

Feeding deterrency in Antarctic marine organisms: bioassays with the omnivore amphipod *Cheirimedon femoratus*

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ABSTRACT: The main predators of Antarctic benthic organisms are vagile invertebrates, including dense amphipod populations. Marked seasonalities of food availability drive consumers to develop opportunistic behaviors, which favors the evolution of defensive chemistry in potential prey. We used the circumpolar omnivorous amphipod *Cheirimedon femoratus* and a new feeding preference assay using alginate caviar-textured food pearls to examine the incidence of lipophilic deterrents in Antarctic benthic organisms. The method showed remarkable discriminatory potential for unpalatable metabolites. We obtained a total of 52 fractions from 40 samples that represented 31 species (including sponges, cnidarians, ascidians, a bryozoan, an echinoderm, a hemichordate and algae) from the Weddell Sea and the South Shetland Islands. Unpalatability was found in 42 extracts from 26 species. The remaining 10 extracts from 7 samples did not exhibit unpalatability, indicating either that deterrents are contained in fractions not tested here, or that alternative defensive traits might protect these organisms. Within the 4 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 *C. femoratus*. Thus, this amphipod could be a significant inducer of chemical protection in its hosts due to the localized and constant contact created by inhabiting and feeding on the same organism. Defense sequestration in specific body-structures, as predicted by the Optimal Defense Theory, was detected in an octocoral sample. We suggest that some of the tested organisms could combine several defensive strategies to prevent predation. Altogether, our results indicate that chemical defenses are broadly used among Antarctic benthic organisms to avoid predation by the opportunistic amphipod *C. femoratus*, and thus, chemical ecology is a key aspect in the functioning of Antarctic ecosystems.

KEY WORDS: *Cheirimedon femoratus* · Antarctic invertebrates · Antarctic algae · Chemical ecology · Omnivorous amphipod · Unpalatability · Feeding preference assays

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INTRODUCTION

Antarctic marine ecosystems are characterized by low temperatures and pronounced seasonality, with periodic limitations of food resources. Despite some

shallow coastal regions (less than approx. 30 m depth) exposed to ice scour and anchor ice, the benthic habitat appears to be physically stable and 'biologically accommodated' (Gutt 2000). Hence, species distribution and abundance in these communities are

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considered to be largely regulated by predatory and competitive interactions (Dayton et al. 1974). The continental shelf of Antarctica houses a rich suspension-feeding macroinvertebrate assemblage comprised of mainly 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 nemertean and asteroid echinoderms, and fish (Richardson 1975, Dearborn 1977, Gutt et al. 2000, Obermuller et al. 2010). These keystone predators, especially sea stars and amphipods, cause intense predation pressure, and are commonly circumpolar in distribution. Thus, sessile and slow-moving organisms from distant Antarctic regions are affected by the foraging activities of these ubiquitous predators (Dayton et al. 1974, De Broyer et al. 2007). The Antarctic biota is very ancient (originating at least 22 million yr ago) and endemic (Gutt 2000). These facts suggest that interactions between species provided many opportunities for the evolution of defensive mechanisms. One common tactic is chemical defense, characterized by the biosynthesis or storage of dietary products with toxic, noxious or unpalatable properties (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 effective against a variety of enemies in order to save energy and mostly addressed towards generalists (Herms & Mattson 1992, Sotka et al. 2009). Moreover, the Optimal Defense Theory (ODT) predicts that chemical defenses should be sequestered in those tissues that are most vulnerable (in terms of fitness) and frequently targeted by predators, and in co-ordination with other defensive mechanisms (Rhoades & Gates 1976). Thus, defenses should be concentrated in the outermost zones, where they would be most effective against a number of predators that first encounter the prey's surface. But in perforated prey (e.g. sponges), in which small grazers may access inner tissues, defenses should also be 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 com-

monly associate temporarily with living substrata (frequently macroalgae and sponges but also others), which are often also their potential (direct or incidental) prey (De Broyer et al. 2001). In tropical areas, these small crustaceans are effective inducers of chemical defense in their associated host and potential prey, because of the intense localized pressure they exert on these species (Cronin & Hay 1996, Toth et al. 2007). Amphipods appear in very high densities off the Western Antarctic Peninsula (up to 300 000 individuals m^{-2} ; Huang et al. 2007), higher than in other latitudes (Nelson 1980). Antarctic amphipods strongly influence the population dynamics of benthic biota, since they feed on a wide array of taxa, including macroalgae, sponges, cnidarians, holothurians, bryozoans, and 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, because they are an important food source for demersal fishes (Richardson 1975).

Feeding deterrents are widespread in Antarctic communities, but the effect of generalist amphipods on the prevalence of these protective metabolites in their prey species has received scarce attention (for reviews see Avila et al. 2008, McClintock et al. 2010), even though these consumers are considered important inducers of defensive chemistry (Cronin & Hay 1996, Toth et al. 2007). The lysianassid amphipod *Cheirimedon femoratus*, with a circumpolar distribution and eurybathic occurrence down to 1500 m depth (De Broyer et al. 2007, Krapp et al. 2008), is described as a voracious, omnivorous, opportunistic scavenger, except for ovigerous females and newly hatched young, which ingest mostly photosynthetic material (algae) during summer (Bregazzi 1972b). In fact, it is a first-arrival feeder at carrion inputs (Smale et al. 2007). As a very abundant generalist feeder (animal, micro- and macroalgal material and detritus) (Bregazzi 1972b), *C. femoratus* is likely a consumer towards which most potential prey inhabiting the Antarctic shelf would address defensive chemistry. *C. femoratus* has reduced swimming abilities and loose host relationships (Bregazzi 1972b, authors' pers. obs.). The present study is the first to use this ubiquitous amphipod as model predator to perform palatability bioassays, with samples coming from variable depths of the scarcely studied Weddell Sea and the South Shetland Archipelago. Since most 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 was to evaluate the

presence of chemical defenses (in the form of unpalatable substances) in Antarctic benthic organisms and to determine the within-body allocation of defenses when possible. Furthermore, a new protocol for feeding preference assays is proposed.

MATERIALS AND METHODS

Selection and sampling of model predator

In our search for an appropriate experimental consumer, we assessed 3 of the most abundant species around the Antarctic Spanish Base (BAE) 'Gabriel de Castilla' on Deception Island ($62^{\circ} 59.369' S$, $60^{\circ} 33.424' W$). Selective trials were performed with the limpet *Nacella concinna*, the sea urchin *Sterechinus neumayeri*, and the amphipod *Cheirimedon femoratus*. The predator needed to be ubiquitous and omnivorous, because extracts from various animals and algae coming from distant locations were being evaluated. Since the limpet and the urchin consistently consumed very little under laboratory conditions—in agreement with previous reports (e.g. Amsler et al. 2005b)—and were not strictly omnivorous, we chose the amphipod as model predator.

Hundreds of individuals of the amphipod *Cheirimedon femoratus* were captured along the shoreline of the Spanish Antarctic Base 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 divers using fishing nets to capture aggregations of individuals associated with the pyroclastic sediment and algae, and with traps baited with sardines and deployed for 48 h, which attracted dense swarms of amphipods. Once the experiments were completed, a few specimens were fixed in 70 % ethanol for taxonomy, and the rest were returned to the sea.

Benthos collection and identification

Marine benthic invertebrate and algal samples of 31 species were collected in the Southern Ocean during 4 Antarctic campaigns: two of them in the eastern Weddell Sea on board the R/V 'Polarstern' during the ANT XV/3 (January to March 1998) and ANT XXI/2 cruises (November 2003 to January 2004); a third on board the R/V 'BIO Hespérides' during the ECOQUIM-2 cruise (January 2006) around the South Shetland Islands; and the fourth during the ACTIQUIM-1 cruise at Deception Island (December 2008 to January 2009; Fig. 1). Sampling was done at a total of 24 stations between 0 m and 1524 m depth by using bottom and Agassiz trawls, an 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 for taxonomical identification 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 $-20^{\circ}C$ for further examination.

Chemical extractions

Invertebrates were dissected for within-body allocation of defensive chemicals when sufficient amounts of sample were available. The sections were cut based on the predictions of the ODT for Antarctic prey, which likely accumulate defenses in the outer layers. Depending on the organism, samples were sectioned into internal (visceral) and external (tunic) tissues in ascidians, external/internal or apical/basal parts in sponges, or polyparium and axial regions in octocoral cnidarians. The invertebrate and algal samples (either dissected or not), consisting of

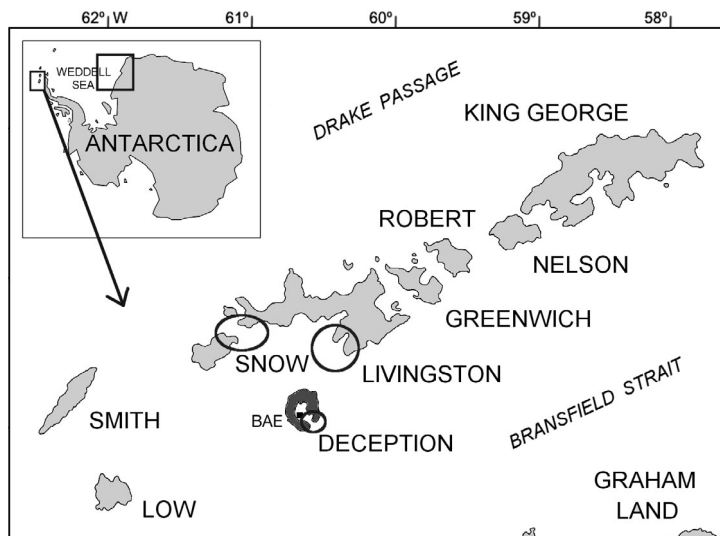


Fig. 1. Main sampling areas (in the 2 squares) for the 4 campaigns on the Antarctic continent, and the South Shetland Archipelago in detail, with sampling points of that particular zone within circles. Dark gray: Deception Island, the location of the Spanish Antarctic Base 'Gabriel de Castilla' (BAE) where the experiments took place

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: brown; O: orange morphotypes

Species	Location	Latitude (S)	Longitude (W)	Gear	Depth (m)
PORIFERA					
Demospongiae					
<i>Isodictya toxophila</i> Burton, 1932	Weddell Sea	70° 57.00'	10° 33.02'	BT	332.8
Hexactinellida					
<i>Anoxycalyx (Scolymastra) joubini</i> Topsent, 1916 (1)	Weddell Sea	71° 06.30'	11° 32.04'	AGT	175.2
<i>Anoxycalyx (Scolymastra) joubini</i> Topsent, 1916 (2)	Weddell Sea	70° 52.16'	10° 43.69'	BT	290.8
<i>Rossella fibulata</i> Schulze & Kirkpatrick, 1910	Weddell Sea	70° 57.00'	10° 33.02'	BT	332.8
<i>Rossella nuda</i> Topsent, 1901	Weddell Sea	71° 4'	11° 32'	BT	308.8
<i>Rossella vanhoffeni</i> (Schulze & Kirkpatrick, 1910)	Weddell Sea	72° 28'	17° 51'	ES	882
<i>Rossella villosa</i> Burton, 1929	Weddell Sea	70° 55.92'	10° 32.37'	AGT	288.0
<i>Rossella</i> sp. 1 (Orange) Carter, 1872	Weddell Sea	70° 55.92'	10° 32.37'	AGT	288.0
CNIDARIA					
Anthozoa					
<i>Alcyonium antarcticum</i> Wright & Studer, 1889	Weddell Sea	70° 56'	10° 31'	BT	337.2
<i>Alcyonium haddoni</i> Wright & Studer, 1889	Deception Island	62° 59.55'	60° 33.68'	SD	9
<i>Alcyonium roseum</i> van Ofwegen, Häussermann & Försterra, 2007	Weddell Sea	71° 17.1'	12° 36'	AGT	416
<i>Primnois antarctica</i> (Studer, 1878) (1)	Weddell Sea	70° 52.75'	10° 51.24'	BT	294.8
<i>Primnois antarctica</i> (Studer, 1878) (2)	Weddell Sea	70° 52.75'	10° 51.24'	BT	294.8
<i>Thouarella laxa</i> Versluys, 1906 (1)	Weddell Sea	71° 4'	11° 32'	BT	308.8
<i>Thouarella laxa</i> Versluys, 1906 (2)	Weddell Sea	70° 52.16'	10° 43.69'	BT	290.8
<i>Thouarella laxa</i> Versluys, 1906 (3)	Weddell Sea	70° 52.75'	10° 51.24'	BT	294.8
<i>Thouarella laxa</i> Versluys, 1906 (4)	Deception Island	63° 02.29'	60° 36.36'	AGT	100.4
<i>Thouarella minuta</i> Zapata-Guardiola & López-González 2009	Weddell Sea	70° 56'	10° 32'	BT	338
<i>Umbellula antarctica</i> Kukenthal & Broch, 1911	Weddell Sea	70° 56'	10° 32'	BT	338
Hydrozoa					
<i>Staurotheca antarctica</i> Hartlaub, 1904	Weddell Sea	72° 51.43'	19° 38.62'	BT	597.6
<i>Symplectoscyphus glacialis</i> (Haderholm, 1904)	Weddell Sea	71° 06.30'	11° 32.04'	AGT	175.2
CHORDATA (Ascidacea)					
<i>Aplidium falklandicum</i> Millar, 1960	Weddell Sea	70° 57.00'	10° 33.02'	BT	332.8
<i>Aplidium fuegiense</i> Cunningham, 1871	Weddell Sea	71° 7'	11° 26'	AGT	228.4
<i>Aplidium meridianum</i> (Sluiter, 1906)	Weddell Sea	70° 56.42'	10° 31.61'	BT	284.4
<i>Synoicum adareanum</i> (B&W) (Herdman, 1902) (1)	Weddell Sea	70° 56'	10° 32'	BT	337.2
<i>Synoicum adareanum</i> (B&W) (Herdman, 1902) (2)	Weddell Sea	70° 55.92'	10° 32.37'	AGT	288.0
<i>Synoicum adareanum</i> (Br) (Herdman, 1902)	Weddell Sea	71° 06.44'	11° 27.76'	AGT	277.2
<i>Synoicum adareanum</i> (O) (Herdman, 1902) (1)	Weddell Sea	70° 55.92'	10° 32.37'	AGT	288.0
<i>Synoicum adareanum</i> (O) (Herdman, 1902) (2)	Weddell Sea	70° 56'	10° 32'	BT	337.2
BRYOZOA					
<i>Isoschizoporella secunda</i> Hayward & Taylor, 1984	Weddell Sea	71° 06.44'	11° 27.76'	AGT	277.2
ECHINODERMATA (Holothuroidea)					
<i>Peniagone vignioni</i> Herouard, 1901	Weddell Sea	70° 47.88'	11° 24.13'	AGT	1524.8
HEMICHORDATA (Pterobranchia)					
<i>Cephalodiscus nigrescens</i> Lankester, 1905	Weddell Sea	70° 56.42'	10° 31.61'	BT	284.4
ALGAE					
Ochrophyta					
<i>Adenocystis utricularis</i> (Bory de Saint-Vincent) Skottsberg, 1907	Snow Island	62° 44.01'	61° 12.2'	SD	1.5
<i>Ascoseira mirabilis</i> Skottsberg 1907	Livingston Island	62° 45'	60° 20'	SD	0.7
<i>Desmarestia anceps</i> Montagne, 1842	Deception Island	62° 59.37'	60° 33.42'	SD	7.5
<i>Desmarestia antarctica</i> Moe & Silva, 1989 with <i>Geminocarpus austrogeorgiae</i> Skottsberg, 1907	Livingston Island	62° 45'	60° 20'	SD	0.7
<i>Desmarestia menziesii</i> J.Agardh, 1848	Deception Island	62° 59.02'	60°35.85'	AGT	109.7
Rhodophyta					
<i>Georgiella confluens</i> (Reisch) Kylin, 1956	Livingston Island	62° 45'	60° 20'	SD	0.7
<i>Gigartina skottsbergii</i> Setchell & Gardner, 1936	Deception Island	62° 59.37'	60° 33.42'	SD	12
<i>Palmaria decipiens</i> (Reinsch) Ricker, 1987	Deception Island	62° 58.59'	60° 40.58'	SD	1.3

several individuals or colonies, were exhaustively extracted with acetone and sequentially partitioned into diethyl ether and butanol fractions. All extraction steps were repeated 3 times, except for the butanol, which was done once at the end. Organic solvents were evaporated using a rotary evaporator. Chemical profiles of the obtained fractions were screened by thin layer chromatography. Only diethyl ether extracts (comprising the most apolar lipophilic metabolites) were used further in the bioassays (Table S1 in the supplement at www.int-res.com/articles/suppl/m462p163_supp.pdf). Butanolic fractions and water residues were not tested here, but were kept for future investigations.

Artificial food preparation

Artificial food was prepared using 2 different protocols. The first one consisted of traditional agar strips on a base of window screen following the method of Hay et al. (1994), except that we reduced the agar proportion from 20 mg ml⁻¹ to 10 mg ml⁻¹ and increased the quantity of food stimulant from 55.6 mg ml⁻¹ to 80 mg ml⁻¹. The second method was new, using alginate caviar-textured pearls made with the Kit Sferificacion® (www.albertyferranadria.com/eng/texturas.html). During pilot trials with *Cheirimon don femoratus*, agar strips had disadvantages compared to alginate pearls: (1) lower consumption rates by *C. femoratus*, resulting in longer experimental times, (2) larger amounts of extract required, and (3) the agar had to be heated to 70°C, possibly resulting in degradation of some compounds in the extracts. Hence, alginate food pearls were selected for our assays. A concentrated blend of powdered dried aquarium food (Phytoplan®, 17 to 19 kJ g⁻¹ dry weight) was chosen as feeding stimulant to maximize consumption rates. During pilot testing of several diets, including vegetarian sources (*Chlorella*, Nori, lyophilized Antarctic seaweeds [*Desmarestia anceps*, *D. menziesii*, *Gigartina skottsbergii*], *Spirulina*-based feed and phytoplankton) and carnivorous sources (krill, Cyclop-eeze mixed zooplankton, fish feed and anchovy paste), Phytoplan® resulted in the highest consumption rates. For the final artificial food recipe, 10 mg ml⁻¹ of 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 next paragraph) were added dropwise with a syringe to a 0.09 M (1%) CaCl₂ (Calcic® of Kit Sferificacion®) water solution, where it gelatinized (i.e. polymerized) to form spheroid

pearls approximately 2.5 mm in diameter. These contained 3.3% protein, 1.36% carbohydrates and 1.3% of lipids (all w/w; based on nutrition facts from Phytoplan®).

Tissue extracts were incorporated in the artificial food via Phytoplan®. Extracts 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 stimulant concentrate prior to being added to the alginate aqueous mix. Extracts were applied to Phytoplan® at natural tissue concentrations, and the factor employed to calculate tissue concentrations of each lipophilic fraction (hereafter referred to as the 'natural concentration') was based on the dry weight. The natural concentration was calculated by dividing the ethereal fraction (EE) by the total dry weight ($DW_T = DW + EE + BE$, where DW is the dry weight of the solid remains of the extracted sample, and BE is the weight of the butanolic fraction). While volume-based normalization is usually applied when dealing with biting predators to calculate the 'defense per unit bite' (e.g. Pisut & Pawlik 2002), concentrations based on biomass employing wet or dry weight are used with non-biting and biting predators (e.g. 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 avoids issues of high variability of weight parameters related to water content in aquatic samples, especially sponges. The relative quantity of each extract at natural concentration was then calculated with respect to the total dry weight of artificial food mix required to produce a whole set of extract-treated pearls for a single experiment (0.03 g alginate + 0.2 g Phytoplan® = 0.23 g). This quantity guarantees the formation of 150 pearls (15 replicates × 10 pearls per replicate) as well as extra pearls for pilot trials investigating feeding-unrelated changes to the pearls (see next subsection). Control food pearls were prepared identically but without extract, adding an equal volume of solvent alone. Control 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 CaCl₂ solution. Pilot trials confirmed that feeding preferences of *Cheirimon don femoratus* did not differ between colored and uncolored pearls ($p = 0.47$), or between red and green colored pearls ($p = 0.47$). Nevertheless, control and treatment food pearls were randomly swapped to green or red colorations throughout the experiments.

Feeding-preference bioassays with amphipods

Live *Cheirimedon femoratus* were maintained in fresh seawater in 8 l aquariums at the BAE and were starved for 3 to 5 d prior to bioassays. Each assay consisted of 15 replicates, which were run in separate 500 ml containers, each filled with seawater, 15 to 20 randomly selected amphipods, and a simultaneous choice of 10 control and 10 extract-treated pearls. Amphipods attached to the pearls and ate individually or in groups of up to 5 specimens per pearl. During pilot trials, we periodically monitored and video-filmed amphipod feeding behavior to determine the time course of the experiments. On average a group of 15 to 20 individuals ingested 10 pearls in about 4 to 5 h. Thus, we terminated assays when at least 5 of the 10 pearls of either food type (control or treatment) had been consumed, or 4 h after food presentation. Amphipods were not re-used in subsequent trials. The number of consumed and unconsumed pearls of each color (control or treatment) was recorded. In pilot trials, food pearls were resistant to degradation during 72 h exposure to seawater. Since our feeding assays were short (4 h), we eliminated the need for control assays without consumers to determine changes unrelated to consumption (Peterson & Renaud 1989).

For each of the 15 replicate containers, the differences in the quantity of ingested control and treated food were calculated. The changes in the amount of the 2 foods in each container are not independent and possess correlated errors. Each replicate is thus represented by a paired result yielding 2 sets of data (treatment and control). Since assumptions of normality and homogeneity of variances were not met, the 2 sets of data were compared using the non-parametric Exact Wilcoxon test in R. Uneaten pearls from completed assays and extra (unused) pearls that had been conserved in seawater were preserved for extraction and analysis by thin layer chromatography, to check for possible alterations in the extracts. No major changes were observed, and since the compounds dissolved in diethyl ether extracts are hydrophobic, there should be little, if any, loss to the water column.

RESULTS

A total of 40 samples (from 31 species, including different morphs of the same species) were tested: 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 2 anthozoan samples and the 8 algal

samples came from shallow coastal areas of the South Shetland Islands (Table 1). No apparent pattern related the incidence of unpalatable chemicals to location or depth. *Cheirimedon femoratus* was very voracious towards Phytoplan[®]-alginate control pearls, which were ingested at extraordinarily high rates (10 alginate pearls in 4 h). Amphipods ate in groups, in accordance with their gregarious behavior, and their feeding preferences were clearly 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.

The incidence of unpalatable defenses in Antarctic benthic organisms against the omnivorous lysianassid amphipod *Cheirimedon femoratus* was very high. Out of a total of 52 lipophilic extracts (from different body regions of the 40 samples), 42 extracts (80.8%; representing 33 samples from 26 species) were unpalatable for *C. femoratus*. The remaining 10 extracts, obtained from 7 samples belonging to 5 different species, did not exhibit any repellent activity (Fig. 2). The 4 major taxonomical groups tested yielded high percentages of significant feeding repellence against the amphipod, with the ascidians showing the highest incidence (91.7% of the ascidian extracts were unpalatable), followed by the sponges (86.7%), cnidarians (85.7%), and algae (75%). Extracts from 1 of the 2 samples of the gorgonian *Primnoisis antarctica*, the hexactinellid poriferan sample *Anoxycalyx (Scolymastra) joubini* 1, the alga *Desmarestia antarctica* (with its epiphyte), the bryozoan, the holothurian and the hemichordate caused no rejection by *C. femoratus*. In fact, 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 over control pearls. For dissected samples, the pennatulacean *Umbellula antarctica* exhibited deterrents in the axial body regions, but the diethyl ether fraction from the polyparium was readily ingested by *C. femoratus*. Moreover, the apical ethereal fraction from the black and white morphotype of the ascidian *Synoicum adareanum* 2 was palatable, while the barely consumed basal-external and visceral extracts were not (Fig. 2, Table S1).

DISCUSSION

Tunicates presented repellents in all the samples tested and had the highest proportion of unpalatable extracts compared with the other taxa, indicating a predominant reliance on organic chemical protec-

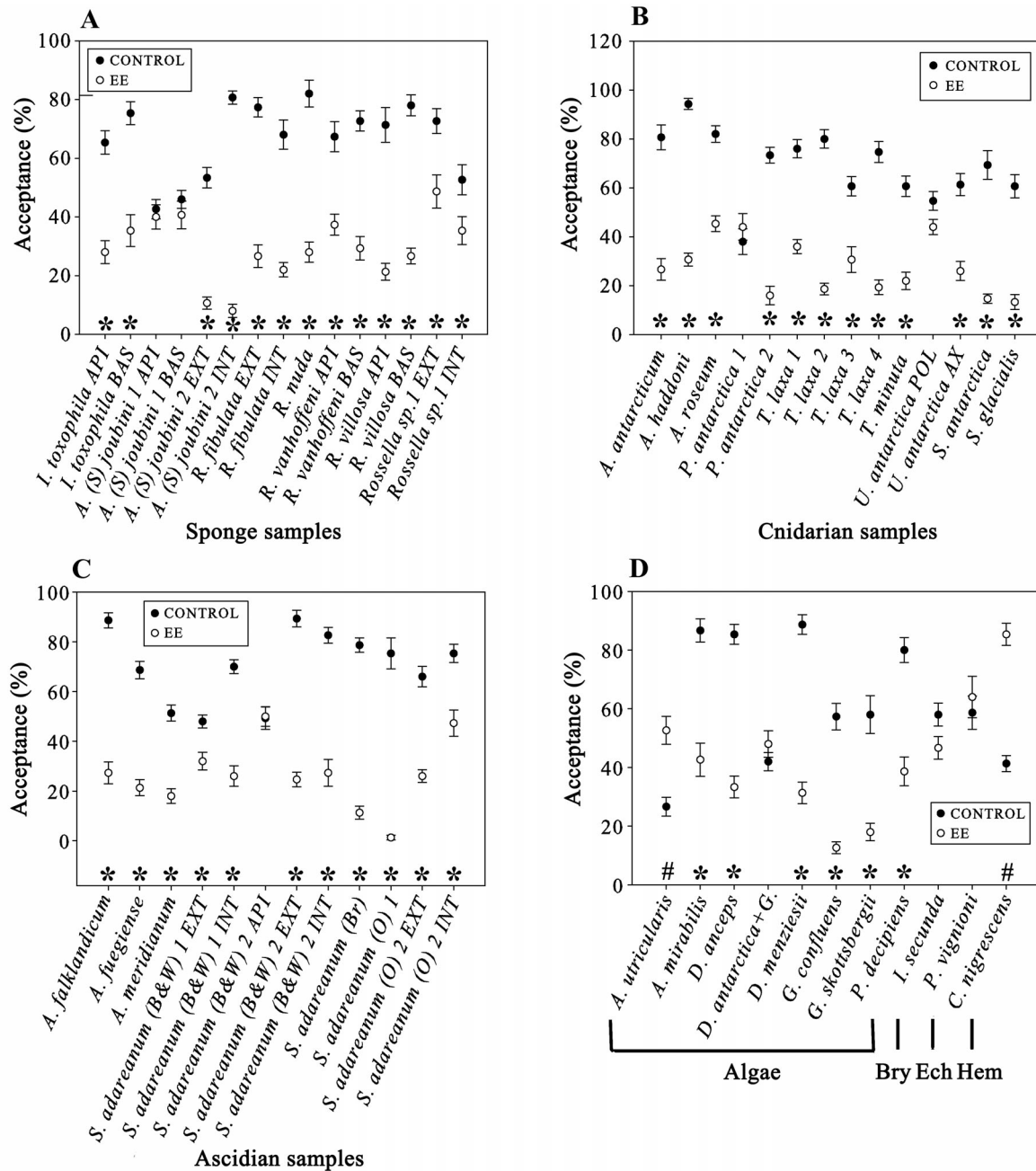


Fig. 2. *Cheirimedon femoratus*. Results of feeding preference bioassays with the amphipod for the 4 major groups assessed: (A) sponges, (B) cnidarians, (C) ascidians (*Synoicum adareanum* morphs—B&W: black & white; Br: brown; O: orange) and (D) algae and other minor groups (Bry: Bryozoa, Ech: Echinodermata; Hem: Hemichordata). EE: ether fraction of sample extract. Assayed body regions—API/BAS: apical/basal; EXT/INT: external/internal; POL/AX: polyparium/axial body-regions. Data: mean \pm SE of the percentage of control and extract-treated food accepted. Significant differences (Exact Wilcoxon test, $p < 0.05$) — (*): control food preferred; (#): treated food preferred

tion. This contrasted with other Antarctic surveys (e.g. Koplóvitz 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, 2 species, *Aplidium falklandicum* and *A. meridianum*, are known to possess the meridi-

ans, defensive alkaloids that cause rejection by 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 intra-specific variability (Varela 2007), and we found 3 morphotypes for *Synoicum adareanum*: black and white, brown and orange. All were significantly unpalatable except for the apical diethyl ether fraction from a black and white morph of *S. adareanum*. This region concentrated siphon mouths and common cloaca, and is where waste matter from digestion accumulates, which could mask the activity of co-occurring deterrent products (Fig. 2, Table S1). Fresh colonies of *S. adareanum* were previously reported to provoke rejection by the fish *Notothenia coriiceps* and by *O. validus*, while crude extracts were preferred by the amphipod *Gondogeneia antarctica* (Koplovitz et al. 2009). Small crustaceans associate with chemically defended substrata including ascidians, obtaining protection from prospective fish predators, as described in *Distaplia cylindrica* with the amphipod *Polycheria antarctica* (McClintock et al. 2009). In this sense, the mentioned Antarctic ascidians may also serve as chemical refuges to benthic amphipod species.

Glass sponges are subject to intense predation by Antarctic invertebrates despite their low energetic value (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 contradict these reports, and all but one of the hexactinellid samples assayed indicated significant unpalatability to our model predator. The palatable extracts from *Anoxycalyx (Scolymastra) joubini* 1 had lower natural concentrations compared to the unpalatable extracts from a conspecific sample, *A. (S.) joubini* 2, suggesting the presence of lower (and insufficient) quantities of repellents in the former sample (Table S1). Hydrophilic and lipophilic extracts from *A. (S.) joubini* were reported to cause strong tube-foot retractions in the spongivorous *Perknaster fuscus* (McClintock et al. 2000). This reaction, however, could be due to the highly specialized diet of this sea star, which rarely ingests any prey other than *Mycale acerata*, and not to the presence of repellents (Dayton et al. 1974). 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. According to the ODT, 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 (Rhoades & Gates 1976). But it might be ineffective towards smaller, biting grazers that can access inner body parts (e.g. of large hexactinellid oscula) through perforations. Amphipods, which occur in high abundance and diversity in association with Antarctic sponges with no obligate host relationships, are clear examples (Kunzmann 1996, Lörz & 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 increased preferences to foods containing extracts (Amsler et al. 2005b, Koplovitz et al. 2009). Contrarily, in our case, the amphipod *Cheirimedon femoratus* might certainly play a role in 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 defense strategy in these groups (Paul 1992, Sammarco & Coll 1992). Our results agree with this assumption, and most of the extracts displayed unpalatability. The only exception was the extract from specimens of one anthozoan sample of *Primnoisis antarctica*, which was consumed by *Cheirimedon femoratus*; however, the highly concentrated extract from a conspecific sample was rejected (Fig. 2, Table S1). This difference in unpalatability is probably due to natural variability in compound concentrations.

Umbellula antarctica is a flower-shaped pennatulacean devoid of sclerites, consisting of a fine stalk connected to a distant prominent polyparium (Pasternak 1962). During dissection, the sample was separated into the more exposed and nutritious polyparium crowns with few (15 to 30) voluminous polyps, and the axis. The polyparium yielded a rich and palatable extract, reflecting a lack of lipophilic deterrents in this region. Thus, defensive agents are either present in more polar fractions, or the prominent autozooids (3 to 4 cm long) are protected by their effectively penetrating nematocysts. In oligotrophic regions,

pennatulid species tend to have fewer but larger autozooids, which practice active macrophagy or carnivory by using their nematocyst system (Dolan 2008). By contrast, the axial stalk of the *U. antarctica* sample (nematocyst-free) did significantly repel *Cheirimedon femoratus*. Also, different chemical profiles were found in the extracts from polyparium and axis in the thin layer chromatography 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. By contrast, both of these strategies co-exist in hydroids (i.e. lipophilic defenses are as common as defensive nematocysts), which is contrary to the OT (Herms & Mattson 1992, Stachowicz & Lindquist 2000, Lindquist 2002). Both hydrozoan extracts tested here revealed unpalatability. In the case of *Symplectoscyphus glacialis* (Sertulariidae), organic repellents are likely required as defense strategy, since the presence of nematocysts is rare. However, species of the Syntheciidae family, like *Staurotheca antarctica*, possess stinging cells (Shostak 1995); thus, the co-existence of both defenses in this species could be considered as redundant.

The ether extracts of the bryozoan and the holothurian tested did not repel *Cheirimedon femoratus* in our experiments. It has been observed that certain large-sized acanthonotozomatid and stilipedid Antarctic amphipods commonly include prey from these taxa in their diet (Coleman 1989a, 1990). However, the species analyzed here seem to possess different defensive mechanisms to avoid consumption. The sea cucumber *Peniagone vignioni*, due to its ability to move and swim, may be less vulnerable to amphipod predation, and might not require chemical defenses (Galley et al. 2008). Similarly, the bryozoan *Isochizoporella secunda* uses alternative defense strategies in the form of a calcified structure (Winston & Bernheimer 1986) and avicularia that can act as traps for small crustaceans (Carter et al. 2010), making unpalatability redundant. The extract from *Cephalodiscus nigrescens* was inactive, even phagostimulatory (Fig. 2). This pterobranch lives sheltered within a secreted reinforced encasement (Ridewood 1907), which likely already provides enough physical protection against predator attacks. The reliance on more polar defensive metabolites, however, cannot be ruled out in any of these organisms.

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 Antarctic graz-

ers (Amsler et al. 2005b). The diethyl ether extract of *D. antarctica* with its epiphyte *Geminocarpus austrogeorgiae* was not repellent in our assay, supporting the assumption that this alga combines acid sequestration with poor organic defenses (Amsler et al. 2005b); however, deterrents might occur in other fractions not tested here. *Adenocystis utricularis* yielded a palatable extract that was preferred by *Cheirimedon femoratus* compared to our controls, similar to results from feeding assays with the amphipod *Gondogeneia antarctica*, but rejection of this alga by sea stars has also been described (Amsler et al. 2005b). However, some of our results disagree with previous findings. For instance, extracts from *Palmaria decipiens* and *Gigartina skottsbergii* were rejected by *C. femoratus* in our assay, but extracts and fresh thalli from these algae have been described as palatable to other amphipods (Amsler et al. 2005b, Huang et al. 2006, Aumack et al. 2010, Bucolo et al. 2011).

In shallow Antarctic marine communities, it has been observed that macroalgal species that host dense amphipod populations in the field are usually rich in feeding deterrents, and are less preferred as prey in laboratory assays. Thus chemically defended algae often harbor high amphipod densities and likely represent refuges (Amsler et al. 2005a,b, Huang et al. 2006, 2007, Zamzow et al. 2010). In contrast to coastal zones, where direct investigations of amphipod habitats may be done by SCUBA diving (e.g. Bregazzi 1972a), determination of temporary host associations on the deeper shelf relies on indirect approaches such as behavioral observations in aquaria of living specimens collected on deep bottoms, or establishment of temporary associations of amphipod species that appear along with potential biosubstrata in sampling trawls (De Broyer et al. 2001). Similarly, our deep invertebrate samples lack information based on direct field observations related to amphipod transitory host associations, since they were collected by trawling.

In benthic ecosystems, biosubstrata represent sources of nutrition for small associated organisms, either because these feed directly on the host, or through casual (indirect) ingestion of host's tissues while foraging on attached detritus and fouling microbiota, such as diatoms. Host organisms also offer structural and/or chemical shelter from predation (Kunzmann 1996, Amsler et al. 2000, De Broyer et al. 2001, Graeve et al. 2001, Amsler et al. 2009, Zamzow et al. 2010). Indeed, amphipods associate with defended invertebrates just as they commonly do with defended algae, because these animals similarly provide chemical refuges from potential preda-

tors (Richardson 1975, Huang et al. 2007, McClintock et al. 2009, Zamzow et al. 2010). Most of the invertebrate samples tested here from the Weddell Sea and South Shetland Islands contain chemical repellents and could replace macroalgae as hosts in deeper aphotic areas with low or absent algal cover. Generalist amphipods, in particular, usually live associated with chemically defended biosubstrata (Poore et al. 2008). This is likely because their generalist diets allow them to feed on several species other than just the host, thus reducing the consumption of high concentrations of host repellents that may be unpalatable or even toxic (Sotka et al. 2009, Paul et al. 2011). Hence, even if defended potential host organisms may be ingested fortuitously while exploiting other resources *in situ* (Graeve et al. 2001), low preference for these tissues can be notable in choice-feeding preference experiments in the laboratory. Some of our results with the opportunistic *Cheirimedon femoratus* could be related to this phenomenon.

When conspecific samples displayed different palatabilities in our assays, the active one was that with the higher extract yields (like in *Anoxycalyx (Scolymastra) joubini* and *Primnoisis antarctica*), suggesting possible higher quantities of repellents. This may be due to different chemical composition of samples collected in stations located in geographical areas far apart from each other (Table 1), subjected to diverse environmental conditions and/or genetic variability (Cutignano et al. 2011). Furthermore, variability in palatability 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 reported (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 marine 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 were kept for future studies. Scavenging lysianassid amphipods have well-developed gustatory gnathopods to typify items chemically and physically while eating (Kaufmann 1994). As a result, necrophagous Antarctic amphipods, including *Cheirimedon femoratus*, are highly sensitive to food cues (Smale et al. 2007), which may explain the exceptional ability of this species to detect repelling chemicals.

The new experimental protocol proposed here evaluated the incidence of chemical defenses against

a significant Antarctic opportunistic consumer, the amphipod *Cheirimedon femoratus*. The test provided many methodological benefits: (1) requirement of small quantities of extract; (2) short experimental duration (4 h); (3) ability to assess both algal and invertebrate samples (since an omnivorous predator was used); (4) ease of interpreting results; (5) simple statistical analysis; (6) lack of heating during artificial food preparation, thus avoiding chemical damage; (7) high discriminatory ability of *C. femoratus* for unpalatable metabolites; and (8) suitability to assess chemical defense in organisms from many Antarctic locations (since a ubiquitous predator was used). Amphipods often provide excellent models for studying feeding behavior since they can be easily manipulated and maintained on artificial diets.

The high proportion of extracts that proved to be unpalatable towards *Cheirimedon femoratus*, with samples coming from a broad depth range of the Weddell Sea and South Shetland Archipelago, may reflect the importance of this amphipod as generalist consumer affecting the evolution of defenses in Antarctic communities. Moreover, this amphipod species has demonstrated to be a very suitable model predator to perform feeding experiments in future research that will enhance our understanding of the ecological interactions in the benthos inhabiting the shelf of the Southern Ocean.

Acknowledgements. 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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Editorial responsibility: James McClintock, Birmingham, Alabama, USA

Submitted: January 30, 2012; Accepted: May 23, 2012
Proofs received from author(s): August 10, 2012