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Distribution of granuloside in the Antarctic nudibranch *Charcotia granulosa* (Gastropoda: Heterobranchia: Charcotiidae)

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Abstract The loss of the shell in nudibranch gastropods has been related to the acquisition of chemical defensive strategies during evolution, such as the use of natural products to deter predation. In the present study, we investigated the origin, location, and putative role of granuloside (**1**), a homosesterterpene lactone, recently isolated from the Antarctic nudibranch *Charcotia granulosa* Vayssière, 1906. Several adults, egg masses, and its bryozoan prey, *Beania erecta* Waters, 1904, were chemically analyzed by chromatographic and spectroscopic techniques. Light- and transmission electron microscopy of the mantle revealed complex glandular structures, which might be associated with the storage of defensive compounds in analogy to mantle dermal formations described in other nudibranchs. Although preliminary in situ repellence bioassays with live specimens of the nudibranch showed avoidance against the Antarctic generalist sea star predator *Odontaster validus*, the specific role of the terpene granuloside requires further investigation. The egg masses do not present granuloside, and the glandular structures are absent in the trochophore larvae. Our results suggest that *C. granulosa* synthesizes granuloside de novo in early stages of its ontogeny, instead of obtaining it from the prey. Considering the wide geographic area inhabited by this slug, this may be advantageous, because natural products produced by the slug will not be affected by fluctuant food availability. Overall, the Antarctic sea slug *C. granulosa* seems to possess defensive strategies that are similar, in terms of production and storage, to nudibranchs from other regions of the world. This species is one of the few cladobranchs investigated so far that present de novo biosynthesis of a defensive compound.

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Introduction

Marine sea slugs are gastropod molluscs traditionally classified as opisthobranchs, although these are currently included in the monophyletic Heterobranchia (including pulmonates). Heterobranch sea slugs are excellent models to understand evolution driven by predation through the study of chemical defenses and the glandular structures involved (Wägele and Klussmann-Kolb 2005; Wägele et al. 2006; Wilson et al. 2013). Nearly all heterobranch taxa contain shelled and naked representatives, besides nudibranchs. Recent phylogenies therefore suggest that shell loss has been acquired several times during the evolution of heterobranchs (Wägele et al. 2014; Zapata et al. 2014). The loss of the shell in sea slugs promoted a panoply of defensive strategies, including the use of chemicals (Avila 1995; Cimino and Ghiselin 2009; Putz et al. 2010). Bioactive metabolites can be either sequestered from the diet (cleptochemicals) or synthesized de novo (e.g., Avila 1995; Gavagnin et al. 2001; Cimino et al. 2004; Putz et al. 2011). It has been widely shown that metabolites present in the

notum (=mantle) of nudibranchs, but not in the digestive tract, are usually involved in chemical defense (Cimino and Ghiselin 2009). Defensive natural products are frequently localized in special glandular structures on the external and most vulnerable parts of the slug (e.g., notum, rhinophores, gills, cerata), displaying anti-predatory activities (Avila and Paul 1997; Wägele et al. 2006; Carbone et al. 2013). These structures can be epidermal and subepithelial glands, or complex glandular structures (see Wägele et al. 2006). Epithelial cells are ultimately responsible for the mucus cover secreted by the slugs. Complex glandular cells, such as mantle dermal formations (MDFs) or similar structures, produce and/or accumulate chemical defenses. These can be found in nudibranchs, cephalaspideans, and sacoglossans. Cladobranch nudibranchs (i.e., with ramified digestive gland) possess terminal sacs for the excretion of digestive products. A special modification of these into cnidosacs is found in some aeolids, where nematocysts from corals are stored and extruded for defense. The strategic allocation compensates the energetic requirements invested for growth, reproduction, and defense, following the postulates of the optimal defense theory (ODT; Rhoades and Gates 1976; Avila et al. 2000; Iken et al. 2002).

Antarctic benthic invertebrates are generally preyed upon by sea stars, which are the dominant predators in shallow waters (Dayton et al. 1974). In order to test for chemical defenses, thus, in situ chemical ecology experiments have been commonly performed using the generalist feeder and ubiquitous sea star *Odontaster validus* (e.g., McClintock et al. 1994; Avila et al. 2000; Iken et al. 2002). However, only four species of Antarctic sea slugs have been chemically analyzed to date, all of them containing defensive natural products in the mantle, used against sympatric predators (McClintock and Baker 1997a; Avila et al. 2000, 2008; Iken et al. 2002; Davies-Coleman 2006). Pteroenone, a polypropionate-derived natural product from the pelagic pteropod *Clione antarctica*, displayed feeding repellence against fish predators (McClintock and Janssen 1990; Yoshida et al. 1995). De novo biosynthesis of bioactive terpene metabolites has been hypothesized for two anthobranch nudibranchs: *Bathydoris hodgsoni* and *Doris kerguelenensis*. Hodgsonal, a sesquiterpene isolated exclusively from the notum and papillae of *B. hodgsoni* (Iken et al. 1998), showed repellence against *O. validus* (Avila et al. 2000). *D. kerguelenensis* was proven to possess a variety of diterpene diacylglycerols in the notum (Gavagnin et al. 1995, 1999a, b, 2003a, b; Diyabalanage et al. 2010), some of them displaying anti-predatory activity against *O. validus* (Iken et al. 2002). These metabolites are synthesized through diverse metabolic routes with a remarkable variability among individuals (Cutignano et al. 2011). This, in combination with molecular phylogenetic analyses, led Wilson et al. (2013) to suggest cryptic speciation driven by predation in this species complex. Finally, the dendronotid *Tritoniella belli* is the only Antarctic

nudibranch investigated so far that obtains its defensive natural product from its food, the stoloniferan soft coral *Clavularia frankliniana*. This is a chimyl alcohol which also displays repellent activity against *O. validus* (McClintock et al. 1994).

Recently, we described a novel homosesterterpene lactone, granuloside (**1**), from the notum of the Antarctic nudibranch *Charcotia granulosa* Vayssière, 1906 (Cutignano et al. 2015). This species is currently assigned to Cladobranchia by having a ramified digestive gland (Wägele et al. 1995; Wägele and Willan 2000; Pola and Gosliner 2010). Cladobranchia are not well investigated yet regarding their chemical ecology. Only a few species from the genera *Melibe* and *Doto* are known to synthesize natural products themselves (see review by Putz et al. 2010, 2011). The family Charcotiidae possesses four Antarctic endemic species—mostly circum-Antarctic—of the genera *Charcotia*, *Pseudotritonia*, and *Telarma*, and one species, endemic from South Africa, of the genus *Leminda* (Wägele 1991). Within this family, only the African monotypic *Leminda millecra* Griffiths, 1985, was chemically analyzed (Pika and Faulkner 1994) and four bioactive sesquiterpenes were described. These compounds are chemically related to metabolites of the octocoral upon which the nudibranch feeds. However, the presence of different octocoral spicules in the digestive tract of *L. millecra* suggested that its diet includes a variety of prey species. This added to the evidence that the nudibranch sequesters its defensive metabolites from different octocoral species (McPhail et al. 2001). In contrast, *Pseudotritonia* and *Charcotia* appear to be specialist bryozoan feeders (Barnes and Bullough 1996). Actually, *C. granulosa*'s diet is species specific to one locally abundant bryozoan, *Beania erecta* Waters, 1904 (Barnes and Bullough 1996). *C. granulosa* was first described from a single specimen of Wandel Island in the western Antarctic Peninsula (Vayssière 1906). More recently, Wägele et al. (1995) redescribed this species from Signy Island (South Orkney Islands, Scotia Sea) including its internal anatomy.

In the present study, we investigated the chemical ecology of *C. granulosa*. We aimed to: (1) localize granuloside (**1**) in the animal tissues; (2) to chemically analyze the egg masses of *C. granulosa* and its prey *B. erecta*, to shed light into the possible origin of granuloside; (3) to describe histologically and ultrastructurally the notum and egg masses of the nudibranch; and (4) to test the feeding repellence of *C. granulosa* through in situ bioassays with the sea star *O. validus*.

Materials and methods

Collection methods

Samples were collected by scuba diving at depths between 5 and 15 m from Deception (62°59.33′S,

60°33.45′W) and Livingston (62°42.7′W, 60°23′W) Islands (South Shetland Islands), during ACTIQUIM-3 (December 2011–February 2012) and ACTIQUIM-4 cruises (December 2012–February 2013) by J. Moles and C. Avila. Additionally, one specimen from Cape Legoupil (63°19.53′S, 57°56.95′W) in the Antarctic Peninsula was collected by J. Moles in the latter campaign. *C. granulosa* specimens were usually found near the bryozoan *B. erecta*, which covered rocks and other substrates from where it was collected. Egg masses of the nudibranch were observed in February, and only a few of them were collected. Samples for chemical investigations were frozen at –20 °C until further analysis. One adult and one egg mass (Fig. 1) were preserved for both histological and cytological analyses (see below).

Histological and ultrastructural analyses

Samples for light microscopy (LM) were preserved in 4 % formaldehyde/sea water, subsequently dehydrated in ethanol and embedded in HEMA (Kulzer; see Wägele 1997). Serial sections (2.5 μm) were stained with Toluidine blue, which specifically stains acidic mucopolysaccharides red to violet, and neutral mucopolysaccharides and nucleic acids, as well as proteins, in various shades of blue. Additionally, histological slides obtained in the same way as described above of *C. granulosa*, *Pseudotritonia gracilidens* Odhner, 1944, and *Telarma antarctica* Odhner, 1934, available from one of the authors (HW) were analyzed for comparison and are discussed herein.
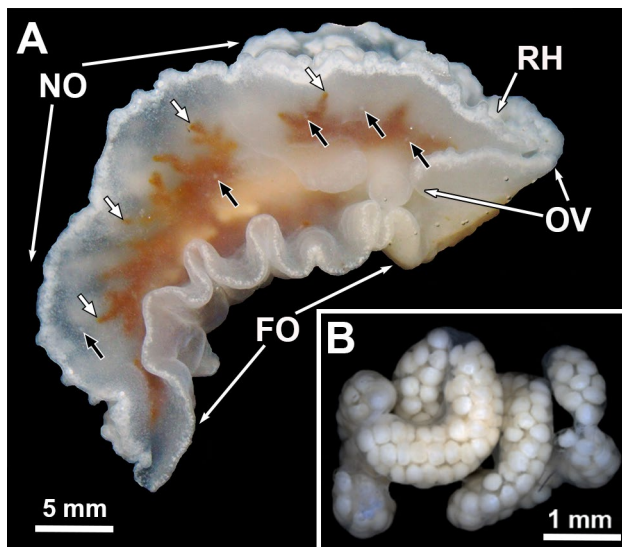

Fig. 1 In vivo photographs of *C. granulosa* collected at Whalers Bay (62°59.33′S, 60°33.45′W; Deception Island). **a** Right lateral view of an adult; *white arrows* show the end of the digestive gland ramifications, *black arrows* show MDF-like structures by transparency; **b** egg mass. *FO* foot, *NO* notum, *OV* oral veil, *RH* rhinophores

Transmission electron microscopy (TEM) was used to describe the ultrastructure of epithelial glands. Fixation of an adult and an egg mass was performed in 2.5 % glutaraldehyde in 0.2 M Millonig's phosphate buffer (MPB) and 1.4 M sodium chloride for 1 h. Samples were then rinsed with MPB for 40 min, post-fixed in 2 % osmium tetroxide in MPB, dehydrated in a graded acetone series, and embedded in Spurr's resin. Ultrathin sections obtained with an Ultracut Reichert-Jung ultramicrotome were mounted on gold grids and stained with 2 % uranyl acetate for 30 min, followed by lead citrate for 10 min (Reynolds 1963). Observations were conducted with a JEOL 1010 transmission electron microscope operating at 80 kV and fitted with a Gatan module for acquisition of digital images at the CCiT (UB).

Chemical analysis

As previously reported, 61 frozen individuals of *C. granulosa* were extracted with acetone (3 × 10 mL) by gentle ultrasound effect (Cutignano et al. 2015). The extracted specimens were later grounded with a mortar and pestle and extracted again by the same procedure. Considering that anatomical dissection of frozen animals is not suitable for this species, the extraction procedure allowed a rough approach to the compounds present in the external and the internal tissues. Two egg masses of the nudibranch from Deception and Livingston Islands were also extracted with acetone. Additionally, methanol extraction of several colonies of the nudibranch's prey, the bryozoan *B. erecta*, was performed. Tiny colonies of this bryozoan were found covering different substrates; they were combined and analyzed together. Organic fractions were evaporated *in vacuo*, and the resulting aqueous suspension was partitioned with diethyl ether (3 × 50 mL) and *n*-butanol (2 × 50 mL). The raw ether extracts were evaluated by SiO_2-TLC (thin-layer chromatography) with petroleum ether/diethyl ether (1:1) and then revealed with sulfate reagent. The organic extracts of egg masses, *C. granulosa* and *B. erecta*, were purified on a silica column using an increasing gradient of petroleum ether/diethyl ether and chloroform/methanol and compared for chemical content. Fractions were analyzed by TLC and ^{1}H-NMR spectroscopy at Servizio NMR at Istituto di Chimica Biomolecolare (ICB).

Feeding repellence assays

Twenty specimens of *O. validus*, ranging 6–10.5 cm in total diameter and collected in proximity of the target nudibranchs, were randomly used in the feeding repellence tests during the ACTIQUIM-4 cruise. Sea stars were distributed in large tanks with current sea water pumped directly from Foster's Bay into the laboratory at the Spanish Antarctic

Base "Gabriel de Castilla" (Deception Island). After 5 days of starvation, ten sea stars were placed individually in small tanks (250 mL) and one living individual of *C. granulosa* was placed under each sea star's mouth. A parallel set of sea stars, with shrimp cubes offered instead, was performed as control (see Avila et al. 2000). Consumption was evaluated after 24 h of the experiment. Statistics were calculated by contrasting the difference in ingestion rates between the living nudibranchs referred to the simultaneous control by applying the Fisher's exact test (Sokal and Rohlf 1995).

Results

Glandular structures

All investigated live animals exhibited a rather transparent epidermis, with the ramified brownish digestive gland shining through (Fig. 1a). The notum epithelium is formed by a unicellular layer of multivacuolated cells (specialized vacuolated epithelium), interspersed with two types of mucus glandular cells (Figs. 2a, 3a, b). These multivacuolated cells were prismatic in shape, had a basal nucleus, and presented microvilli all over the apical part. Cilia were seldom observed between the microvilli and might actually belong to another cell type. The mid to apical part contained abundant elongate to "sausage-like" shaped vacuoles with an electron-lucent substance. Sometimes a less electron-lucent central material (spindle) was present. Mucus gland cells with dark violet-stained contents (acid mucopolysaccharides) had granules in different degrees of condensation (Fig. 2a, f), usually being highly electron-dense (Fig. 3c, d). Secretion granules were sometimes homogeneously fused when exocytosis occurred (Fig. 2a). These mucus cells were occasionally extending subepidermally, but were strictly related to the epidermis. Additionally, cup-like macrovacuolated cells presenting a basal nucleus surrounding a huge vacuole were also present. This vacuole stained light blue (neutral mucopolysaccharides) and had a fibrillar appearance under TEM (Fig. 3b).

Special glandular structures with unusual characteristics were observed within the epidermis, but also extending far into the notum tissues. These glands resemble the MDFs described from doridoidean species and are therefore called "MDF-like" structures herein (sensu Wägele et al. 2006). A total of 65 and 71 MDF-like structures were found, mainly in the notum, in the two specimens investigated here. They were abundant in the dorsal papillae and notal edge. Additionally, they were also present in the oral veil and at the base of the rhinophores (see black arrows in Fig. 1a). MDF-like structures analyzed measured 100.2 ± 14.33 μm (mean $\pm$ SD) and were spherical in shape (Fig. 2b, d). They were composed of surrounding epithelial tissue with

cells containing a highly active nucleus (Fig. 2c, d). The surrounding epithelium presented cells full of cisternae of rough endoplasmic reticulum (RER) and vacuoles in formation (Fig. 3e, f). These cells contained a substance(s) that stained homogenously light blue (LM) or exhibited a granulose appearance under TEM. The substance(s) was stored in large vacuoles with variable electron-dense properties (Fig. 3f). Vacuoles were observed to fuse occasionally, forming larger droplets. Lipid droplets were also present within the vacuoles (Fig. 3f). Some of the MDF-like structures were observed open to the exterior, through a channel composed of epidermal cells, often with high density of mucus glandular cells (Fig. 2b, e). The content was still surrounded by a membrane when transported outside (Fig. 2b, e). The exudation channel was not observed in all MDF-like structures found.

Similarly to other Cladobranchia, the digestive gland in the family Charcotiidae is ramified (see white arrows in Fig. 1a) with terminal sacs in the tip of some of its diverticula. Terminal sacs lie subepithelially and consist of greatly enlarged cells containing very large non-staining vacuoles in *C. granulosa* (Fig. 2f) and *T. antarctica* (Fig. 2h), while *P. gracilidens* presents big cells with big bluish vacuoles (Fig. 2g). Further analysis of some histological preparations of *P. gracilidens* and *T. antarctica* revealed similar epithelial cells to those described above for *C. granulosa*, but no MDF-like structures were found there.

Egg masses of *C. granulosa* were laid during February 2012 and 2013, attached to rocks near the prey, the bryozoan *B. erecta*. They were cylindrical, capsule-filled strings, attached repeatedly along the outer mucous cover, thus conferring an irregularly arranged coiled appearance (Fig. 1b). Eggs measured 304.77 ± 17.18 μm in diameter and were surrounded by a thin membranous layer, probably albumen (Fig. 4a–c). The eggs and the albumen layer were surrounded by a compact mucoid layer, thus forming a capsule. The capsules were surrounded additionally by an outer, thin and translucent mucus layer. Both egg masses prepared for LM and TEM were in an early stage of development, i.e., trochophore larva (Fig. 4b). Several blastomeres with big nuclei were found containing abundant proteid yolk platelets, with some probably being digested (Fig. 4d, e). Mature platelets had a distinct layered cortex from a less electron-dense homogeneous central core, and some of them were aggregated (Fig. 4f). Several lipid droplets, smaller and sparser than the proteid platelets, were observed. Glucogen at different degrees of aggregation was observed widespread in the cytoplasm (Fig. 4f). Some metaphase nuclei were seen in LM. Blastomeres from the apical tuft of the larva had several flagella. Each flagellum had a centriole containing two kinetochores, a basal body, and a distinct basal plate anchored to the blastomere (Fig. 4g).

Fig. 2 Histological sections of *C. granulosa* at light microscopy (**a**–**f**), *P. gracilidens* (**g**), and *T. antarctica* (**h**). **a** Notal epithelium. **b** MDF-like structure connected to the outside through a channel (*asterisk*). **c** Possible formation of a MDF-like structure, where different secretory cells surround an internal vacuolated matrix. **d** MDF-like structure protruding its content to the outside, showing one of its surrounding cells with a large nucleus (*asterisk*). **e** Two MDF-like structures, one being released (*asterisk*). **f** Terminal sac of *C. granulosa* near the epidermis showing vacuolated cells. **g** Terminal sac of *P. gracilidens* presenting bluish-staining vacuoles (*asterisk*). **h** Detail of epithelium and terminal sac of *T. antarctica*. *dd* digestive diverticulum, *lum* lumen, *mac* macrovacuolated cell, *muc* glandular mucus cell, *smc* specialized multivacuolated cell, *vc* vacuolated cells
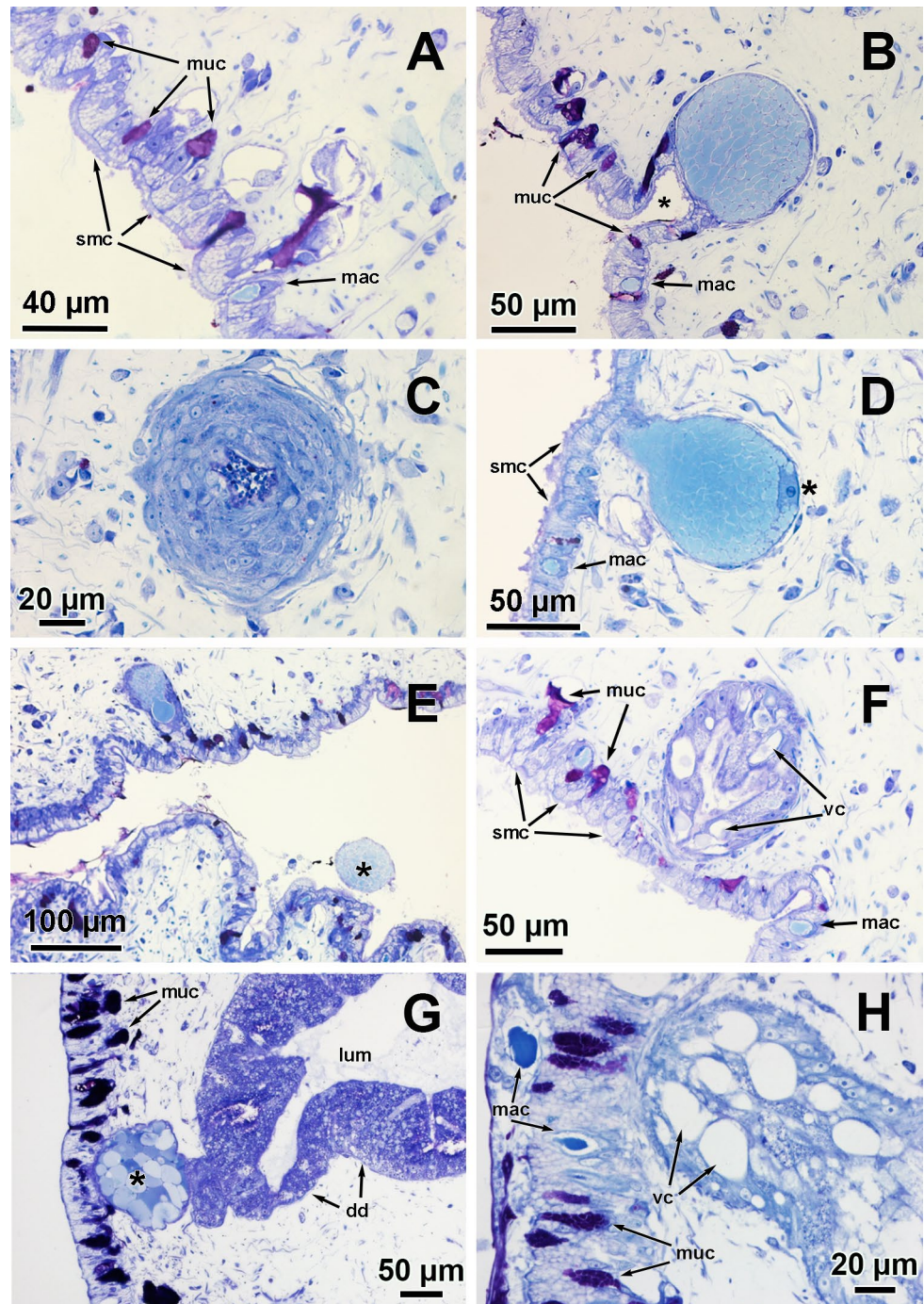
Origin and role of granuloside

Ether extracts of the "outer" and "inner" tissues of *C. granulosa*'s adult specimens, egg masses, and the prey *B. erecta* evaluated by TLC showed differences in their chemical pattern (see Fig. 5 for a schematic representation). The extract of the external part of *C. granulosa* contained granuloside (**1**), as previously reported (Cutignano 2015). The terpene **1** was absent in the "inner" organs of the animal. Using the same procedures (chromatographic purification of the ether extracts and NMR characterization of the obtained fractions), neither eggs, nor the bryozoan prey revealed the presence of granuloside or any precursor of the terpene skeleton (Fig. 6).

With regard to the feeding assays, there was no consumption of live individuals of the nudibranch *C. granulosa* by the sea star *O. validus*, whereas all shrimp food cubes were eaten in the parallel control (Fisher's exact test: p value = 0.000).
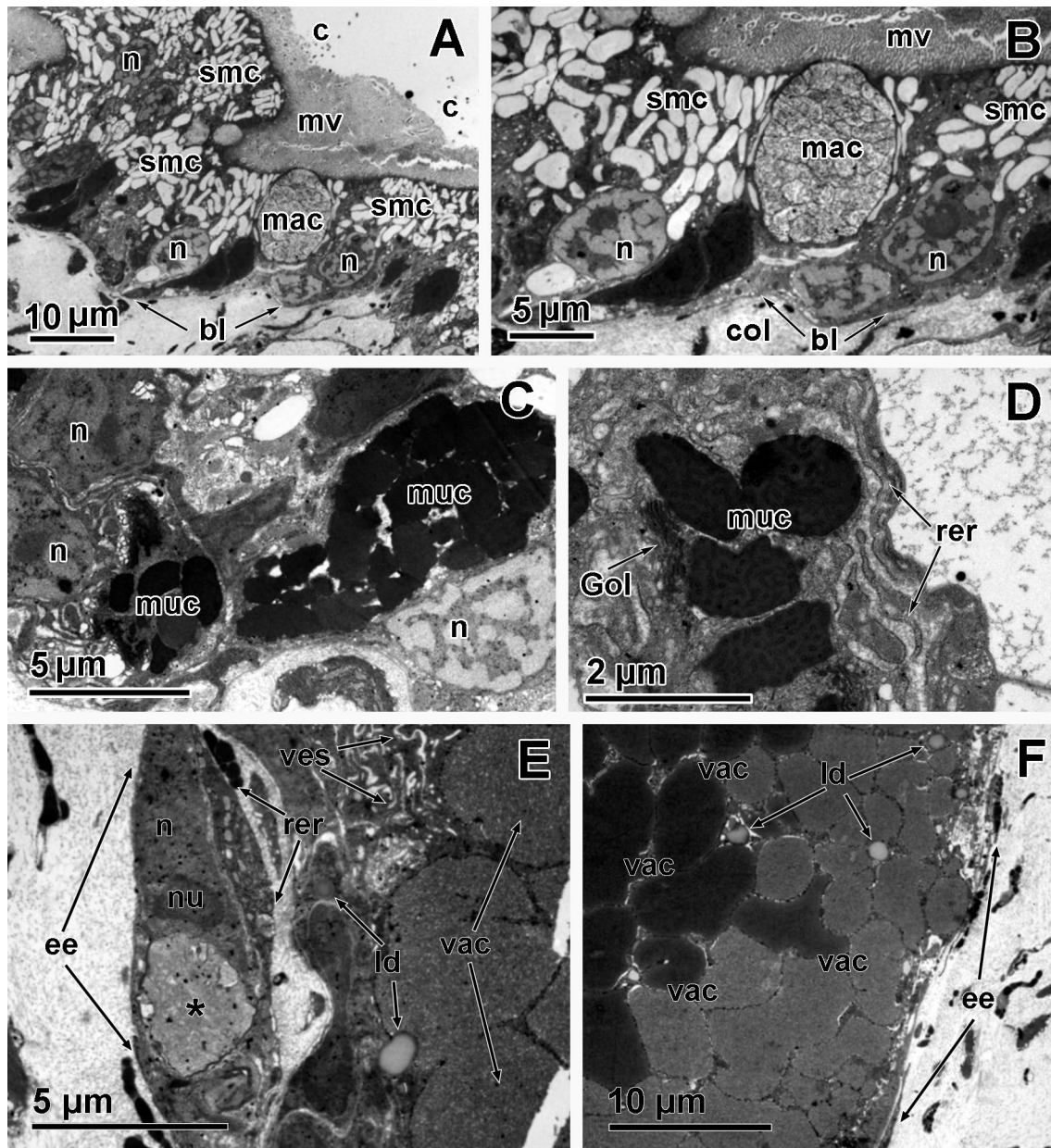
Fig. 3 Transmission electron microscopy micrographs of *C. granulosa* epithelium. **a** General view of the epithelium. **b** Close view of multivacuolated, mucus glandular, and macrovacuolated cells. **c** Detail of glandular mucus cells. **d** Internal mucus granules being produced. **e** External epithelium of the MDF-like structure, showing a vacuole in formation (*asterisk*). **f** Detail of vacuoles from a MDF-like structure, more or less electron dense. *bl* basal lamina, *c* cilia, *col* collagen, *ee* external epithelium, *Gol* Golgi apparatus, *ld* lipid droplets, *mac* macrovacuolated cell, *mv* microvilli, *muc* glandular mucus cell, *n* nucleus, *rer* rough endoplasmic reticulum, *smc* specialized multivacuolated cell, *vac* vacuoles, *ves* vesicles

Discussion

Charcotia granulosa has been recorded in Adelaide, Livingston, Signy, and Wandel Islands, as well as in the Ross Sea (Vayssière 1906; Wägele et al. 1995; Arnaud et al. 2001; Barnes and Brockington 2003; Shields et al. 2009). Our specimens were collected at Deception and Livingston Islands and at the northern part of the Antarctic Peninsula. Thus, now

its geographic distribution covers the South Orkney Islands, South Shetland Islands, and Western Antarctic Peninsula until the Ross Sea. This may indicate a circum-Antarctic distribution, related to that of its prey, the bryozoan *B. erecta* (OBIS 2014). The present study shows that *C. granulosa* is protected against the sea star *O. validus*, a predator commonly found sympatrically in shallow-water Antarctic benthic communities (Dayton et al. 1974; Moles et al. 2015a).
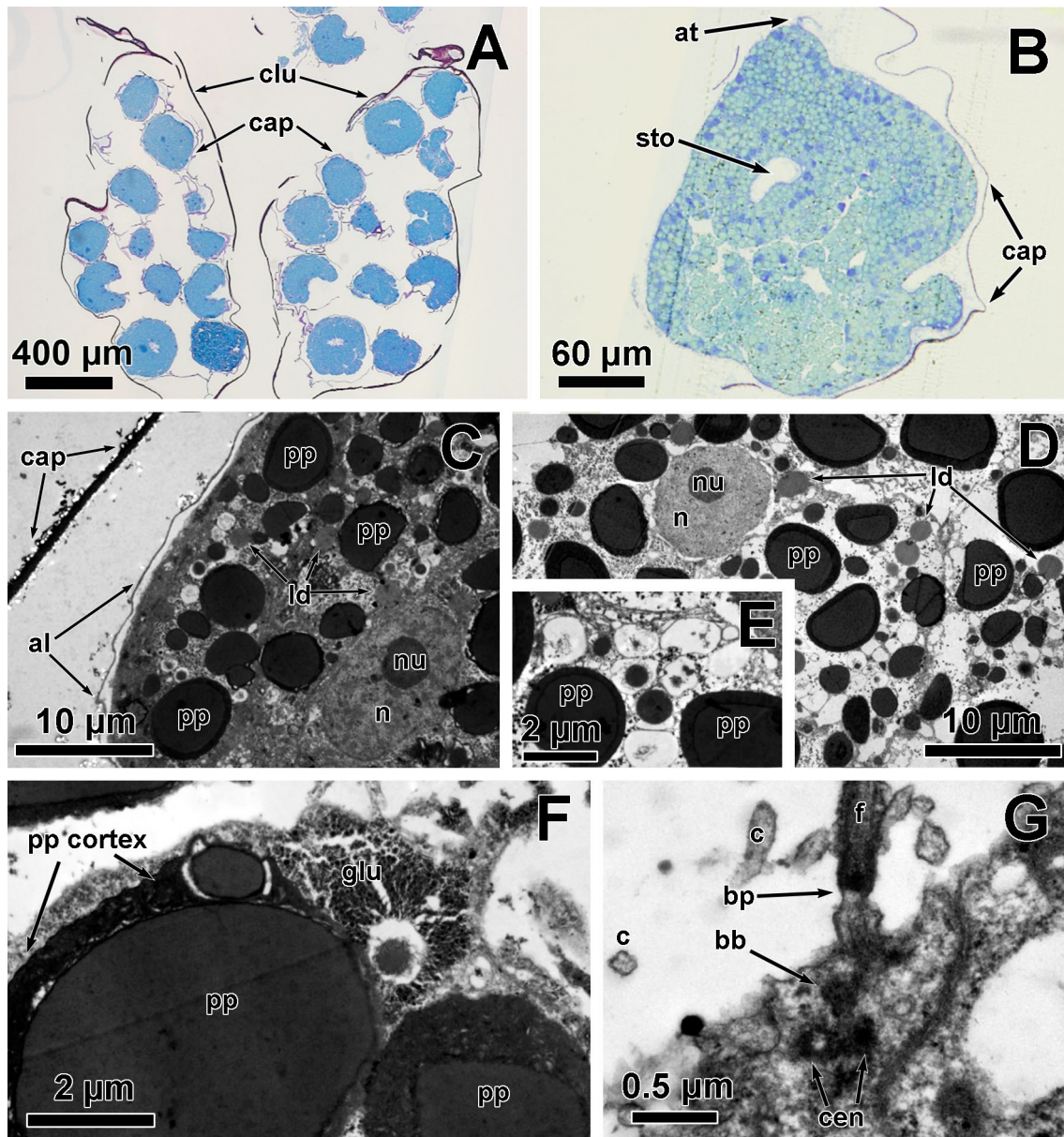
Fig. 4 Light- and transmission electron microcopy micrographs of *C. granulosa*'s egg masses. **a** Cross section of egg string showing embryos' capsule and outer clutch. **b** Trochophore larva. **c** Detail of albumen and capsule layers of trochophore. **d** Blastomere with proteid yolk platelets and lipid droplets. **e** Close-up of proteinaceous platelets in different degrees of digestion. **f** Detail of glucogen clusters inside blastomere. **g** Flagellum insertion in the apical tuft of the trochophore. *al* albumen layer, *at* apical tuft, *bb* basal body, *bp* basal plate, *c* cilia, *cap* capsule, *cen* centriole, *clu* a coil of the clutch, *f* flagellum, *glu* glucogen, *ld* lipid droplets, *nu* nucleolus, *n* nucleus, *pp* proteid platelet, *sto* stomodeum

Vacuolated cells were found throughout the epithelium of the notum and foot of *C. granulosa*, resembling the so-called specialized vacuolated epithelium found generally in nudibranchs (see Wägele 1998; Wägele and Willan 2000). Vacuolated cells were suggested to play a role in the uptake of soluble substances, especially in the digestive system, which is only found in cladobranchs (Schmekel 1982). Recent investigations showed that these cells possess an internal spindle of chitinous nature, which may act

reducing damage from cnidarian nematocyst attacks (Martin et al. 2007a). Thus, the possession of the special vacuolated epithelium in the digestive tract presumably is related to the sequestration of nematocysts in members of the Cladobranchia. As seen for other nudibranchs that do not feed on cnidarians (Wägele 1998; Martin et al. 2007b), this specialized epithelium is only developed in the most external and vulnerable parts exposed to nematocyst aggressions, but not in the digestive tract. This could be the case
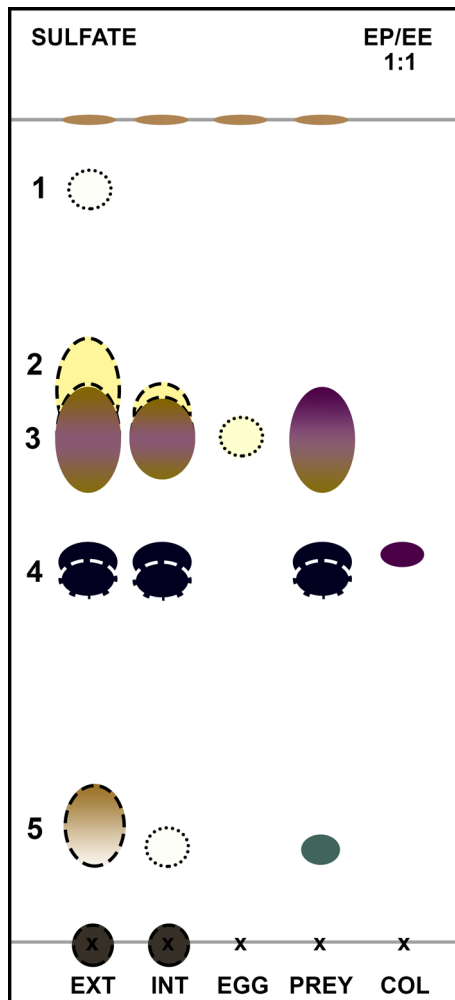
Fig. 5 Schematic diagram of the SiO$_2$-TLC comparing ether extracts of *C. granulosa*: external part (*EXT*), internal part (*INT*), egg mass (*EGG*), and its prey, the bryozoan *B. erecta*, using cholesterol (*COL*) as a reference. *Dashed lines* indicate UV–visible products. Petroleum ether/diethyl ether (1:1) was used as eluent and sulfate reagent to reveal organic spots. Main spots are as follows: *1* UV–visible uncharacterized terpene. *2* Fatty acids. *3* Sterols. *4* $\Delta^{5,7}$ sterols (UV–visible). *5* Granuloside (*brown* UV–visible)
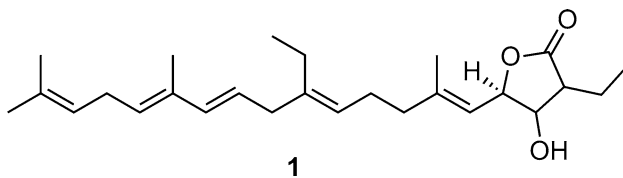

Fig. 6 Structure of granuloside (**1**)

of *C. granulosa* since it feeds on bryozoans (Barnes and Bullough 1996; authors pers. obs.). Two typical nudibranch cell types (macrovacuolated and mucus glandular cells) in the epidermis probably secrete acid and neutral mucus and

are thus responsible for the slime secreted by *C. granulosa*. A structural protection in the form of masses of intracellular grains in vacuolated epithelial cells, together with mucous secretions, may be a first physical protection in *C. granulosa* against parasites, microbes, and even cnidarian attacks (Avila and Durfort 1996; Wägele et al. 2006; Martin et al. 2007b). However, a specialist ectosymbiont copepod has recently been discovered living on the notum of *C. granulosa* (Moles et al. 2015b).

The term "MDF-like" structures, sensu Wägele et al. (2006), is used here for the glandular structures described in *C. granulosa*, because they had an opening to the outside, lacked the muscular clot and the surrounding muscular layer typical of the MDF, and because of their diameter (approximately 100 μm). MDF and MDF-like structures have been proven to store natural products for defensive purposes (e.g., García-Gómez et al. 1990; Avila et al. 1991; Avila 1995; Avila and Paul 1997). They are widely distributed in Chromodorididae, storing defensive natural products accumulated from their sponge diet (e.g., Avila et al. 1991; Fontana et al. 1994). However, Wägele et al. (2006) found these structures also in other nudibranchs, such as Dorididae and Triophinae, and even in some cephalaspideans and sacoglossans. This adds more evidence to the current hypothesis of Wägele et al. (2006), suggesting that complex glandular structures (i.e., MDF and MDF-like) may have constraints concerning structure—and therefore function—since they are found widespread in completely unrelated heterobranch taxa. The number and location of MDF-like structures in the most vulnerable parts of *C. granulosa* (i.e., rhinophores, notal edges) suggest a defensive role against predators (following the postulates of the ODT; Rhoades and Gates 1976).

Contrastingly, the charcotiids *P. gracilidens* and *T. antarctica* did not present the complex glandular MDF-like structures of *C. granulosa*, although scarce material was available. Thus, we cannot completely discard its presence; in fact the three species studied had the same type of epithelial and subepithelial singular glandular cells. However, there were clear differences concerning the terminal sacs typical of the Charcotiidae. Terminal sacs are saccular structures placed at the terminations of the diverticula of the digestive gland in some charcotiid, arminid, embletonid, and aeolid cladobranchs. The presence of terminal sacs represents an apomorphic state within Cladobranchia and is considered homologous to the Aeolidoidea cnidosacs (Wägele and Willan 2000). These authors suggested an excretory function of the terminal sacs, since they directly connect the lumen of the digestive gland to the epidermis, and they present huge vacuolar cells. In fact, dendronotacean species, such as *Hancockia*, present several small cnidosacs in each cerata which is open to the exterior to expulse nematocysts (Martin et al. 2009). In our study,

Telarma and *Charcotia* specimens presented similar terminal sacs, as mentioned above; thus, we suggest an excretory function for them. *P. gracilidens*, instead, presented huge vacuoles staining bluish. These vacuoles resemble the MDF-like structures of *C. granulosa*, but they are structurally different, and its origin is endodermal (not ectodermal like the MDF-like structures); therefore, they are not attached to the epidermis. Again, functional constraints related to the need to exude substances might have led to the similar morphology, although their developmental origin is different.

Granuloside (**1**) was isolated from the lipophilic extract of the external part (i.e., notum and foot) of the nudibranch *C. granulosa*. Since the compound was absent in the gut contents and the digestive gland, we suggest that the putative defensive homosesterterpene is not of dietary origin. Accordingly, neither terpene **1** nor any other related molecule was present in its specialist prey, the bryozoan *B. erecta*, collected together with the nudibranch. In several Antarctic bryozoans, anti-predatory strategies have been shown to vary from physical to chemical protection (Figuerola et al. 2013). In *B. erecta*, probably the huge and abundant "bird's beak" avicularia provide adequate mechanical protection (Hayward 1995). From our data, granuloside (**1**) was unequally distributed between skin and inner organs of the nudibranch and was absent in its common prey; therefore, de novo biosynthesis is suggested. Moreover, the presence of numerous RER cisternae, active nuclei, and vesicles in the surrounding epithelial tissue of the MDF-like structures provides evidence of an active synthesis. Further biosynthetic experiments with isotopic labeled precursors are needed to confirm both the de novo biosynthesis and the metabolic terpenoidic route. Although the origin of some particular secondary metabolites in some molluscs has been associated with bacterial symbionts (Davis et al. 2013; Lin et al. 2013), in our study, LM and TEM did not reveal the presence of associated bacteria in the tissue structures under study. Thus, production of granuloside by symbiont partnership seems not to be supported. Finally, further bioassays with the isolated compound should determine whether granuloside is ultimately responsible for the chemical repellence in *C. granulosa* against *O. validus*. However, the chemical lability of the molecule will make the ecological assays difficult.

Some previous studies demonstrated that egg masses and embryos of some invertebrates were chemically protected (McClintock and Baker 1997b; Benkendorff et al. 2001). Here, the egg masses analyzed did not present granuloside or any related terpene, neither the specialized structures supposed to store the chemicals. The high number of proteinaceous platelets in the trochophore larva suggests a provision of food for postembryonic development, after the veliger stage (Morrill 1964). The rather thin mucus layers of the egg masses, which probably are degraded much faster than those of, for example, the sympatrically occurring *D. kerguelenensis* (Wägele 1989, 1996), also indicate a rather short developmental time within the egg clutches. Thus, after a relatively short intracapsular period of time, *C. granulosa* juveniles probably hatch anatomically complete, except for the reproductive system. It seems that the egg clutches of *C. granulosa* provide enough physical protection at this stage, and subsequent juvenile stages of the nudibranch may further develop MDF-like structures and produce granuloside, as described for MDFs in chromodorid nudibranchs (Wägele et al. 2006).

The present study on the Antarctic nudibranch *C. granulosa* is a multidisciplinary approach to chemical ecology with microscopical, ultrastructural, ecological, and chemical methods. *C. granulosa* from Antarctica offers evidence of synthesizing and delivering of natural products as a defensive strategy against the sea star *O. validus*. We suggest a non-dietary origin of the homosesterterpene granuloside in this charcotiid species, which is likely de novo biosynthesized in early juvenile stages. Our findings together with literature data indicate that, additional to Dotidae and Tethydidae, Charcotiidae is the third cladobranch family where members seem to rely on de novo biosynthesis of natural compounds (Putz et al. 2010, 2011). De novo biosynthesis allows the species to be independent from diet for obtaining their defensive compounds. In addition, we provide the first description of *C. granulosa* spawn, showing that egg masses do not contain granuloside. A physical protection of the clutch together with a fast development is assumed to be the strategy to protect early intracapsular development, reducing the exposition time to predators.

Although single glandular cells are commonly found and widespread in the family, no evidence of MDF-like structures has been found so far in members of the genera *Pseudotritonia* and *Telarma*. Further histological analyses as well as chemical studies are needed to unravel the relationships and life strategies among congeners of Charcotiidae; also, further biosynthetic studies with stable-labeled precursors could provide indication about the origin of the linear homosesterterpene **1** in the nudibranch *C. granulosa*.

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