and δ 77.0 for carbon). ESIMS and HRESIMS were measured on a Micromass Q-TOF Micro spectrometer coupled with a HPLC Waters Alliance 2695. The instrument was calibrated by using a PEG mixture from 200 to 1000 MW. Silica gel chromatography was performed using precoated Merck F_{254} plates and Merck Kieselgel 60 powder. HPLC purification was carried out on a Shimadzu LC-10AD liquid chromatograph equipped with a UV SPD-10A wavelength detector.

Collection and Extraction of the Animal Material. Specimens of *A. grandis* were collected in the Western Weddell Sea (Antarctica) at 597.6 m depth during the ANT XXI/2 cruise of *R/V Polarstern* (AWI; Bremerhaven, Germany), from November 2003 to January 2004, using a bottom trawl. The biological material was immediately frozen at −20 °C and then transferred to the laboratory in Spain. Subsequently the sample was extracted with acetone (25 mL × 3). The organic solvent was removed under reduced pressure, and the residual water was partitioned with Et_2O and subsequently with *n*-butanol. An aliquot of the Et_2O extract (16.7 mg) was used for the ecological tests. The remaining part (365 mg) was transferred to ICB in Naples (Italy) and chemically analyzed. A voucher specimen was fixed in 10% formalin for taxonomical determination, and it is stored at Dept. of Animal Biology (Invertebrates), University of Barcelona (sample code #1152).

Purification of Compounds 2−10. An aliquot (183 mg) of the Et_2O extract of *A. grandis* was fractionated on Sephadex LH-20 chromatography using a mixture of $CHCl_3$/MeOH (1:1) as eluent to yield three fractions: A (18.7 mg), B (5.3 mg), and C (17.4 mg). Fraction A was chromatographed on a silica gel column (light petroleum ether/Et_2O gradient), affording pure compounds **2** (1.3 mg), **3** (0.5 mg), and **4** (0.6 mg) and a mixture, which was separated on preparative TLC (SiO_2, C_6H_6/light petroleum ether, 8:2) to give compounds **5** (1.2 mg) and **6** (1.3 mg). Fraction B was submitted to preparative TLC (SiO_2, light petroleum ether/Et_2O, 9:1) to give pure **7** (2.8 mg) and a mixture (3.0 mg), which was further purified by preparative TLC (SiO_2, C_6H_6/light petroleum ether, 8:2) to yield compound **8** (0.7 mg). Fraction C was subjected to reversed-phase HPLC using a Supelco Discovery C18 column (25 cm × 10 mm, particle size = 5 μm) eluted with a 20 min gradient from 80 to 100% CH_3OH in H_2O (flow rate 2 mL/min) to give pure compounds **9** (0.5 mg) and **10** (1.0 mg).

4,12-Bis-*n*-butanoylalcyopterosin O (2): colorless oil; UV (CH_2Cl_2) λ_{max} (log ϵ) 226 (3.57) nm; IR (liquid film) ν_{max} 2929, 1745, 1173 cm^{-1}; ^{1}H and ^{13}C NMR in Tables 3 and 2; HRESIMS *m*/*z* 397.2298 (calcd for $C_{23}H_{34}O_4Na$, 397.2355).

13-Acetoxy-12-acetylalcyopterosin D (3): colorless oil; UV (CH_2Cl_2) λ_{max} (log ϵ) 228 (4.07), 233 (3.56) nm; IR (liquid film) ν_{max} 1752, 1246, 1019 cm^{-1}; ^{1}H and ^{13}C NMR in Tables 4 and 2; HRESIMS *m*/*z* 375.1338 (calcd for $C_{19}H_{25}O_4ClNa$, 375.1339).

4,12-Bis(acetyl)alcyopterosin O (4): colorless oil; UV (CH_2Cl_2) λ_{max} (log ϵ) 227 (3.64) nm; IR (liquid film) ν_{max} 2935, 1768 cm^{-1}; ^{1}H and ^{13}C NMR in Tables 3 and 2; HRESIMS *m*/*z* 341.1718 (calcd for $C_{19}H_{26}O_4Na$, 341.1729).

12-Acetyl-13-*n*-butanoxyalcyopterosin D (5): colorless oil; UV (CH_2Cl_2) λ_{max} (log ϵ) 227 (4.12) nm; IR (liquid film) ν_{max} 2956, 1738, 1227 cm^{-1}; ^{1}H and ^{13}C NMR in Tables 4 and 2; HRESIMS *m*/*z* 403.1645 (calcd for $C_{21}H_{29}O_4ClNa$, 403.1652).

12-Acetyl-4-*n*-butanoylalcyopterosin O (6): colorless oil; UV (CH_2Cl_2) λ_{max} (log ϵ) 227 (4.34) nm; IR (liquid film) ν_{max} 2923, 1739, 1237 cm^{-1}; ^{1}H and ^{13}C NMR in Tables 3 and 2; HRESIMS *m*/*z* 369.2038 (calcd for $C_{21}H_{30}O_4Na$, 369.2042).

12-Acetylalcyopterosin D (7): colorless oil; UV (CH_2Cl_2) λ_{max} (log ϵ) 226 (3.57) nm; IR (liquid film) ν_{max} 1739, 1224, 1024 cm^{-1}; ^{1}H and ^{13}C NMR in Tables 1 and 2; HRESIMS *m*/*z* 317.1286 (calcd for $C_{17}H_{23}O_2ClNa$, 317.1284).

12-*n*-Butanoylalcyopterosin D (8): colorless oil; UV CH_2Cl_2) λ_{max} (log ϵ) 226 (3.36) nm; IR (liquid film) ν_{max} 2969, 1738, 1227 cm^{-1}; ^1H and ^{13}C NMR in Tables 1 and 2; HRESIMS *m/z* 345.1581 (calcd for $C_{19}H_{27}O_2ClNa$, 345.1567).

13-Hydroxyalcyopterosin D (9): colorless oil; UV (CH_2Cl_2) λ_{max} (log ϵ) 227 (3.69) nm; IR (liquid film) ν_{max} 2935, 1229 cm^{-1}; ^1H and ^{13}C NMR in Tables 4 and 2; HRESIMS *m/z* 291.1128 (calcd for $C_{15}H_{21}O_2ClNa$, 291.1128).

Alcyopterosin P (10): colorless oil; $[\alpha]_D$ −822.9 (*c* 0.07, $CHCl_3$); UV (CH_2Cl_2) λ_{max} (log ϵ) 227 (3.72) nm; IR (liquid film) ν_{max} 2929, 1765, 1073 cm^{-1}; ^1H and ^{13}C NMR in Tables 1 and 2; HRESIMS *m/z* 287.0790 (calcd for $C_{15}H_{17}O_2ClNa$, 287.0815).

Acetylation of 9. 13-Hydroxyalcyopterosin D (**9**, 0.5 mg) was dissolved in dry C_5H_5N (0.5 mL) and treated with Ac_2O (two drops) at room temperature for 8 h. After evaporation, the residue was filtered on a Pasteur pipet-SiO_2 column (light petroleum ether/Et_2O) to give the diacetyl derivative **3** (0.5 mg).

Methanolysis of 10. Alcyopterosin P (**10**, 1.0 mg) was dissolved in anhydrous MeOH (1 mL), and an excess of Na_2CO_3 was added. The solution was stirred at room temperature for 4 h and filtered, and the solvent evaporated. The crude product was purified on a Pasteur column (light petroleum ether/Et_2O), affording 0.8 mg of pure **12**: $[\alpha]_D$ −18.9 (*c* 0.08, $CHCl_3$); ^1H NMR ($CDCl_3$) δ_H 7.14 (1H, s, H-8), 4.57 (1H, s, H-1), 3.97 (3H, s, −OMe), 3.52 (2H, m, H_2-4), 3.18 (2H, m, H_2-5), 2.90 (1H, d, J = 16 Hz, H-10a), 2.54 (1H, d, J = 16 Hz, H-10b), 2.37 (3H, s, H_3-13), 1.18 (3H, s, H_3-15), 1.02 (3H, s, H_3-14); ESIMS $(M + Na)^+$ *m/z* 319.

Preparation of MTPA Esters. (*R*)- and (*S*)-MTPA-Cl (10 μL) and a catalytic amount of DMAP were separately added to two different aliquots of the alcohol **12** (1.0 mg each) in dry CH_2Cl_2 (0.5 mL), and the resulting mixtures were allowed to stand at room temperature for 12 h. After the usual workup the reaction mixtures were purified on preparative TLC (SiO_2, light petroleum ether/Et_2O, 7:3), affording pure (*S*)- and (*R*)-MTPA esters of **12**, respectively.

(*S*)-MTPA ester (12a): selected ^1H NMR values ($CDCl_3$) δ_H 7.17 (1H, s, H-8), 6.22 (1H, s, H-1), 3.80 (3H, s, −OMe), 3.65 (2H, m, H_2-4), 3.56 [(3H, s, −OMe (MTPA)], 3.22 (2H, m, H_2-5), 2.90 (1H, d, J = 16 Hz, H-10a), 2.48 (1H, d, J = 16 Hz, H-10b), 2.40 (3H, s, H_3-13), 1.02 (3H, s, H_3-15), 0.95 (3H, s, H_3-14).

(*R*)-MTPA ester (12b): selected ^1H NMR values ($CDCl_3$) δ_H 7.14 (1H, s, H-8), 6.19 (1H, s, H-1), 3.84 (3H, s, -OMe), 3.58 (2H, m, H_2-4), 3.36 [3H, s, -OMe (MTPA)], 3.14 (m, 2H, H_2-5), 2.92 (1H, d, J = 16 Hz, 1H, H-10a), 2.53 (1H, d, J = 16 Hz, 1H, H-10b), 2.38 (3H, s, H_3-13), 1.15 (3H, s, H_3-15), 1.07 (3H, s, H_3-14).

Biological Assays. Individuals of the Antarctic omnivorous predator the sea star *Odontaster validus* were collected in the South Shetland Islands (Livingston and Deception Is.) on board the *B/O Hespérides* during January 2006 for feeding−repellence assays. Experiments took place at the Spanish Base "Gabriel de Castilla" in Deception Island, Antarctica, during the same period. Dry Et_2O extracts from specimens of *A. grandis* were diluted in solvent (Et_2O) and coated into shrimp pieces. These shrimp pieces were then presented to the sea stars. Natural concentration (as that obtained from the soft coral) was used for the tests. After 24 h the number of shrimp pieces eaten out of a total of 10 pieces was compared in treatment versus control experiments. The tests were carried out following the detailed methodology reported in previous works.[12,13]

Acknowledgment. This research was developed in the frame of the ECOQUIM (REN2003-00545 and REN2002-12006-E ANT) and ECOQUIM-2 (CGL2004-03356/ANT) projects and financed by the Ministry of Education of Spain (MEC) and the Ministry of University and Research of Italy (MUR). L.N.-P. was consecutively supported by PharmaMar S.A., an I3P (CSIC) grant, and a FPU Fellowship from MEC during this study. We thank O. Iannicelli, M. Garofalo, S. Taboada, J. Vázquez, and M. Ballesteros for their help, as well as A. Ramos, W. Arntz, T. Brey, and the staff of the *R/V Polarstern* and the *BIO Hespérides* research vessels, and the Spanish Antarctic Base "Gabriel de Castilla" for all their logistic support. The NMR spectra were recorded at the ICB NMR Service, the staff of which is gratefully acknowledged. Thanks are due to Mr. R. Turco for preparing the structures and tables.

Supporting Information Available: ^1H, ^{13}C NMR and HMBC spectra of compounds **2**−**10** are available free of charge via the Internet at http://pubs.acs.org.

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NP900162T

ANNEX II

CARBONE M, NÚÑEZ-PONS L, CASTELLUCCIO F, AVILA C and GAVAGNIN M. 2012. Rossinone-related meroterpenes from the Antarctic ascidian *Aplidium fuegiense*. ***Tetrahedron*** 68:3541-3544.

Tetrahedron 68 (2012) 3541–3544

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Rossinone-related meroterpenes from the Antarctic ascidian *Aplidium fuegiense*

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ARTICLE INFO

Article history:
Received 4 January 2012
Received in revised form 17 February 2012
Accepted 5 March 2012
Available online 13 March 2012

Keywords:
Marine natural products
Meroterpenoids
Quinones
Ascidian
Antartic

ABSTRACT

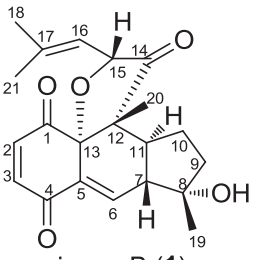
The chemical analysis of the ascidian *Aplidium fuegiense* resulted in the isolation of three novel meroterpenoids **2**–**4**, structurally related to the main co-occurring known rossinone B (**1**). The structures of the new compounds were determined by interpretation of spectroscopic data. Compounds **1**–**4** were found to be selectively localized in the viscera of the ascidian.

1. Introduction

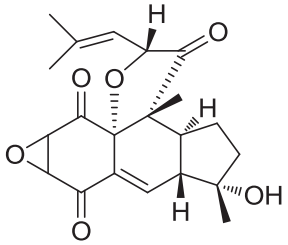
The group of ascidians is one of the most compelling sources of metabolites both of chemical and biomedical interest in the marine environment.[1] In particular, the species belonging to the genus *Aplidium* (family Polyclinidae) are recognised as prolific producers of bioactive natural products exhibiting an extensive structural variability and including non-nitrogenous compounds, such as prenyl hydroquinones and prenyl quinones, and nitrogenous metabolites, like nucleosides, peptides and a high variety of alkaloids.[2,3]

As a part of our continuing search for biologically active secondary metabolites of marine organisms from distinct geographical areas,[4–8] we have investigated the chemistry of the ascidian *Aplidium fuegiense*, collected in Antarctica. Previous studies on Antarctic *Aplidium* species have resulted in the discovery of meridianins, which are a group of indole alkaloids showing a potent cdk inhibitor activity, and displaying feeding repellence towards sympatric sea stars.[9–11] Moreover, bromoindole derivatives, the aplicyanins, with strong cytotoxic and antimitotic activities,[12] as well as meroterpenes, such as rossinone B (**1**), exhibiting anti-leukaemic, antiviral and anti-inflammatory properties have been also reported in southern ascidian representatives of this genus.[13]

We describe here the isolation of three novel meroterpenes (**2**–**4**) structurally related to the co-occurring known metabolite rossinone B (**1**), from the ether extract of the visceral part of *A. fuegiense*. This extract exhibited significant feeding-deterrent activity against the generalist Antarctic asteroid predator *Odontaster validus*.



rossinone B (**1**)



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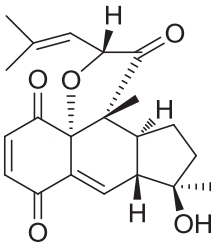

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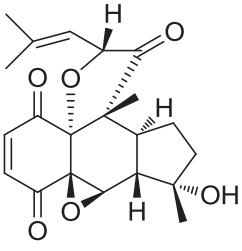

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doi:10.1016/j.tet.2012.03.013

2. Results and discussion

The ascidian *A. fuegiense* was collected by dredging in the Weddell Sea (Antarctica), during the December–January of 2003–2004. The biological material was frozen at −20 °C and transferred to the laboratory in Spain where it was later dissected into external (tunic) and internal (viscera) parts. The two sections were separately extracted with acetone. In a further Antarctic campaign in December–January 2008–2009, the Et_2O soluble portion of the acetone extracts of the two parts were tested in feeding-repellence tests against *O. validus*. Both extracts showed strong deterrent activity. Subsequently, these extracts were transferred to the laboratory in Italy for the chemical analysis. Comparative TLC of the extracts revealed different secondary metabolite pattern for the two anatomical sections. In particular, a main spot at R_f 0.50 along with other spots at R_f 0.10–0.60 (light petroleum ether/Et_2O, 2/8) were selectively present in the viscera of the animal. With the aim to identify these components, the visceral extract (117.0 mg) was submitted to further purification steps including silica-gel column and preparative TLC as well as reverse-phase HPLC chromatography (see Experimental). Four structurally related pure molecules were recovered: rossinone B (**1**, 9.0 mg), the main compound, and three minor metabolites 2,3-epoxy-rossinone B (**2**, 1.0 mg), 8-*epi*-rossinone B (**3**, 1.1 mg) and 5,6-epoxy-rossinone B (**4**, 0.5 mg). Compound **1** had been already reported from an unidentified Antarctic *Aplidium* species,[13] whereas compounds **2**–**4** were not previously described.

Preliminary NMR spectroscopic analysis of the minor co-occurring compounds **2**–**4** revealed the presence of a meroterpene carbon skeleton, the same as **1**, characterized by an uncommon molecular architecture with a 6-6-5 tricyclic core. The structure of rossinone B (**1**) has been recently confirmed by biomimetic total synthesis.[14] Comparison of MS data indicated that compounds **2** and **4** contained an additional oxygen atom with respect to **1** whilst compound **3** was an isomer of **1** having the same molecular formula.

The ^{1}H NMR spectrum of 2,3-epoxy-rossinone B (**2**) closely resembled the spectrum of rossinone B (**1**) (Table 1). The relevant difference was in the chemical shift of the AB quartet due to the quinone protons resonating at higher fields [δ_H H-2: 3.75 (d, J=3.5 Hz) in **2**, 6.79 (d, J=10.5 Hz) in **1**; δ_H H-3: 3.80 (d, J=3.5 Hz) in **2**, 6.90 (d, J=10.5 Hz) in **1**]. Bearing in mind that the molecular formula of **2** ($C_{21}H_{28}O_5$) contains an additional oxygen with respect to **1**, it was suggested the presence of an epoxy ring at C-2/C-3 in the structure of **2**. Accordingly, the ^{13}C NMR spectrum of **2** displayed two additional sp^3 carbons at δ_C 55.5 (C-2) and 56.4 (C-3) in the place of the quinone resonances of **1** at δ_C 139.0 (C-2) and 141.2 (C-3) (Table 2). The remaining part of **2** was suggested to be identical with **1** including the relative stereochemistry of chiral centres on the basis of the strong similarity of both proton and carbon NMR resonances with those of **1** (Tables 1and 2). Analysis of a series of NOE experiments confirmed this suggestion (see Supplementary data). Unfortunately, no steric effect was observed between either H-2 or H-3 with other protons of the structure preventing the definition of the orientation of the epoxide ring with respect to the plane of the molecule. Thus, this stereochemistry remained undetermined. All proton and carbon resonances of 2,3-epoxy-rossinone B (**2**) were assigned by 2D NMR experiments (see Supplementary data) and reported in Tables 1and 2.

Table 1
^{1}H NMR data[a–c] of compounds **1**–**4**

H	**1**[c]	**2**	**3**	**4**
	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1	—	—	—	—
2	6.79, d (10.5)	3.75, d (3.5)	6.79, d (10.3)	6.94, d (10.3)
3	6.90, d (10.5)	3.80, d (3.5)	6.91, d (10.3)	7.05, d (10.3)
4	—	—	—	—
5	—	—	—	—
6	7.44, d (1.9)	7.12, br s	7.48, d (1.5)	3.78, app. s[d]
7	2.06, dd (12.4, 1.9)	2.01, d (12.5)	2.48, dd (12.7, 1.5)	1.80, m
8	—	—	—	—
9	1.92, m	1.95–2.08, m	1.80–2.18, m	1.90–1.80
10	1.53, m	1.43–1.58, m	1.38–1.45, m 1.52–1.58, m	1.45–1.60 m
11	2.67, m	2.45–2.62, m	2.05–2.15, m	2.48–2.58, m
12	—	—	—	—
13	—	—	—	—
14	—	—	—	—
15	4.55, d (8.8)	4.64, d (8.8)	4.53, d (8.4)	4.85, d (8.9)
16	5.07, br d (8.9)	5.03, br d (8.8)	5.07, br d (8.4)	5.08, br d (8.9)
17	—	—	—	—
18	1.76, d (1.1)	1.75, br s	1.76, br s	1.77, br s
19	1.54, s	1.50, s	1.40, s	1.51, s
20	1.10, s	1.02, s	1.10, s	0.93, s
21	1.73, d (1.1)	1.70, br s	1.73, br s	1.70, br s

[a] The spectra were recorded in $CDCl_3$ at 400 MHz and 600 MHz.
[b] Assignments made by ^{1}H–^{1}H COSY, HSQC and HMBC (J=10 Hz) experiments.
[c] Values from Ref. 13.
[d] H-6 exhibited a very small J (0.32 Hz), which was measured by acquiring the high resolution spectrum of δ 3.30–5.50 region (0.003426 Hz/pt).

Table 2
^{13}C NMR data[a–d] of compounds **1**–**4**

C	**1**[d]	**2**	**3**	**4**
1	190.3	n.d.	189.9	191.0
2	139.0	55.5	139.0	140.0
3	141.2	56.4	141.4	141.7
4	185.0	191.8	184.4	193.9
5	134.6	134.3	133.3	55.9
6	144.2	145.0	144.0	61.4
7	50.5	49.7	50.5	48.5
8	78.0	78.0	78.3	79.3
9	40.2	40.2	40.5	39.8
10	21.1	21.0	20.9	21.1
11	39.5	39.3	39.5	34.4
12	49.3	49.2	48.9	49.8
13	82.8	83.8	82.8	83.7
14	213.0	212.2	212.3	212.2
15	77.2	77.4	77.4	77.0
16	118.6	118.7	117.8	118.5
17	142.5	142.3	143.0	142.7
18	25.9	25.9	25.6	25.9
19	27.1	27.1	26.7	27.4
20	8.8	8.7	8.6	11.0
21	18.7	18.9	18.6	18.7

[a] The spectra were recorded in $CDCl_3$ at 300 MHz.
[b] Assignments made by HSQC and HMBC (J=10 Hz) experiments.
[c] qC δ's measured by indirect detection.
[d] Values from Ref. 13.

3-*Epi*-rossinone B (**3**), with the molecular formula $C_{21}H_{28}O_4$, was isomeric with the main co-occurring rossinone B (**1**). Analysis of the ^{1}H–^{1}H COSY spectrum of **3** revealed the presence of the same spin systems as **1** (Table 1) indicating identical substitution patterns. Thus the two molecules had to differ in the configuration of one or more chiral centres. The carbon values of **3** were quite similar to those of **1** (see Table 2) suggesting that the stereochemistry of the 7,11- and 12,13-junctions were the same as **1**. Instead, significant differences were observed in the proton values of both H-7 and H-11 [δ_H H-7: 2.06 in **1**, 2.48 in **3**; δ_H H-11: 2.67 in **1**; 2.10 in **3**] that could be explained by the diverse steric influence of the hydroxyl group in the two compounds. The analysis of a series of NOE experiments conducted on both compounds **3** and **1** was very indicative (see Supplementary data). In particular, in compound **3** the angular proton H-7 had NOE interactions only with H_3-20 whereas in rossinone B (**1**) H-7 showed steric effects with both H_3-19 and H_3-20 according to the β-orientation of the two methyl groups with respect to the plane of the molecule. This suggested that in **3** the

methyl at C-8 should be α-oriented as it was further supported by the diagnostic NOE effect observed between H-11 and H_3-19. Thus **3** was the C-8-epimer of rossinone B. Full proton and carbon assignments are reported in Tables 1and 2.

5,6-Epoxy-rossinone B (**4**) was obtained in very small quantities. The ^{1}H NMR spectrum immediately revealed the lack of the double bond in ring B with respect to co-occurring compounds **1**–**3**. In fact, the signal due to the olefinic proton H-6 observed for rossinones **1**–**3** was replaced in **4** by an apparent singlet at δ_H 3.80 assigned to a proton, which was correlated in the HQSC spectrum to a CH carbon resonating at δ_C 61.4. Analysis of the HMBC experiment revealed a diagnostic correlation between H-3 (δ_H 7.05) and an additional sp^3 carbon resonance at δ_C 55.7, which was attributed to the angular quaternary carbon C-5 directly connected to the quinone moiety. Accordingly, due to the absence of the conjugated double bond, C-4 quinone carbonyl in **4** was observed down-field shifted (δ_C 193.9) with respect to **1** (δ_C 185.0) and **3** (δ_C 184.4). Thus, taking into account the additional oxygen atom required by the molecular formula of **4** and the unsaturation degrees to be satisfied, an epoxide ring was located at C-5/C-6 being the remaining part of the molecule almost the same as **1**(Tables 1and 2). Significant differences were only observed in C-8 and C-20 carbon values according to the presence of the additional substituent in ring B. The connection with the ring C was supported by diagnostic HMBC correlations between C-7 and both H-6 and H-11. As the vicinal coupling between H-6 and H-7 was not observed in the ^{1}H–^{1}H COSY spectrum due to the very small coupling constant, the ^{1}H INADEQUATE experiment was performed on compound **4** definitively evidencing this correlation. With the exception of the epoxide cycle, the overall relative stereochemistry of **4** was suggested to be the same as **1** by the similarity of NMR carbon and proton values (Tables 1and 2). Selected diagnostic NOE difference experiments confirmed this assignment (see Supplementary data). The relative configuration of C-5 and C-6 was suggested analyzing the molecular models of the two possible—α- and β-epoxide—stereoisomers by Chem3D computer program. The J_{H6-H7} value (0.32 Hz, see Table 1) indicated that the dihedral angle between the two protons had to be about 90°. This geometry was observed in the isomer exhibiting the β-oriented epoxide thus supporting the proposed structure **4**.

3. Conclusion

The secondary metabolite pattern of the ascidian *A. fuegiense* was characterized by the presence of meroterpenoids, according to the literature data for the genus *Aplidium*. Three new members, rossinones **2**–**4**, have been added to this interesting class of cyclic prenyl quinones. These compounds, along with the structurally related main metabolite rossinone B (**1**), were found in the viscera extract of *A. fuegiense* that showed strong activity in the repellency assay on *O. validus*. In situ ecological experiments on the pure isolated compounds **1**–**4** are still in progress to verify if the deterrent activity of the extract could be in part ascribed to the meroterpenoid content. Biosynthetically, cyclic prenylated quinones, such as rossinone B (**1**) are suggested to derive from the corresponding linear hydroquinone derivatives,[14] which have been reported to co-occur in the natural sources.[13] Interestingly, neither acyclic hydroquinones nor putative quinone-containing precursors of rossinones **1**–**4** were detected in *A. fuegiense* extract.

4. Experimental section

4.1. General procedures

TLC plates (Merck Silica Gel 60 F_{254}) were used for analytical TLC and Merck Kieselgel 60 was used for preparative column chromatography. HPLC purification was carried out on a Shimadzu apparatus equipped with an LC-10ADVP pump and an UV SPD-10AVP detector by using reverse-phase semi-preparative column (250×10 mm, Phenomenex, Kromasil C18). 1D and 2D NMR spectra were acquired in $CDCl_3$ (shifts are referenced to the solvent signal) on a Bruker Avance-400 operating at 400 MHz, using an inverse probe fitted with a gradient along the *Z*-axis and a Bruker DRX-600 operating at 600 MHz, using an inverse TCI CryoProbe fitted with a gradient along the *Z*-axis. ^{13}C NMR spectra were recorded in $CDCl_3$ (*d* values are reported to the solvent signal) on a Bruker DPX-300 operating at 300 MHz, using a dual probe. Optical rotations were measured on a Jasco DIP 370 digital polarimeter. IR spectra were measured on a Biorad FTS 155 FTIR spectrophotometer. Both ESIMS and HRESIMS spectra were recorded on a Micromass Q-TOF microinstrument.

4.2. Collection and extraction of the animal material

Specimens of *A. fuegiense* were collected in the Western Weddell Sea (Antarctica) at a depth of 597.6 m, during the ANT XXI/2 cruise of R/V Polarstern (AWI; Bremerhaven, Germany), from November 2003 to January 2004, using a bottom trawl. The biological material was immediately frozen at -20 °C transferred to the laboratory in Spain, and later on sent to the ICB (Italy) where it was dissected into external (tunic) and internal (viscera) parts. The two sections were separately processed. Afterwards both sample parts were homogenized with a pestle and extracted with acetone (150 mL×3) by using ultrasound vibration. The organic solvent was removed under reduced pressure and the residual water was partitioned with Et_2O and subsequently with *n*-butanol. An aliquot of the Et_2O extracts (10.1 mg visceral and 2 mg tunic) were used for the ecological tests. The remaining part (of visceral and 72.1 mg of tunic) was chemically analyzed. A voucher specimen was fixed in 10% formalin for taxonomical determination and it is stored at the Dept. of Animal Biology (Invertebrates), University of Barcelona (Sample code #1093).

4.3. Purification of compounds 1–4

A portion of the visceral Et_2O extract (117.0 mg) was fractionated on silica-gel column using light petroleum ether with increasing amounts of Et_2O as eluent, yielding four meroterpenoids containing fractions (A–D). Fraction A (4.2 mg) was subjected to reverse-phase HPLC purification using a Supelco Discovery C18 column (25 cm×10 mm, particle size=5 μm) eluted with methanol/water 7:3 (flow rate=2 mL/min) to give pure compounds **2** (1.0 mg). Fraction B (14.8 mg) was submitted to preparative TLC (SiO_2, light petroleum ether/Et_2O, 2:8) to give compounds **1** (9.0 mg). Finally, fractions C (1.1 mg) and D (0.5 mg) were directly analyzed by ^{1}H NMR showing to contain pure compounds **3** and **4**, respectively.

*4.3.1. Rossinone B (**1**).* Colourless oil; R_f (20% petroleum ether/diethyl ether) 0.50; −2.2 (*c* 0.82, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 204 (4.20), 225 (4.21); ^{1}H and ^{13}C NMR data see Tables 1and 2.[13] ESIMS *m*/*z* 379 $(M+Na)^+$; HRESIMS: $(M+Na)^+$, found 379.1518. $C_{21}H_{28}NaO_4$ requires 379.1521.

*4.3.2. 2,3-Epoxy-rossinone B (**2**).* Colourless oil; R_f (20% petroleum ether/diethyl ether) 0.60; (*c* 0.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 205 (4.08); ^{1}H and ^{13}C NMR data see Tables 1and 2. ESIMS *m*/*z* 395 $(M+Na)^+$; HRESIMS: $(M+Na)^+$, found 395.1459. $C_{21}H_{28}NaO_5$ requires 395.1471.

*4.3.3. 3-Epi-rossinone B (**3**).* Colourless oil; R_f (20% petroleum ether/diethyl ether) 0.35; 0 (*c* 0.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 204 (4.03), 225 (4.00); ^{1}H and ^{13}C NMR data see Tables 1and 2.

ESIMS *m/z* 379 $(M+Na)^+$; HRESIMS: $(M+Na)^+$, found 379.1532. $C_{21}H_{28}NaO_5$ requires 379.1520.

*4.3.4. 5,6-Epoxy-rossinone B (**4**).* Colourless oil; R_f (20% petroleum ether/diethyl ether) 0.20; (*c* 0.05, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 205 (3.89); ^1H and ^{13}C NMR data see Tables 1and 2. ESIMS *m/z* 395 $(M+Na)^+$; HRESIMS: $(M+Na)^+$, found 395.1433. $C_{21}H_{28}NaO_5$ requires 395.1471.

4.4. Biological assays

Individuals of the Antarctic omnivorous sea star *O. validus* were collected in Deception Island (South Shetland Is.) on board of B/O Hespérides during January 2006 for feeding-repellence assays. Experiments took place at the Spanish Base (BAE) 'Gabriel de Castilla' in Deception Is., Antarctica, during the same period. Each assay consisted of ten sea stars, each presented to one shrimp item. For the treatment tests, shrimp cubes containing Et_2O extracts from specimens of *A. fuegiense* at the natural concentration (on a dry weigth basis), were presented to the sea stars. For the control tests the shrimps were treated with solvent (Et_2O) alone. After 24 h, the number of shrimp pieces eaten out of the 10 replicates was statistically contrasted in treatment versus control assays (Fisher's Exact tests). The tests were carried out following the methodology reported in previous studies.[15,16]

Acknowledgements

We wish to thank the R/V Polarstern, BIO Hespérides, BIO Las Palmas and the BAE 'Gabriel de Castilla' crews for their support during the Antarctic cruises. Funding was provided by the Ministry of Science and Education of Spain through the ECOQUIM and ACTIQUIM Projects (REN2003-00545, REN2002-12006E ANT, CGL2004-03356/ANT and CTM2010-65453/ANT), and PRIN-MIUR 2009 Project 'Natural products and bioinspired molecules interfering with biological targets involved in control of tumour growth'. Also thanks are due to J. Vázquez, D. Melck, S. Taboada, B. Figuerola, F. J. Cristobo and M. Varela for laboratory and field work.

Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2012.03.013. These data include MOL files and InChiKeys of the most important compounds described in this article.

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ANNEX III

RODRÍGUEZ J, NÚÑEZ-PONS L, NIETO RM, JIMÉNEZ C and AVILA C. *in prep*. Identification of a new group of minoritary indole alkaloids of the meridianin series from the crude extract of the Antarctic ascidian *Aplidium falklandicum* by mass spectometry.

Identification of a new group of minoritary indole alkaloids of the meridianin series from the crude extract of the Antarctic ascidian *Aplidium falklandicum* by mass spectrometry. *in prep.*

Se reunieron varios extractos etéteros de la muestra *Aplidium falklandicum* 1, con el fin de obtener suficiente cantidad para la detección de meridianinas minoritarias. Los extractos se disolvieron en metanol y se introdujeron en una columna de 20 cm de altura y 4 cm de diámetro de Sephadex LH-20 y para la separación se utilizó una mezcla de metanol:diclorometano 1:1. Después de eluir la columna con 1.5 L de mezcla y tras seguimiento de la columna mediante cromatografía en capa fina, se obtuvieron 8 fracciones denominadas MC1-MC8 que se sometieron por separado a un análisis de HPLC-MS/MS. Para ello cada fracción se sometió a separación mediante una columna C18 utilizando un gradiente de acetoniltrilo-agua con un 0.1% de ácido fórmico.

En las fracciones MC1-MC2-MC3 no se detectaron meridianinas mediante espectrometría de masas. Sólo las fracciones MC4-MC8 presentaban meridianinas.

HPLC-MS de la fracción MC4

La fracción MC4 mostró dos picos de HPLC cuyos espectros de masas de alta resolución [M+H]+ a m/z 245.06 y 247.06 (tiempos de retención 10.15 y 13.51 min.) eran indicativos de dos compuestos isómeros con fórmulas $C_{12}H_8ClN_4$. Además presentaron el grupo isotópico típico de un solo átomo de cloro con intensidades correspondientes a los isótopos ^{35}Cl y ^{37}Cl en una relación 2:1. A estos dos compuestos les denominamos meridianinas I, e I', y proponemos sus estructuras (Fig. 1). Junto a estos picos se detectaron otros a 12.34 y 15.03 min. asignados a compuestos con fórmulas $C_{12}H_8Br_2N_4O_2$ (meridianina U) y $C_{12}H_8BrClN_4$ (meridianina K).

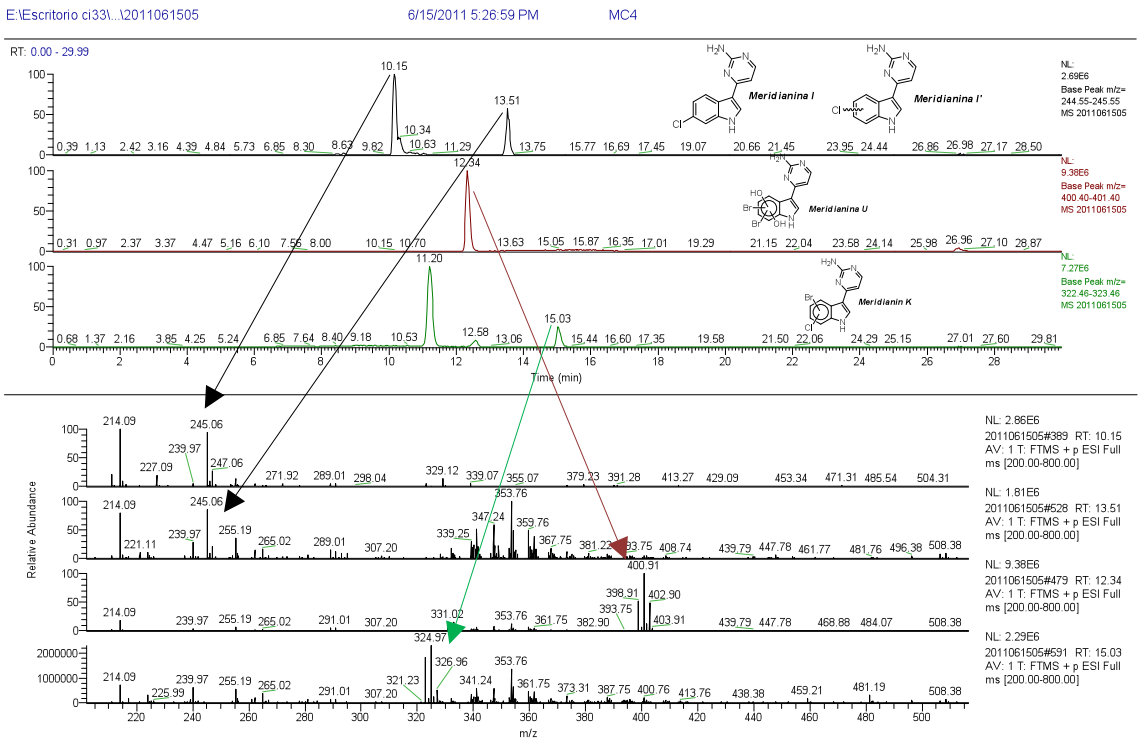


Fig. 1 HPLC-MS de la fracción MC4 mostrando los picos de las meridianinas I, I', U, K

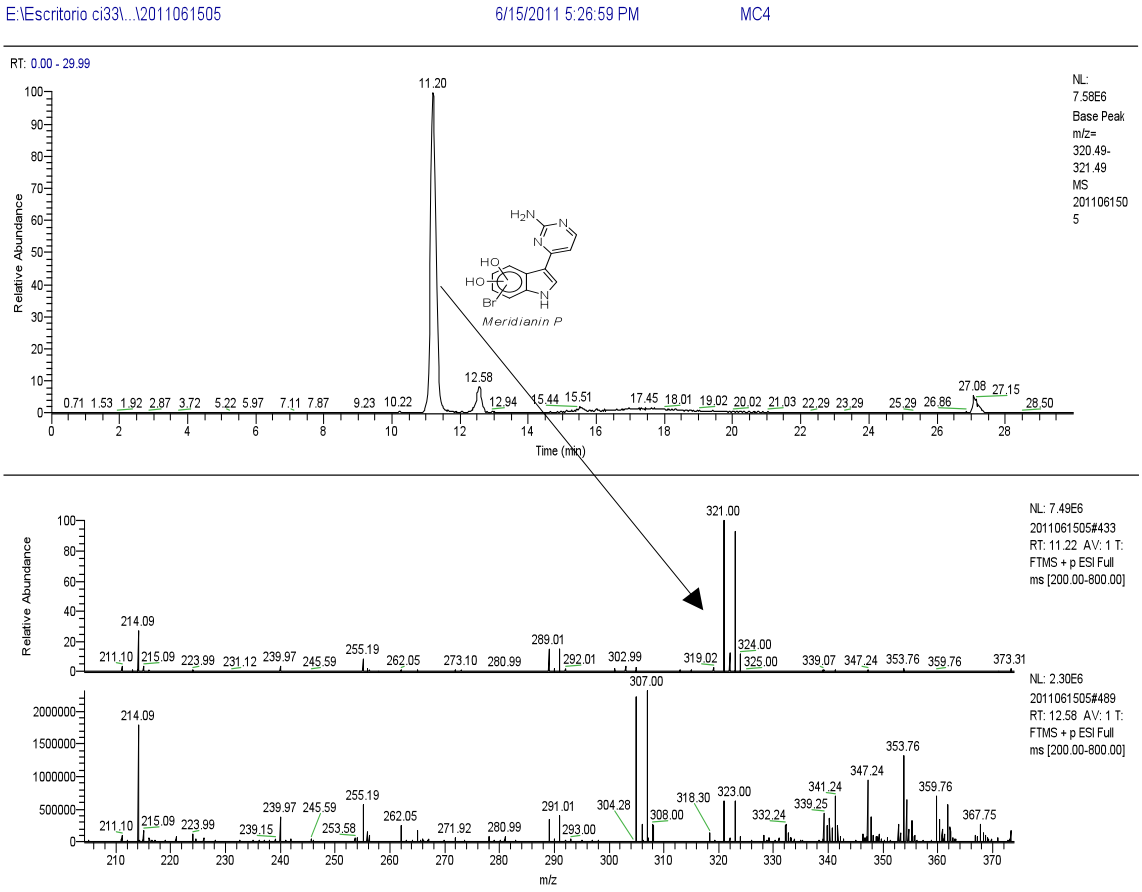
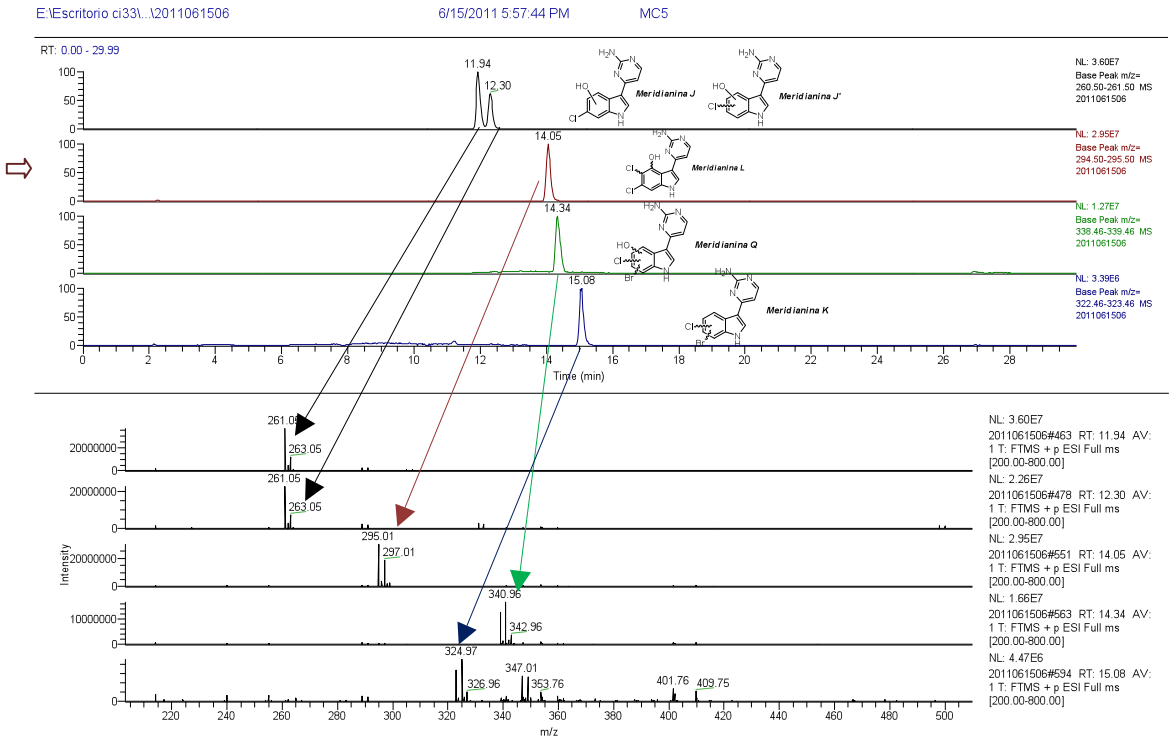


Fig 2. HPLC-MS de la fracción MC4 indicando el pico de la meridianina P

El pico a 11.20 min. con fórmula $C_{12}H_9BrN_4O_2$ se asignó a una nueva meridianina P (Fig. 2).

HPLC-MS de la fracción MC5



De la fracción MC5 se detectaron dos picos cromatográficos a 11.94 y a 12.30 min. correspondientes a dos meridianinas isoméricas con fórmulas $C_{12}H_8ClN_4O$ que se asignaron tentativamente a las estructuras que se indican en la figura. De otros tres picos se observaron picos [M+H]+ a m/z 295.01/297.01 (asignados a meridianina L), m/z 338.96/340.96/342.96 (asignados a meridianina Q), y m/z 322.97/324.97/326.97 (asignados a meridianina K ya observada en la fracción MC-4)

HPLC-MS de la fracción MC6

Se observaron las meridianinas J, J', L y otra isomérica a Q (que hemos denominado Q') y otra de fórmula $C_{12}H_9Br_2N_4O$ (picos m/z 382.91/384.91/386.91) a la que hemos llamado meridianina R (Fig. 4).

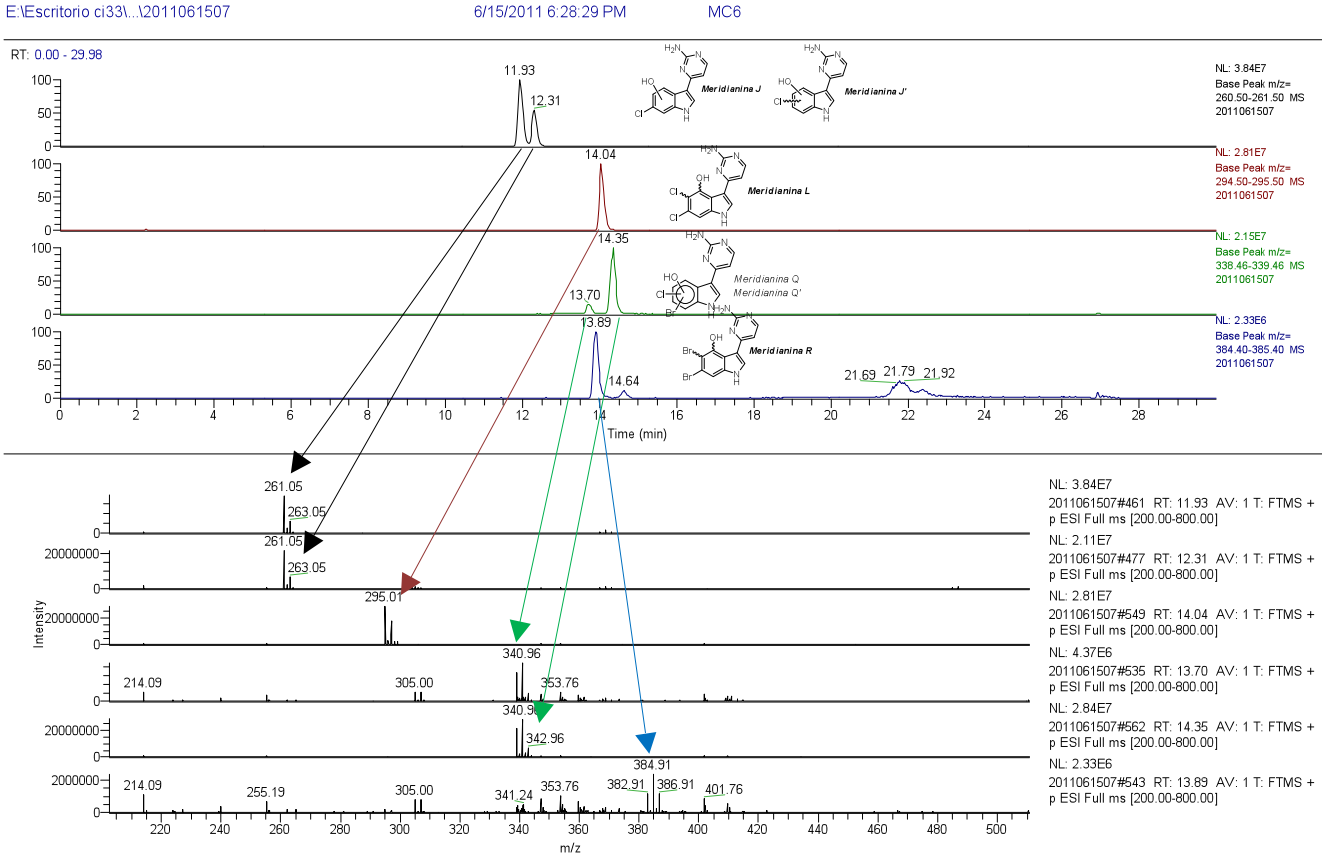


Fig. 4 HPLC-MS de la fracción MC6 mostrando los picos de las meridianinas J, J', L, Q, Q' y R

HPLC-MS de la fracción MC7.

Se detectaron la meridianina R y otro isómero de ésta, al que hemos denominado R' (Fig. 5).

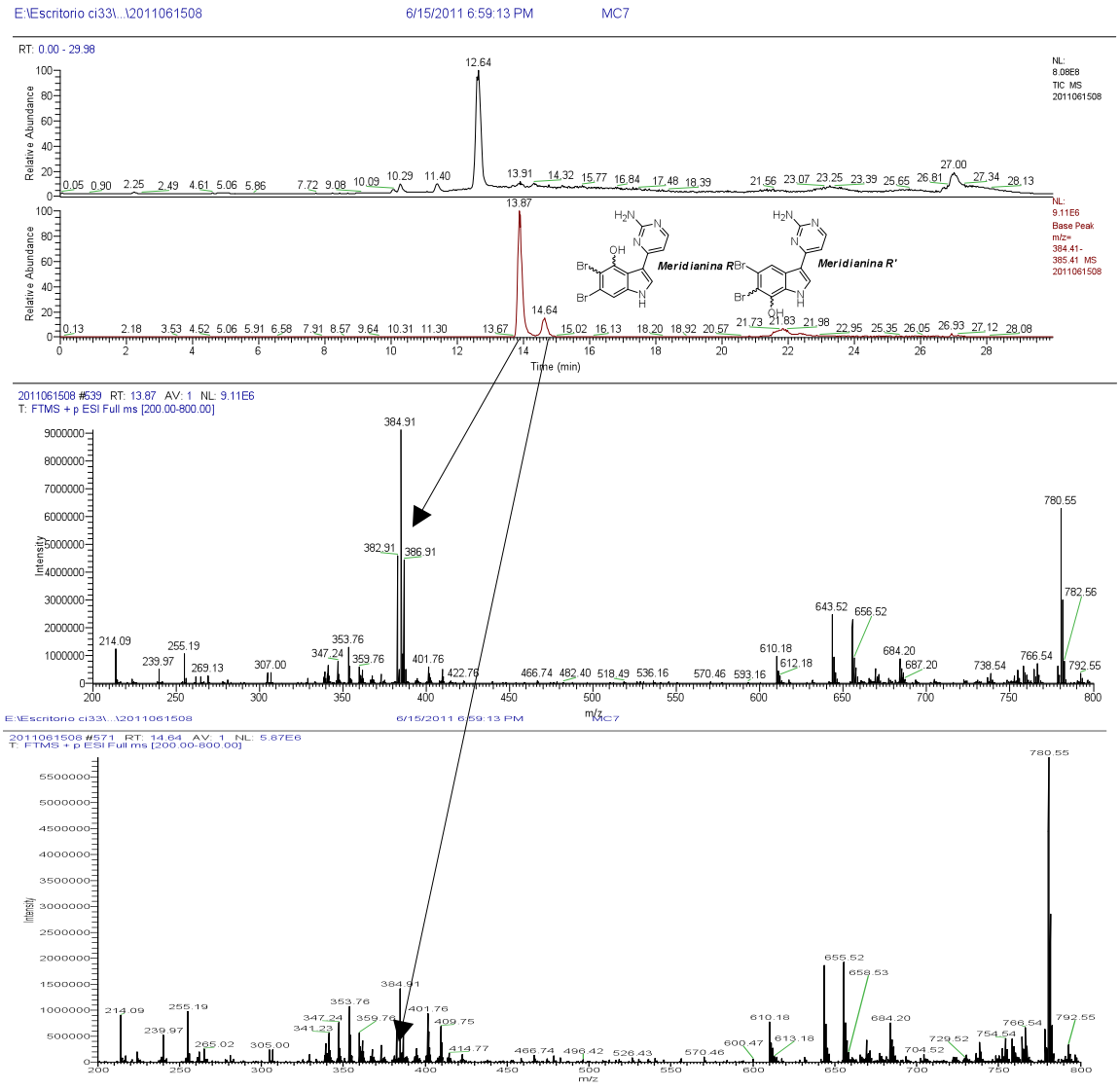


Fig. 5 HPLC-MS de la fracción MC7 mostrando el pico de las meridianinas R y R'

HPLC-MS de la fracción MC8. Dímeros de meridianinas

En esta última fracción se detectaron picos con masas más altas que nos indicaron la presencia de posibles formas diméricas de las meridianinas. Se trataba de dímeros derivados de las meridianinas mayoritarias (A, B, E ó F), de los cuales se detectaron dos que pueden tener la estructura que se indica a continuación (Fig. 6).

Posible estructura del dímero de meridianina B ó E

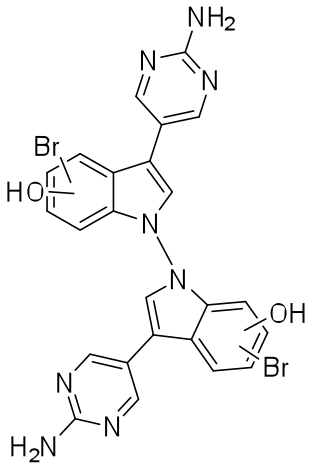


Fig. 6 Posible estructura de uno de los dímeros de las meridianinas B o E de la fracción MC8.

Este era el cromatograma del pico de detección de este dímero. Se podía observar que los picos cromatográficos de los iones (M+H)+ y $(M+2H)^{+2}$ aparecían en el mismo tiempo de retención por lo que son del mismo compuesto. Esto confirmaba la presencia de una sustancia dímera (Fig. 7).

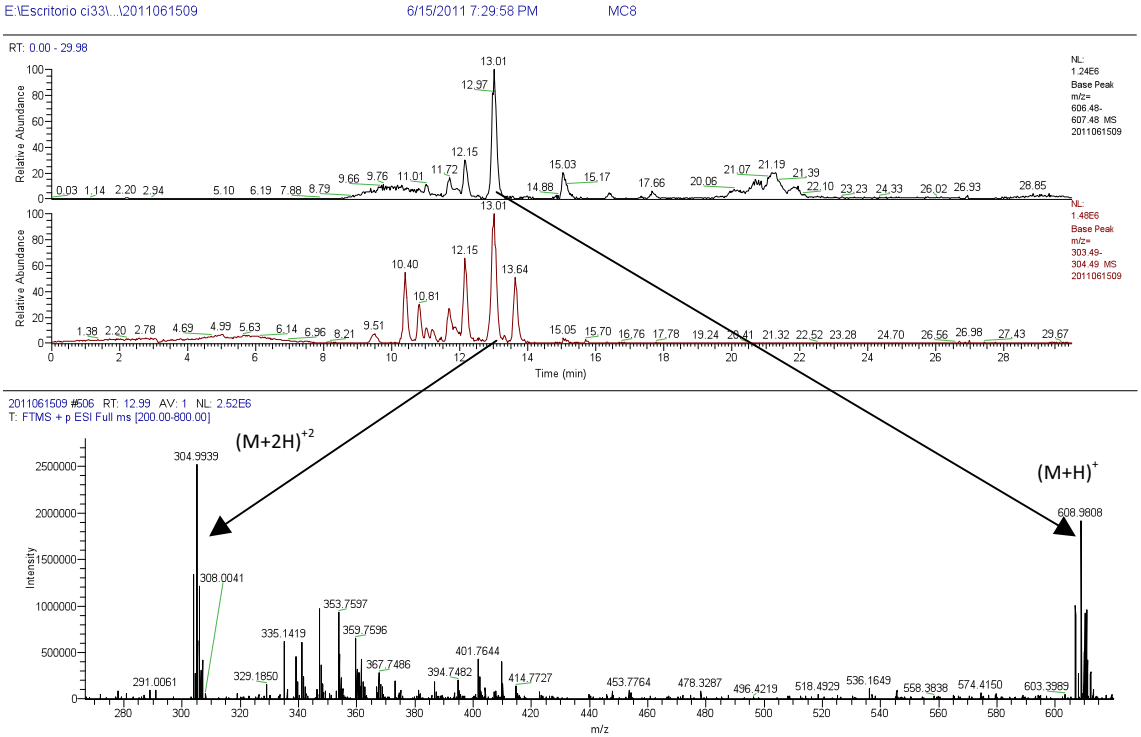


Fig. 7 Cromatograma de la fracción MC8 mostrando los picos de uno de los dímeros de meridianinas

También el dímero de la meridianina A se pudo detectar en MC8 por sus espectros de LC-masas (Fig. 8 y 9).

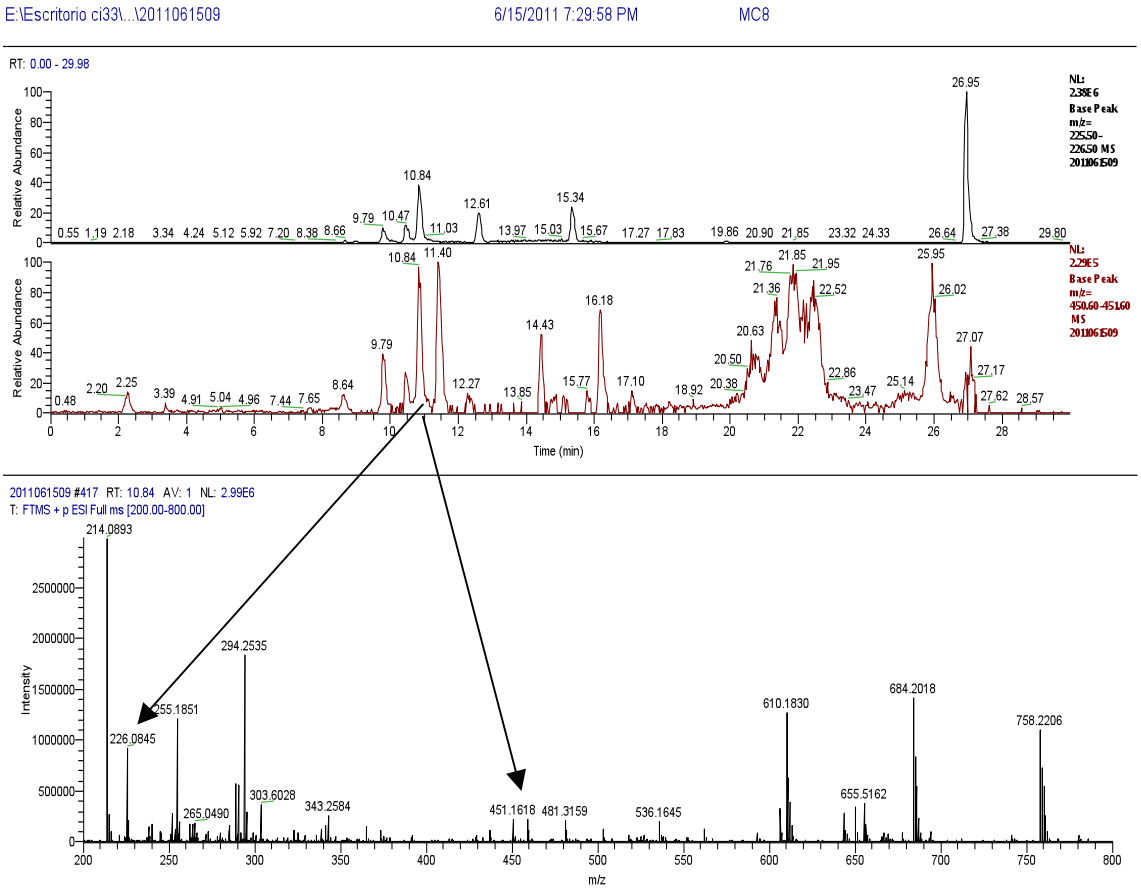


Fig 8. Espectro LC-MS de la fracción MC8 donde se indican los picos del dímero de la meridianina A

Posible estructura del dímero de meridianina A

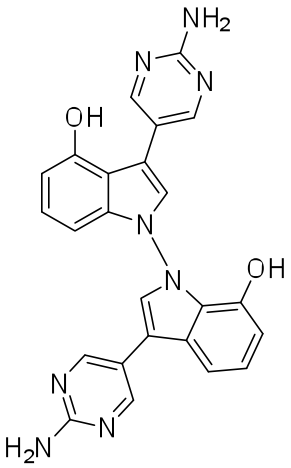
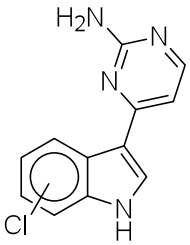


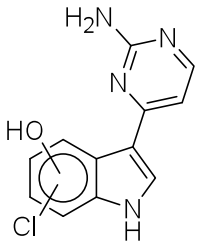
Fig 9. Posible estructura molecular del dímero de meridianina A de la fracción MC8

Estas estructuras propuestas son tentativas, ya que la espectrometría de masas no nos indica las posiciones de los grupos funcionales. A continuación se presentan todas las meridianinas detectadas y sus posibles estructuras (Fig. 10):

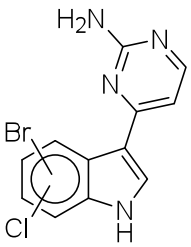
Estructuras propuestas de las nuevas meridianinas



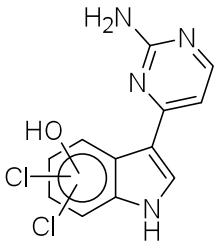
Meridianina I
Meridianina I'



Meridianina J
Meridianina J'

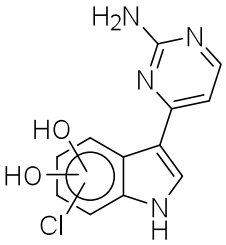


Meridianina K

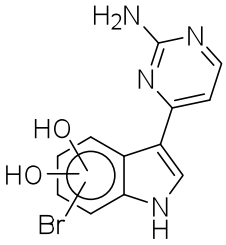


Meridianina L

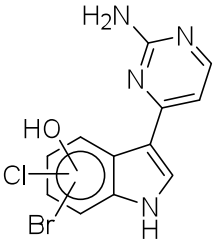
Fórmula	$C_{12}H_8ClN_4$	$C_{12}H_8ClN_4O$	$C_{12}H_8BrClN_4$	$C_{12}H_8Cl_2N_4O$
Pico $[M+H]^+$	245.0594	261.0543	322.9699	295.0153



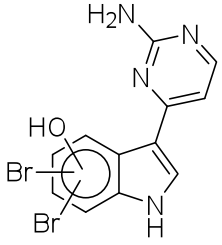
Meridianina O



Meridianina P

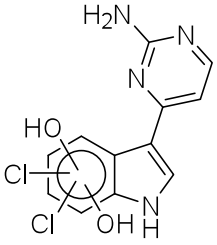


Meridianina Q
Meridianina Q'

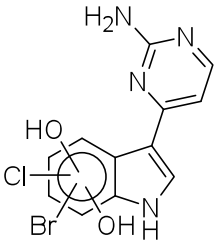


Meridianina R
Meridianina R'

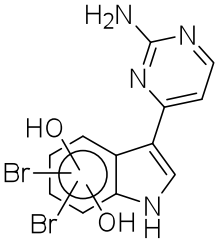
Fórmula	$C_{12}H_9ClN_4O_2$	$C_{12}H_9BrN_4O_2$	$C_{12}H_8BrClN_4O$	$C_{12}H_9Br_2N_4O$
Pico $[M+H]^+$	277.0492	320.9987	338.9648	382.9143



Meridianina S



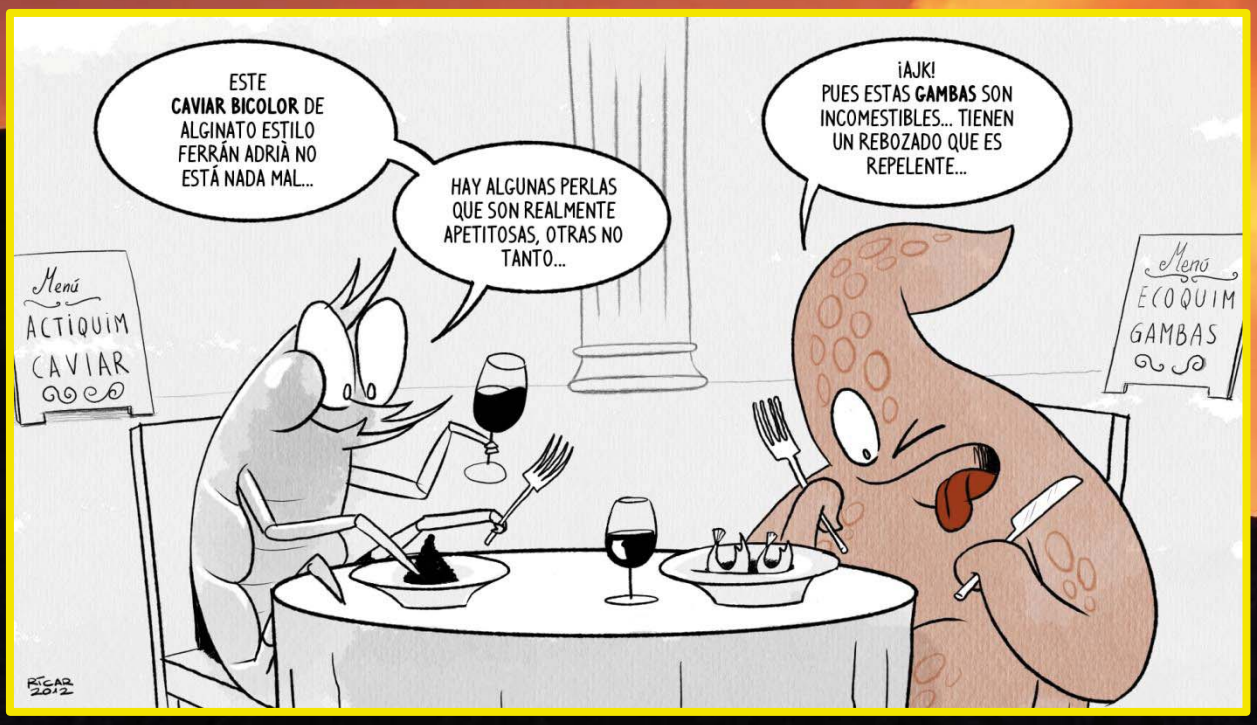
Meridianina T



Meridianina U

Fórmula	$C_{12}H_8Cl_2N_4O_2$	$C_{12}H_8BrClN_4O_2$	$C_{12}H_8Br_2N_4O_2$
Pico $[M+H]^+$	311.0103	354.9597	398.9092

Fig. 10 posibles estructuras de todas las meridianinas detectadas en nuestro estudio con extractos etéreos de la muestra de la ascidia colonial antártica *Aplidium falklandicum* 1



Nunca fue fácil ser depredador en la Antártida... pero tampoco presa, y por eso hay que defenderse... y por eso hay que comer de todo... Si fuésemos todos especialistas, raramente llegaríamos a un equilibrio, y no habría razón de inventar mecanismos de supervivencia, sería un poco aburrido... Propongo un brindis por los depredadores oportunistas y generalistas... ¡Por ellos!

LAU