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ORIGINAL PAPER

Feeding repellence in Antarctic bryozoans

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Abstract The Antarctic sea star *Odontaster validus* and the amphipod *Cheirimedon femoratus* are important predators in benthic communities. Some bryozoans are part of the diet of the asteroid and represent both potential host biosubstrata and prey for this omnivorous lysianassid amphipod. In response to such ecological pressure, bryozoans are expected to develop strategies to deter potential predators, ranging from physical to chemical mechanisms. However, the chemical ecology of Antarctic bryozoans has been scarcely studied. In this study we evaluated the presence of defenses against predation in selected species of Antarctic bryozoans. The sympatric omnivorous consumers *O. validus* and *C. femoratus* were selected to perform feeding assays with 16 ether extracts (EE) and 16 butanol extracts (BE) obtained from 16 samples that belonged to 13 different bryozoan species. Most species (9) were active (12 EE and 1 BE) in sea star bioassays. Only 1 BE displayed repellence, indicating that repellents against the sea star are mainly lipophilic. Repellence toward *C. femoratus* was found in all species in different extracts (10 EE and 12 BE), suggesting that defenses against the amphipod might be both lipophilic and hydrophilic. Interspecific and intraspecific variability of bioactivity was occasionally detected, suggesting possible environmental inductive responses, symbiotic associations, and/or genetic variability. Multivariate analysis revealed similarities among species in relation to bioactivities of EE and/or BE. These findings support the hypothesis that, while in some cases alternative chemical or physical mechanisms may also provide protection, repellent compounds play an important role in Antarctic bryozoans as defenses against predators.

Keywords *Odontaster validus* · *Cheirimedon femoratus* · Chemical ecology · Chemical defense · Deception Island

Introduction

The continental shelf of the eastern Weddell Sea and other Antarctic regions are characterized by presence of diverse, well-structured benthic communities, dominated by eurybathic suspension feeders such as sponges, gorgonians, bryozoans, and ascidians (Dayton et al. 1974; Teixidó et al. 2002; Figuerola et al. 2012a). The establishment of the Antarctic Circumpolar Current led to the isolation of the Antarctic continent, approximately 20 million years ago, allowing benthic species to co-evolve in habitats under low and relatively stable temperatures, and extreme seasonality of primary production (Clarke 1992; Arntz et al. 1997; Clarke et al. 2004; Dayton et al. 1994). Antarctic marine benthic communities below the limit of anchor ice and ice scour (300- to 400-m depth) are influenced by intense biotic interactions (Dayton et al. 1974; Orejas et al. 2000), which constitute important driving forces in controlling population structure (Pawlik 2012). Such ecological relationships favor the evolution of chemical defenses to avoid predation, competition (either for food or space), and/or fouling, producing a complex web of chemically mediated ecological interactions (Avila et al. 2008; McClintock et al. 2010; Figuerola et al. 2012b).

Generalist echinoderm and crustacean predators occupy high trophic levels in Southern Ocean communities (Dearborn et al. 1983; Huang et al. 2007). Antarctic echinoderms are the dominant mobile megafaunal taxa in terms of abundance and diversity, showing a high diversity along the shelf, slope, and deep-sea plains (Brandt et al. 2007). In

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particular, the common omnivorous sea star *Odontaster validus* (Koehler, 1906), which has a varied diet, drives intense predation events (Dayton et al. 1974; McClintock et al. 2010). This ubiquitous eurybathic sea star, found all around the Southern Pole down to 2,900 m depth, has been chosen previously as a model generalist predator to conduct feeding repellence bioassays using different methodologies (McClintock et al. 1992, 1993; McClintock 1994; Slattery and McClintock 1995; McClintock and Baker 1997; Avila et al. 2000; Mahon et al. 2003; Peters et al. 2009; Núñez-Pons et al. 2012a; Taboada et al. 2013; see also http://iobis.org/mapper/). Consistent with the high predation pressure exerted by this keystone asteroid, recent studies have demonstrated the presence of feeding repellents in crude organic extracts of most taxonomic groups of Antarctic invertebrates (Avila et al. 2008; McClintock et al. 2010; Figuerola et al. 2012b; Taboada et al. 2013).

A high diversity (estimated at more than 600 species) and huge densities of opportunistic crustacean amphipods are found on the sea bottom of the eastern shelf of the Weddell Sea, while the diversity of scavenger species in the continental slope (1,000–3,000 m) appears to be richer than elsewhere in the world (Dauby et al. 2001b; Brandt et al. 2007; Huang et al. 2007; De Broyer et al. 2011). One of the causes of this abundance and success is related to their ability to switch feeding strategies, ranging from suspension-feeding to scavenging, depending on food availability (Dauby et al. 2001b). Many gammaridean species from the Weddell Sea show a broad diet spectrum on benthic invertebrates, such as bryozoans, cnidarians and sponges, suggesting a lack of prey specialization (Dauby et al. 2001b). These sessile invertebrates, besides representing potential prey for amphipods, can also be potential host biosubstrata, offering a large variety of microhabitats and thus available structural and chemical refuges from predation, mainly by fish and starfish (Oshel and Steele 1985; De Broyer et al. 1999, 2001; Dauby et al. 2001a; Huang et al. 2008). *Cheirimedon femoratus* (Pfeffer, 1888; Gammaridea: Lysianassidae) is an opportunistic omnivore with a circumantarctic and eurybathic distribution (from 0 to 1,500 m depth), frequently found associated with macroinvertebrates (De Broyer et al. 2007; Krapp et al. 2008). This scavenger amphipod is described as a generalist feeder, preying upon invertebrates, algae and detritus (Bregazzi 1972), and it has been recently used as a model consumer in feeding repellence assays (Núñez-Pons et al. 2012b).

In the Southern Ocean, Bryozoa is a rich and varied faunal group, with many new species described in recent years (Kuklinski and Barnes 2009; Hayward and Winston 2011; Figuerola et al. 2013). Antarctic bryozoans are often characterized by having circumpolar distributions, broad bathymetric ranges, and for showing high specific richness compared to tropical areas, e.g., Hawaii (Hayward 1995; Clarke and Johnston 2003; Figuerola et al. 2012a). The most successful living order of bryozoans is the Cheilostomatida, which shows a high proportion of endemism (56 %) in Antarctic waters (Barnes et al. 2009; Griffiths 2010). Cheilostomatida are characterized by box-like zooids, reinforced with calcium carbonate skeletons, and an operculum protecting the orifice through which the animal extends its ring of tentacles (lophophore; Hayward 1995). Most cheilostomes have developed a high degree of zooidal polymorphism, the polymorphs specialized in different tasks including feeding (autozooids), cleaning, and protecting colonies (e.g., avicularia and vibracula) and reproduction (ovicells) (Winston 2009). Moreover, bryozoans are known to produce natural products, such as alkaloids and terpenoids (hydrophilic and lipophilic, respectively), with deterrent properties including antifouling and antipredation (Al-Ogily and Knight Jones 1977; Lopanik et al. 2004; Gray et al. 2005; Sharp et al. 2007). Thus, bryozoan colonies represent convenient model systems for studying diverse defensive strategies. Until now, the global bryozoan species richness is quite elevated, 5869 spp. (Bock and Gordon 2013); however, their role in benthic communities focused on their natural products has been understudied (Sharp et al. 2007; Blunt et al. 2012). In particular, the chemical ecology of Antarctic bryozoans remains poorly studied, even though they are a rich and diverse phylum (Winston and Bernheimer 1986; Lebar et al. 2007; Figuerola et al. 2012b; Taboada et al. 2013; unpublished results from the authors).

In order to establish the role of bryozoan natural products from the Weddell Sea area, the sea star *O. validus* and the amphipod *C. femoratus* were selected as model predators to conduct feeding repellence assays that aimed to: (1) evaluate the presence of natural products that may serve as repellents against these relevant Antarctic predators, and (2) compare the intraspecific and interspecific variability of bioactivity in selected species. Moreover, we discussed the possible protection provided by the different potential defensive systems, both chemical and physical, in Antarctic bryozoan colonies.

Material and methods

Collection and identification of bryozoan samples

Antarctic bryozoan samples (16) belonging to 13 different species were collected between 273.6 and 351.6 m depth during the ANT XXI/2 (November 2003–January 2004) cruise on board the R/V Polarstern, from the Alfred Wegener Institute for Polar and Marine Research (Bremerhaven, Germany). Samples were collected using Agassiz Trawl and Bottom Trawl in the eastern Weddell Sea, Antarctica (Table 1). Each bryozoan sample comprised several colonies obtained from each collecting station. Bryozoan colonies were sorted on deck, photographed, and a voucher portion of each was

Table 1 Sample collection data, skeleton, defensive structures, and bioactivity of ether and butanol extracts from Antarctic bryozoans

Species	Latitude (S)	Longitude (W)	Depth (m)	Skeleton	Defensive structures	Feeding repellence		Feeding preference	
						EE	BE	EE	BE
Bostrychopora dentata (Waters, 1904)	70°57.00′	10°31.61′	284.4	Rigid	Paired, lateral oral avicularia; one or more avicularia on frontal wall	++	-	+	++
Camptoplites angustus (Kluge, 1914) (1)	70°50.75′	10°28.01′	281.2	Flexible	Three kinds of bird's head avicularia, pediculate; autozooid with two large spines	++	-	-	++
C. angustus (Kluge, 1914) (2)	70°50.78′	10°28.51′	273.6	Flexible	Three kinds of bird's head avicularia, pediculate, autozooid with two large spines	+++	+	-	+++
Camptoplites bicornis (Busk, 1884)	70°56.83′	10°32.61′	338	Flexible	Four kinds of bird's head avicularia, pediculate; autozooid with two short spines	++	-	-	++
Camptoplites tricornis (Waters, 1904) (1)	70°56.67′	10°32.05′	302.4	Flexible	Four kinds of bird's head avicularia, pediculate; autozooid with three large spines	+++	-	-	+++
C. tricornis (Waters, 1904) (2)	70°57.33′	10°33.86′	351.6	Flexible	Four kinds of bird's head avicularia, pediculate; autozooid with three large spines	+	-	++	-
Dakariella dabrowni (Rogick, 1956d)	70°57.00′	10°33.02′	332.8	Rigid	Adventitious avicularia	-	-	++	-
Isoschizoporella secunda Hayward and Taylor, 1984	71°06.44′	11°27.76′	277.2	Rigid	Suboral avicularium	+	-	-	++
Isosecuriflustra tenuis (Kluge, 1914)	70°52.75′	10°51.24′	294.8	Flexible	Avicularia along branch margins	+	-	-	+
Klugella echinata (Kluge, 1914)	70°56.83′	10°32.61′	338	Flexible	Avicularia at proximal end of autozooid; autozooid with three to nine pairs of spines	++	-	+++	-
Melicerita obliqua (Thornely, 1924)	71°06.44′	11°27.76′	277.2	Rigid	-	+	-	+	++
Nematoflustra flagellata (Waters, 1904)	70°56.42′	10°31.61′	284.4	Flexible	Vibraculum at the distal end of autozooid	-	-	++	++
Notoplites drygalskii (Kluge, 1914) (1)	70°57.11′	10°33.32′	337.2	Flexible	Two kinds of avicularia; autozooid with spine at distal end only to bifurcation of colony	+	-	++	++
N. drygalskii (Kluge, 1914) (2)	71°04.30′	11°33.92′	308.8	Flexible	Two kinds of avicularia; autozooid with spine at distal end only to bifurcation of colony	+	-	++	+
Smittina Antarctica (Waters, 1904)	71°06.44′	11°27.76′	277.2	Rigid	Suboral avicularium	-	-	++	-
Systenopora contracta Waters, 1904	71°06.44′	11°27.76′	277.2	Rigid	Two kinds of avicularia	-	-	+++	++

Results of three ether extracts (EE) of *B. dentata*, *I. secunda*, and *I. tenuis* come from Taboada et al. (2013). Colonies were defined here as flexible (weakly calcified: capable of being bent) or rigid (heavily calcified: resistant to the deformation). Feeding repellence: tests using *Odontaster validus*. Feeding preference: tests using *Cheirimedon femoratus*

EE ether extract, *BE* butanol extract

Significance: + $p<0.05$, ++ $p<0.005$, +++ $p<0.0001$, - not significant

fixed in 70 % ethanol for further taxonomic studies at the Faculty of Biology, University of Barcelona. Hayward's (1995) guide was used for species identification, and a detailed description of structural defensive devices was made by microscopical observations. All colonies were stored at −20 °C for further chemical ecology experiments.

Collection of experimental sea star and amphipod predators

Living individuals of the voracious eurybathic Antarctic sea star *O. validus* were sampled at Port Foster Bay in Deception Island, South Shetland Islands (62°59.369′S, 60°33.424′W), during three campaigns: ACTIQUIM-1 (December 2008–January 2009), ACTIQUIM-2 (January 2010), and ACTIQUIM-3 (January–February 2012). Hundreds of sea stars were collected by scuba diving on sea bottoms ranging from 3- to 15-m depth, and of the amphipod *C. femoratus* between 2- and 7-m depth by scuba diving using fishing nets, along the coastline of the Antarctic Spanish Base (BAE) during the ACTIQUIM-3 campaign (January 2012).

Chemical extractions of bryozoan samples

Bryozoan colonies were extracted with acetone at room temperature by using a mortar and pestle and then sequentially partitioned into ether extracts (EE) and butanol extracts (BE).

A water residue was also obtained and conserved frozen for further study. All fractionation steps were repeated three times, except for the butanol partition, which was done only once. Organic solvents were evaporated under reduced pressure, resulting in dry EE and BE, and aqueous residues. EE (comprising the most apolar lipophilic metabolites) and BE (polar hydrophilic metabolites) were used for bioassays at their corresponding natural concentrations, according to dry weight calculations (see below).

Physical defenses versus chemical defenses

The total number of polymorphs (spines, avicularia, and/or vibracula) per cm^2 was counted in three different portions of colonies for 13 species, and the mean value for each was considered for the analysis. With the purpose of knowing if a relationship exists between the density of physical defenses and the level of the repellent activity of each species for the two experiments, the Pearson correlation coefficient was calculated. The repellent activity was here referred as the number of active extracts in the two experiments, ranging from 0 active extracts to 4 (EE and BE in the two experiments).

Feeding repellence assays with the asteroid *O. validus*

The common omnivorous sea star *O. validus* has been already used in feeding repellence studies by our group as a generalist model Antarctic predator (e.g., Avila et al. 2000; Núñez-Pons et al. 2012a; Taboada et al. 2013). Sea stars, 6.5–10.5 cm in diameter, were acclimated for 5 days in large tanks, with seawater pumped directly from the sea, before being used in the feeding repellence assays. EE and BE from Antarctic bryozoans were diluted into diethyl ether and methanol, respectively, and then coated uniformly onto shrimp-based food items until the solvent had evaporated, following the methodology previously described (Avila et al. 2000; Taboada et al. 2013). Sea stars were individually accommodated in 2.5 l tanks, and later shrimp cubes (5×5×5 mm, 13.09±3.43 mg of dry weight) with or without extract were offered (10 replicates each test). Control feeding shrimp cubes were only treated with solvent. The natural concentration was calculated as in previous studies by our group (e.g., Núñez-Pons et al. 2012b), by dividing dry ether or butanol partitions (EE or BE) by the total dry weight (TDW=DW+EE+BE, where DW is the dry weight of the solid remains of the extracted sample). We used dry weight for our calculations because this parameter avoids deviations related to water content, which is very variable in aquatic samples. After 24 h, the number of eaten food units was recorded for each test, and uneaten items were frozen. Thin layer chromatography (TLC) of the remaining food cubes was used subsequently to demonstrate the permanence of the compounds on the shrimp cubes. Feeding repellence was statistically evaluated by using Fisher's exact test for each experiment referred to the simultaneous control (Sokal and Rohlf 1995). After the experiments, the sea stars were brought back alive to the sea.

Feeding preference assays with the amphipod *C. femoratus*

The ubiquitous and opportunistic amphipod *C. femoratus* (Bregazzi 1972; De Broyer et al. 2007) was chosen for our experiments, following the protocol recently described by Núñez-Pons and coauthors (2012b). Artificial caviar-textured food items 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 alginate solution with a drop of green or red food coloring (see below), and introduced into a syringe. The mixture was then added dropwise into an aqueous 0.09 M (1 %) $CaCl_2$ solution, where it gelatinized to form spheroidal pearls, approximately 2.5 mm in diameter. For treatment pearls, extracts were dissolved in a minimum volume of extract to totally wet the powdered food and the solvent was evaporated, resulting in a uniform coating of extract prior to being added into the alginate aqueous mixture. The relative quantity of each extract was calculated according to the natural concentration in a dry weight basis attending to the explanations exposed above. Control pearls were prepared similarly but with solvent alone. Alive organisms were maintained in large 8 l aquariums and were starved for 3–5 days. Each assay consisted of 15 replicate containers filled with 500 ml of seawater and 15 amphipods each, which were offered a simultaneous choice of 10 treatment and 10 control extract-free pearls of different color (20 food pearls in total: 10 control and 10 extract-treated), green or red. The colors for treatment and control pearls were randomly switched throughout the experiment; furthermore, previous trials had confirmed the null effect of different color on feeding preferences ($p=0.47$, n.s.). The assays ended when approximately 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 one eighth of its original size. Finally, statistics were calculated to determine feeding preference of extract-treated pearls relative to the paired extract-free controls to establish repellent activities. Each replicate was represented by a paired result yielding two sets of data (treatments and controls). Since assumptions of normality and homogeneity of variances were not met, our data were compared using nonparametric procedures by applying the Exact Wilcoxon test with R-command software. Uneaten treatment food pearls were preserved for extraction and analyzed 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.

Comparison of activity between species

Nonmetric multidimensional scaling (NMDS) ordination analyses were performed using Vegan software (R version 2.15.2) in order to assess the similarities of bryozoan species in relation to: (a) the type of active extract (EE and/or BE) and (b) the type of feeding repellence (sea star and/or amphipod). Binary data (active/inactive) were used to make the distance matrix using the Bray–Curtis similarity index. The Bray–Curtis index was chosen as it is among the most widely employed indices, being equivalent to the Sörensen index for presence/absence matrices (Clarke et al. 2006; Legendre and Legendre 2012). The resulting similarity matrix was analyzed by NMDS, where the first two dimensions were plotted. The distance between dots indicates their similarity measured by stress values. A stress value of less than 0.1 indicates that the plot accurately represents similarities, while a stress value greater than 0.3 indicates that the points are close to being randomly placed (Clarke 1993). In order to verify statistical support in the defined groups, we performed an analysis of similarity (ANOSIM, Global *R* statistic), which does not require normal distribution data. The ANOSIM randomization test compares the within and between group similarity of elements measured by the Bray–Curtis index and calculates a global *R* statitistic. The resulting *R* value ranges between 0 and 1, with high values indicating a large degree of discrimination among groups (Clarke and Green 1988).

Results

Bryozoan species collected showed different levels of skeletal calcification, diversity in zooid density, and zooid types (spines, avicularia, and vibracula; Fig. 1). Colonies were then classified as flexible (weakly calcified, e.g., *Klugella echinata*) or rigid (heavily calcified, e.g., *Bostrychopora dentata*). A total of 16 EE and 16 BE obtained from 16 bryozoan samples, representing 13 species with rigid or flexible skeletons and several defensive structures, were tested at their natural concentration in both types of feeding assays. Our study demonstrates that all species tested exhibit some kind of repellent activity against the sea star *O. validus* and/or the amphipod *C. femoratus* (Table 1). All species of the genera *Camptoplites* and *Notoplites* exhibited repellent compounds against both predators. Their repellent defenses were found in both EE and BE, indicating the presence of defensive compounds in both lipophilic and hydrophilic extracts. However, some species did not show activity in all extracts (EE and BE). In the case of *Camptoplites tricornis* and *K. echinata* only EE displayed activity in both experiments.

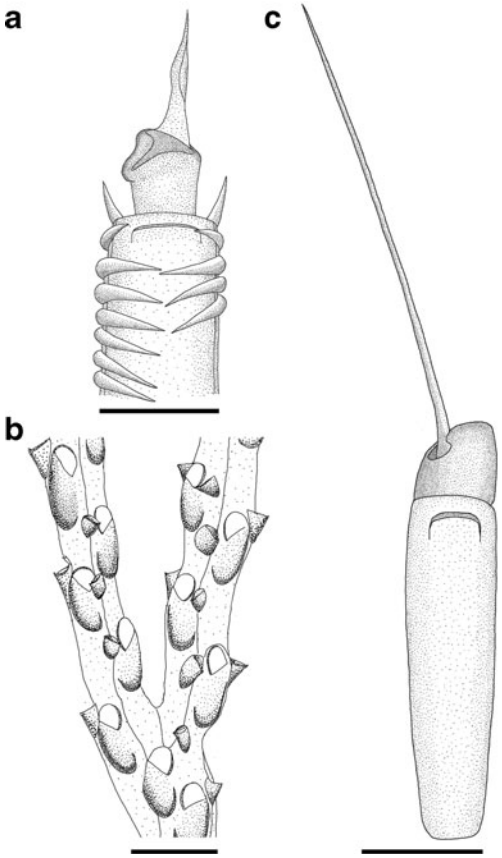

Fig. 1 Presumed defensive structures of some Antarctic bryozoans. **a** Autozooid of *Klugella echinata* with frontal membrane overarched by a variable number of marginal spines, and with columnar avicularium situated at the proximal end. **b** Sessile avicularia, with sharply hooked mandible, in a part of a branched colony of *Notoplies drygalskii*. **c** Avicularium (vibraculum) with a whip-like seta situated at the distal end of autozooid of *Nematoflustra flagellata*. Scale bar: 0.5 mm

Physical defenses versus chemical defenses

Bryozoan colonies with diverse levels of flexibility and/or defensive structures show different mechanical and/or chemical defensive strategies (Table 1). In this sense, most of the species tested may employ a variety of defensive mechanisms. *Dakariella dabrowni*, *Smittina antarctica*, and *Systenopora contracta*, with rigid, well-calcified skeleton, did not show deterrent activity toward the asteroid *O. validus*. *Melicerita obliqua*, a species without physical defensive structures and without calcified frontal shield (anascan cheilostome), showed repellent activity in both experiments. However, considering all the studied species, no correlation existed between the number (density) of polymorphs (spines, avicularia, and vibracula) and the chemical defenses ($r=0.427$, $p=0.099$; Fig. 2).

Feeding repellence assays with the asteroid *O. validus*

O. validus rejected 9 of the 13 bryozoan species (69 %) that were presented to them as EE, indicating the presence of

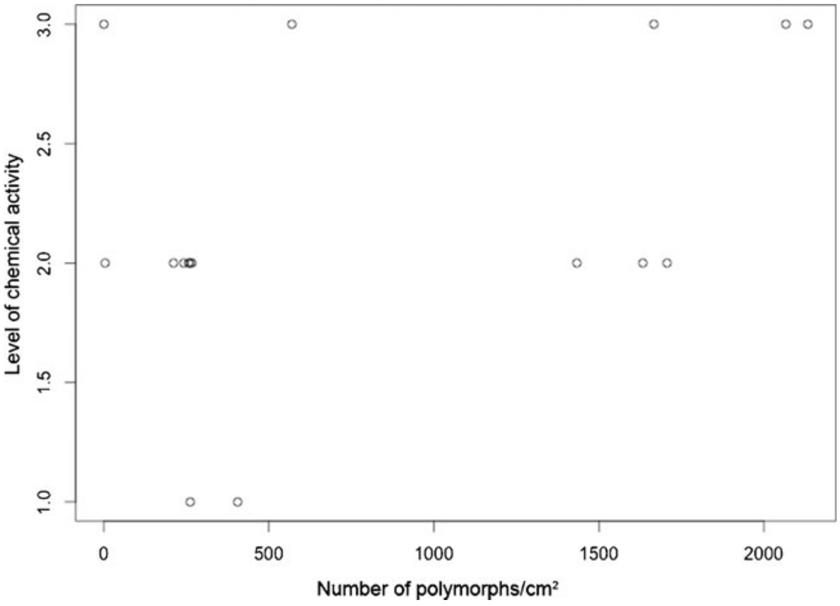

Fig. 2 Relationship between physical (spines, avicularia, and/or vibracula) and level of chemical defenses (referred to active extracts of each sample for the two experiments) for 16 samples ($r = 0.427$, $p = 0.099$)

lipophilic defensive metabolites (Fig. 3). In contrast, only 1 BE from the sample *Camptoplites angustus* (2) was repellent to the sea star. The flexible bryozoan *Nematoflustra flagellata* and the rigid bryozoans *D. dabrowni*, *S. antarctica*, and *S. contracta* did not show any activity. Intraspecific and interspecific variability was found in the genus *Camptoplites*, even when the samples came from relatively close areas and similar depths.

Feeding preference assays with the amphipod *C. femoratus*

All bryozoan species tested caused repellence to the amphipod, either with both EE and BE, or with one of them (Fig. 4). Actually, most of the lipophilic (62 %) and hydrophilic extracts (75 %) repelled *C. femoratus*, and six species displayed activity in both extracts. Interspecific variability was found again in the genus *Camptoplites*, whereas intraspecific differences were detected in *C. angustus*, *C. tricornis*, and *Notoplites drygalski*. Colonies of the genus *Camptoplites* showed repellence only in the hydrophilic extracts except for *C. tricornis* (2).

Comparison of activity between species

Comparing both tests, four species were the most active: *B. dentata*, *C. angustus* (2), *M. obliqua*, *N. drygalskii* (1) and

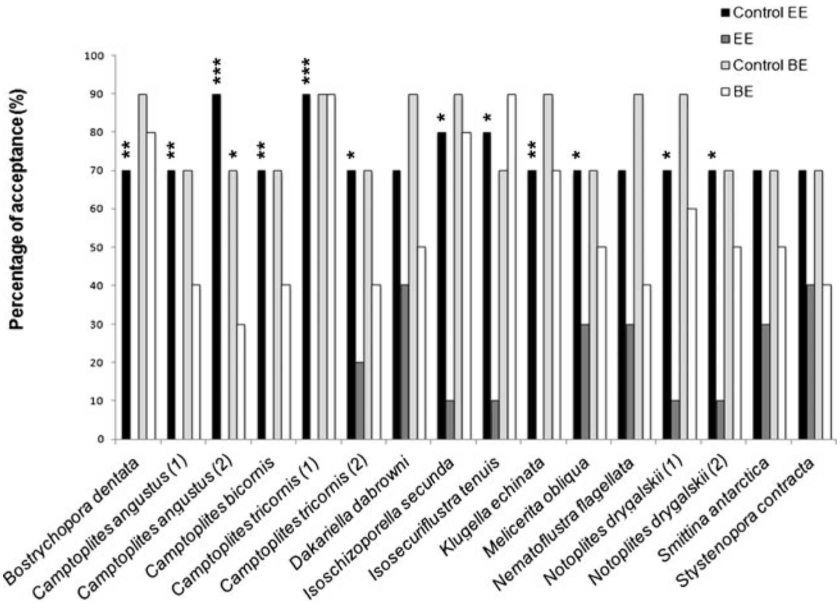

Fig. 3 Feeding repellence assays, with ether (*EE*) and butanol (*BE*) extracts from Antarctic bryozoans against the sea star *Odontaster validus*, showing the paired results of control- and extract-treated shrimp cubes for each test (10 replicates for each test), expressed as the percentage of acceptance. Significant differences: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$ with control as the preferred food (Fisher's exact test). The results of the EE of *B. dentata*, *I. secunda*, and *I. tenuis* come from Taboada et al. (2013)

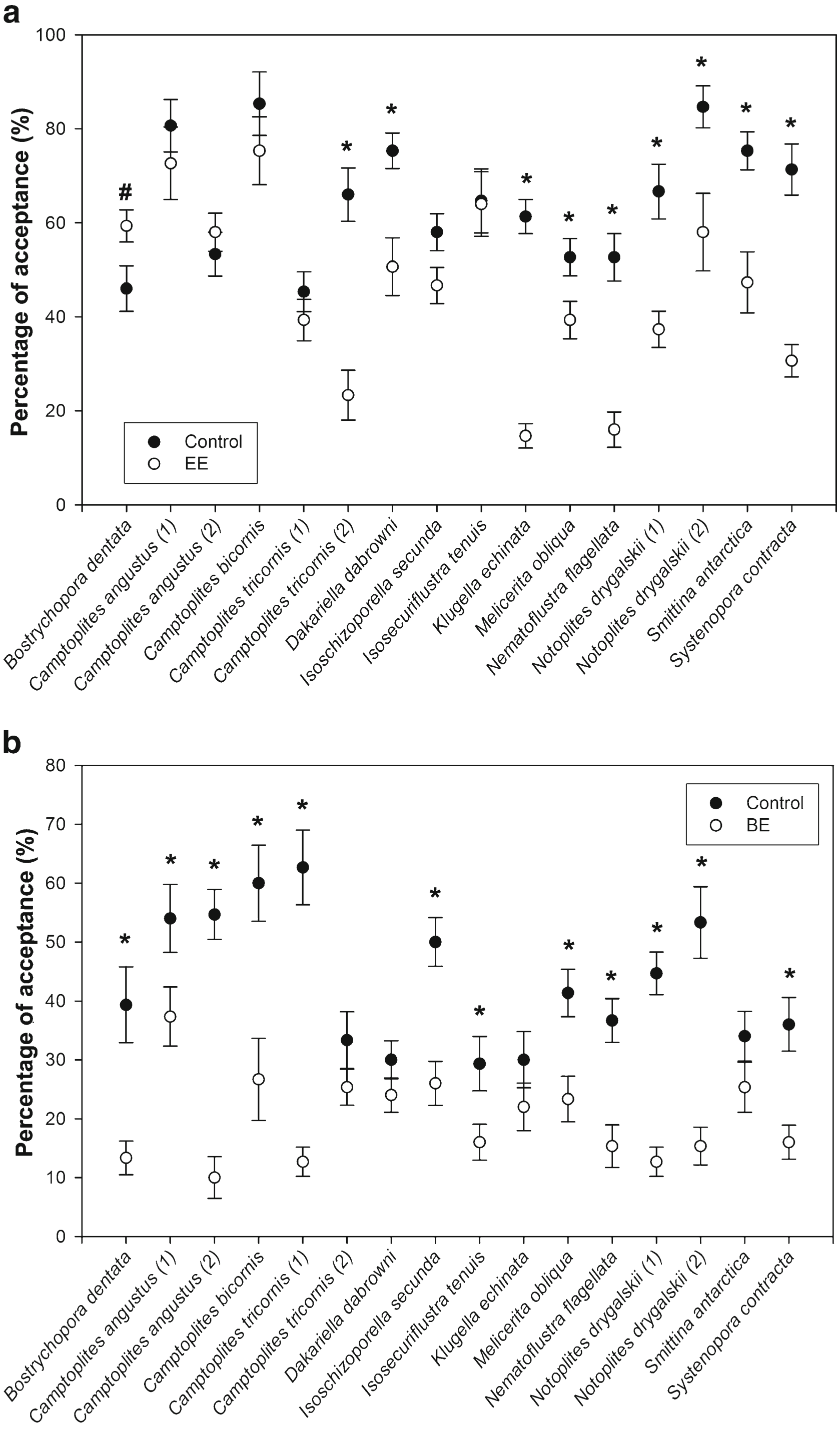

Fig. 4 Feeding preference bioassays against the amphipod *Cheirimedon femoratus* with ether (*EE*) and butanol (*BE*) extracts from Antarctic bryozoans, showing the paired results of control- and extract-treated foods (15 replicates for each test) with the mean percentage of acceptance and standard error bars. Significant differences: * control pearls as food preferred; # treated pearls as preferred food

(2). These species displayed activity in three experiments: EE in the feeding repellence and both extracts (EE and BE) in the preference bioassays. The only exception was *C. angustus* (2), where both extracts were active in the feeding repellence, and the BE in the preference bioassay. Eight species showed activity in two extracts [*C. angustus* (1), *C. bicornis*, *C. tricornis* (1), *I. secunda*, and *I. tenuis*, in the EE toward the sea star and the BE toward the amphipod; *C. tricornis* (2) and *K. echinata* in both EE, *N. flagellata* and *S. contracta* in both extracts toward the amphipod] and two species (*D. dabrownii* and *S. antarctica*) only displayed activity in one (EE toward amphipod; Table 1).

Low stress values (<0.03) of the NMDS indicated a good representation in the two-dimensional ordination (Clarke 1993). Five groups were discriminated by the NMDS analysis in relation to the bioactivity of bryozoan species of both

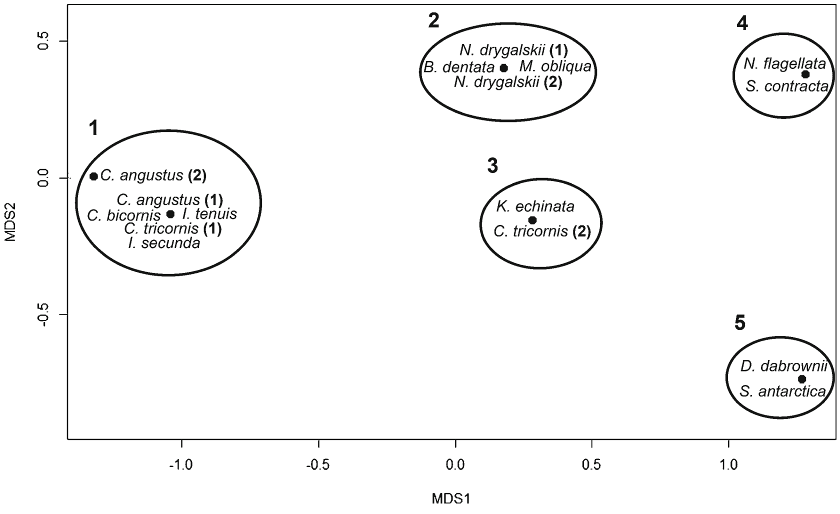

Fig. 5 Nonmetric multidimensional scaling (NMDS) ordination plot of different bryozoan species in relation to the number of their active ether and butanol extracts in the two experiments of repellent defense. *Circles* correspond to grouped species (Groups 1–5) in relation to number and type of active extracts in both bioassays (stress <0.03)

experiments (Fig. 5): Group 1 with five species [*C. angustus* (1) and (2), *C. bicornis*, *C. tricornis* (1), *Isoschizoporella secunda*, and *Isosecuriflustra tenuis*] characterized by displaying activity in the EE [and in the BE in the case of *C. angustus* (2)] against the starfish and in the BE toward the amphipod; Group 2 with three species [*B. dentata*, *N. drygalskii* (1) and (2), and *M. obliqua*] displaying activity in the EE against the starfish and all extracts in preference bioassays; Group 3 including two species [*C. tricornis* (2) and *K. echinata*] showing activity only in the EE; Group 4 represented by two species (*N. flagellata* and *S. contracta*) that reveal activity only toward the amphipod in both extracts; finally, Group 5 comprising two species (*D. dabrownii* and *S. antarctica*) displayed repellence only in preference bioassays (EE). A significant difference in activity between groups was found (ANOSIM Global $r=0.92$, $p=0.001$).

Discussion

Feeding repellence against generalist predators

Most species in our study appear to be readily defended against at least one of the two abundant predators, the sea star *O. validus* and the amphipod *C. femoratus*, under laboratory conditions, thus suggesting the importance of chemical defenses against predation in Antarctic bryozoans. In Antarctica, sessile organisms are subjected to intense predation pressure, which has led to the development of chemical defenses in a high percentage of species (Lebar et al. 2007; Avila et al. 2008; McClintock et al. 2010). Nonetheless, bryozoans have been poorly investigated in terms of their chemical ecology so far (Avila et al. 2008). To the best of our knowledge, only 17 EE have been tested previously in feeding bioassays against *O. validus* and only one against *C. femoratus* (Figuerola et al. 2012b; Núñez-Pons et al. 2012b; Taboada et al. 2013), and no BE have ever been tested before this study.

Feeding repellent responses were more frequent in the assays with *C. femoratus* than toward the sea stars, suggesting that bryozoans might require more protection against potential opportunistic host amphipods, which may exert a higher localized pressure (Núñez-Pons et al. 2012a). Unpublished results from our group also propose that the amphipod test seems to be more discriminative for feeding repellence when comparing both assays. However, chemical defenses might be ineffective in preventing the sea stars from attacking the bryozoans. Moreover, the implicated metabolites responsible for the activity appear to be present in both the hydrophilic as well as the lipophilic extracts. The extended activity recorded in our hydrophilic extracts contrasts with previous studies using different species of amphipods, which were more (or only) susceptible to lipidic defenses (Amsler et al. 2009; Koplovitz et al. 2009). However, both lipophilic and hydrophilic defenses against amphipods are common in macroalgae. Regarding the chemistry of cold-water bryozoans, alkaloids and terpenes have been isolated from the circumpolar bryozoan *Flustra foliacea*, discouraging predators, being this a typical function of these types of compounds (Paul 1992; Lebar et al. 2007; Sharp et al. 2007). In addition, one alkaloid, tambjamine, was isolated from the Antarctic bryozoan *Bugula longissima* (Lebar et al. 2007). For the species studied here, chemical analyses are in progress in order to identify the natural products responsible for these activities.

Our results suggest interspecific and intraspecific variability in the genera *Camptoplites* and *Notoplites*, although this variability could exist also for other genera not tested yet at this level. Geographical and/or temporal variability in the production of secondary metabolites induced by particular local environmental pressures, or genetic variation between populations, could be at the origin of these differences, as it seems to happen in other marine taxa such as sponges (Duckworth and Battershill 2003; Page et al. 2005), soft corals (Puglisi et al. 2002), gorgonians (Roussis et al. 2000), and molluscs (Cutignano et al. 2011). Regarding local predation pressures, some bryozoans produce physical defenses (spines) induced by the presence of predators such as the cosmopolitan bryozoan *Membranipora membranacea* (Iyengar and Harvell 2002). This fact could also be linked to the intraspecific variation found. Likewise, different geographical chemotypes have been detected in the bryozoan *Amathia wilsoni* (Blackman and Matthews 1985; Morris and Prinsep 1999). Other kinds of tambjamines, similar to those previously mentioned, were originally isolated from various marine sources including bryozoans, ascidians, and their nudibranch predators and also bacteria (Carte and Faulkner 1983; Lindquist and Fenical 1991; Blackman and Li 1994). The similarity of bryozoan natural products with other metabolites found in marine and terrestrial environments supports the idea of a broad evolutionary retention of these types of compounds, or perhaps that some compounds isolated from Bryozoa might have a symbiotic origin (Sharp et al. 2007). Symbiotic bacteria have been proposed to be responsible for the production of numerous natural products found in invertebrates (e.g., Piel et al. 2005), although this has only seldom been demonstrated. For instance, the bryozoan *Bugula neritina* harbors different strains of bacteria responsible for producing bryostatins in a single specific geographical location (Davidson and Haygood 1999). Thus, geographical and seasonal variations of associated bacterial communities may also cause the intraspecific and interspecific differences in chemical profiles (Anderson et al. 2010). More studies are needed to confirm this fact in these species.

The omnivorous sea star *O. validus* feeds on a highly diverse assortment of benthic invertebrates and algae (McClintock et al. 1994) including some bryozoans as part of its regular diet (Dayton et al. 1974). Although bryozoans are clonal organisms with extensive regenerative potential, they cannot survive after major injuries of the colonies caused mainly by large predators (e.g., sea stars, echinoids; Winston 2009). Thus, defensive strategies against such predators are expected to be developed. Our results showed that *C. angustus* (2) seems to combine feeding repellents of different nature toward *O. validus* appearing in both EE and BE. Instead, other bryozoan samples, which cause significant levels of feeding repellence, are mostly defended by the presence of deterrents in their lipophilic extracts. In previous studies of Antarctic sponges, lipophilic extracts were also reported to show higher tube-foot retraction responses in the spongivorous sea star *Perknaster fuscus* compared to hydrophilic extracts (McClintock et al. 2000). In recent studies, we demonstrated the existence of repellent compounds against *O. validus* in the EE of 10 out of 17 Antarctic bryozoans (mostly different species to our study, except the EE of *B. dentata*, *I. secunda*, and *I. tenuis*), showing that bryozoans are a very active phylum with extended deterrent activities (Figuerola et al. 2012b; Taboada et al. 2013).

The generalist amphipod *C. femoratus* was deterred by all bryozoan species tested here. In previous studies, a single bryozoan EE was tested from the species *I. secunda* resulting suitable (Núñez-Pons et al. 2012b). In our results, only the BE (which contains polar substances) of this species displayed repellence, which suggests that the active compound is of hydrophilic nature. Although predation by smaller zooid-level predators, such as amphipods, is generally sub-lethal in colonial organisms (Winston 2009), chemical defenses are frequently developed in clonal organisms to avoid future attacks. In addition, deterrents against amphipods are frequently reported in other Antarctic sessile invertebrates (Núñez-Pons et al. 2012a). Certain amphipod species are considered specialist bryozoan predators at the zooid level, feeding on individual autozooids (Lidgard 2008). For instance, the amphipod *Gnathiphimedia mandibularis* has adapted its mandibles for crushing hard items such as the skeletons of some bryozoan species, which are its principal food source (Coleman 1989). Therefore, repellent activities reported here could be useful to prevent transient consumers like amphipods, which may feed directly, sporadically, or accidentally on bryozoan colonies; amphipods may also disrupt feeding currents due to this activity and their huge numbers (De Broyer et al. 2001). Similar consequences are caused by amphipod populations associating with sponges, as suggested by Amsler and colleagues (2009).

Diverse bryozoan shapes and structures provide many potential substrates for amphipods to be used as nurseries or as physical or chemical refuges from predation (Winston 2009). In fact, benthic amphipod species from the eastern Weddell Sea are often found on bryozoan colonies (De Broyer et al. 2001). Among the most frequent bryozoan morphologies found, are those with erect rigid or flexible laminar (e.g., *N. flagellata*, *I. tenuis*, and *I. secunda*) and bush-like forms (e.g., species of *Camptoplites* and *N. drygalskii*). While laminar species grow in a two-dimensional fashion, the bush forms provide three-dimensional substrates (e.g., genera *Bugula* and *Camptoplites*) with internal space where small crustaceans may hide from predators and feed on debris, diatoms, and other small organisms (Carter 2008). *C. femoratus*, as many other benthic amphipods, is a bottom-dweller with reduced swimming capacity. It is found regularly associated with living substrata, mainly macroalgae but also invertebrates (Núñez-Pons et al. 2012b, personal observations). Thus, we propose that the use of repellent compounds to avoid settlement of transient populations of *C. femoratus* on bryozoan colonies may be useful to avoid direct or occasional predation, as well as filtration disruptions, as discussed above.

Relationships between deterrent activity and physical defenses

Most bryozoan species seem to show a combination of chemical and physical defenses (spines, avicularia, and/or vibracula) without a clear relationship between the density of the polymorphs and the level of chemical activity. This suggests that these traits are neither complementary mechanisms of protection nor redundant defenses and that they could add other purposes not yet investigated. For example, although *K. echinata* and *N. drygalskii* seem to be more vulnerable in having membranous frontal membranes (McKinney et al. 2003), these species possess both chemical strategies and physical defenses (avicularia and spines). Furthermore, the lack of chemical defenses against *O. validus* in *D. dabrowni*, *S. antarctica*, and *S. contracta* could be related to the presence of likely physical defensive mechanisms, such as their thick skeletons, proposed to be comparable with echinoid spines or bivalve shells (Hayward 1995). In fact, several studies have demonstrated trade-offs between physical and chemical defenses in some benthic invertebrates, leading to an optimization of energy addressed to either one of the two mechanisms of protection (Stachowicz and Lindquist 2000; Hines and Pawlik 2012). Some encrusting cheilostome bryozoans, such as *D. dabrowni*, are also able to form hard aggregates capable of resisting damage by predators (Best and Winston 1984; Winston 1986). Species from this group of Antarctic bryozoans typically show abundant and diverse forms and sizes of avicularia also, with an apparent protective function, as exposed in the recent description of a new Antarctic species (Figuerola et al. 2013). Avicularia are specialized zooids with a hypertrophied operculum (mandible), lacking a feeding role. Although the functions of various avicularia are still unknown, morphological and behavioral studies suggest physical protection, cleaning, and defensive functions against predators or grazers (Winston 1986, 1991). In particular, their mandibles are rapidly closed by adduction, discouraging potential predators (Winston 1986). Additionally, avicularia may have different forms and sizes hypothesized to enable capture particular predators (Silén 1977). A highly specialized form is the often-polymorphic so-called “bird's-head” avicularia, which, in Antarctica, is only found in the genus *Camptoplites*, represented in the present study by three species: *C. angustus*, *C. bicornis*, and *C. tricornis* (Hayward 1995). Several observations on living colonies show that the mandible may capture small items like appendages of small-sized potential predators including amphipods (Winston 1986, 1991, 2009; Carter et al. 2010). Likewise, other studies document the capture of gammarid amphipods by the bird's-head avicularia of *Bugula* (Forbes 1938; Kaufmann 1971). These trapped organisms may end up dying and, over time, decaying, attracting bacteria, thus providing potential additional food sources for bryozoans (Winston 1991). In addition, the long slender peduncles of the avicularia of *Camptoplites* sway slowly back and forth across the frontal surface of the colony branches, carrying any intruding organism toward the edge of the colony (Winston 2010). Another feature of the three species of *Camptoplites* studied here is the presence of three or four kinds of avicularia and spines. Despite these structures of protection, flexible *Camptoplites* species combined the presence of repellents against both the sea stars and the amphipods, probably increasing the defensive capacity against the amphipods. Similarly, *N. flagellata*, which is weakly calcified but possesses physical deterrents in the form of vibracula, showed repellent activity against the amphipod. Vibracula are modified zooids with an operculum in the form of a long seta, which act producing the vibration of small organisms. Presumably, this movement triggers a wave that provokes the movement of setae over the colony surface carrying settling organisms outside the branches (Hayward 1995; Winston 2010).

In summary, the presence of physical defenses, such as avicularia, spines, and vibracula, together with a highly efficient regeneration potential, and the presence of deterrent metabolites, may significantly increase predation protection and ecological success beyond that offered by each defensive strategy separately. Similarly, in hydroids, the cooperation of nematocysts with chemical defenses has been reported (Stachowicz and Lindquist 2000). There are cases though, such as *M. obliqua*, where the lack of apparent physical protection and the lightly calcified frontal walls, providing less resistance to grazing predators (McKinney et al. 2003), are likely compensated by effective chemical defenses. Actually, this species showed significant repellent activity in the assays toward both sea star and amphipods.

Similarities between species in relation to their bioactivities

Most species of *Camptoplites*, *I. secunda*, and *I. tenuis* had similar deterrent bioactivity against the two generalist predators, as suggested by our NMDS analysis. These species, except for *I. secunda*, exhibit a flexible skeleton, being apparently physically defenseless to predators (Hayward 1995), and thus requiring chemical defenses. Additionally, the active compounds of these species were of different nature, either lipophilic or hydrophilic. *B. dentata*, *N. drygalskii* (1) and (2), and *M. obliqua* formed a group characterized by displaying feeding repellence strategies in most extracts. In particular, *N. drygalskii* (1) and (2) also possess flexible colonies, and therefore the use of chemical protection is presumably needed. Conversely, *M. obliqua* has a rigid skeleton but lacks other defensive structures and accordingly possesses effective chemical defenses. The other rigid species, *B. dentata*, has diverse sessile avicularia; however, these are small and could possibly not be effective enough to discourage the predators tested in this study. The flexible colonies of *C. tricornis* (2) and *K. echinata* were grouped together, since both caused chemical deterrence against the two predators, thus compensating for their lack of rigidity. In contrast, *N. flagellata* and *S. contracta* displayed activity in both extracts only against the amphipod. Finally, *D. dabrowni* and *S. antarctica* were grouped together for being rejected by *C.*

femoratus in only one extract, but these species possess a hard skeleton, which likely may constitute an important physical defense against predation such as that exerted by *O. validus*.

Concluding remarks

The diversity and high frequency of defensive activity against two sympatric predators showed in our study, along with the high number of morphological defensive structures, demonstrate the importance of defenses against common predators in Antarctic bryozoans. In this sense, understanding prey–predator interactions in Antarctica is a key aspect in these stable ecosystems, where biological factors are the main driver structuring benthic communities (Dayton et al. 1974). Our study suggests that chemical defenses seem to be as common as physical defensive devices among Antarctic bryozoans, indicating that two or more different strategies may significantly increase protection in this group of invertebrates. Moreover, some species showed only chemical or physical defenses, suggesting potential resource trade-offs. To date, research in marine natural products and their role in an ecological context have focused mostly on phyla such as Porifera, Cnidaria, and Mollusca (Apeltans et al. 2012; Blunt et al. 2012). In the case of Antarctic bryozoans, there are very few chemical ecology studies (Avila et al. 2008), although some cold-water bryozoans were reported to harbor pharmacologically interesting bioactive products (Lebar et al. 2007). This study represents a step-forward to the knowledge on Antarctic chemical ecology, particularly in the group of the bryozoans. However, further studies are needed to better understand the complexity of chemically mediated Antarctic ecological interactions involving this phylum and to identify their natural products.

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