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ORIGINAL PAPER

Mechanisms involved in pearlfish resistance to holothuroid toxins

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Abstract Holothuroids produce triterpenoid saponins that act as chemical defenses against predators and parasites. These saponins interact with sterols of the plasma membranes, inducing the formation of pores and then cell lysis. To avoid such harms from their own saponins, holothuroids present specific sterols in their tissues. Despite the noxious cytotoxic effect of their chemical defenses, holothuroids host various associates that display specific adaptations to resist to saponin toxicity. Among them, symbiotic carapid fishes (i.e., pearlfishes) are resistant to ichthyotoxic saponins as they display no stress response and a survival time 45 times longer than free-living fishes without any specific gill adaptation. The present study aims at discovering the resistance mechanism(s) developed by carapids by addressing 3 hypotheses: carapids have (1) a mechanical barrier against the toxin constituted by a larger secretion of mucus than other fishes, (2) a bioactive barrier against the toxins constituted by a mucus effective on saponins and (3) a Δ^7sterol tissue composition mimicking holothuroids that enable them to resist to saponins. First experiments showed that the mucus has no effective impact on saponin chemical structures. Mass spectrometry analyses showed that carapids, similarly to non-symbiotic fishes but contrary to their hosts, present a Δ^5sterol tissue composition. However, two different procedures have shown that carapids produce six to ten times more mucus than control fishes, suggesting that a great quantity of mucus can protect carapids from their host's saponins and acts as a mechanical barrier against toxins. Therefore, these results provide a new understanding of the carapids–holothuroids relationship.

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Introduction

Defense mechanisms are a common tool enabling organisms to survive against predators, parasites or abiotic factors (Benndorf 1987). They can be either active or passive; either chemical, mechanical or behavioral; and may play a substantial role in the organisms survival. In marine ecosystems, chemical defense is particularly important with a large range of active secondary metabolites (Cimino and Ghiselin 2001). Among echinoderms, holothuroids and asteroids produce cytotoxic metabolites called saponins (Yamanouchi 1955; Burnell and Apsimon 1983) that are present in many organs. In some holothuroids, they are especially concentrated in Cuvierian tubules that are long sticky tubules expelled through the cloacum to immobilize predators (Flammang 2002). Saponins, initially discovered in terrestrial plants (Kofler 1927; Bader and Hiller 1987; Li et al. 2006), consist of one or more sugar chains

(usually containing glucose, quinovose, 3-O-methylglucose, 3-O-methylxylose or xylose), linked to a hydrophobic aglycone (terpenoid or steroid) (Francis et al. 2002), conferring an amphiphilic character associated with a toxic activity on cells. Their cytotoxic effect generally arises from their interactions with sterols at the level of the double bond in position 5, especially with the cholesterol contained in plasma membranes (Popov et al. 1983). The membranotropic effect resides then in the formation of numerous 40–50 Å pores, the release of potassium and *in fine* the cell lysis (Glauert et al. 1962; Mackie et al. 1977; Kalinin et al. 1996; Armah et al. 1999; Stonik et al. 1999; Maier 2008; Augustin et al. 2011). This membrane destruction mechanism seems particularly efficient for sensitive organs composed of thin cell layers like fish gills (Nigrelli 1952; Bakus 1968; Eeckhaut et al. 2015).

However, several organisms are able to avoid this defense mechanism, including asteroids and holothuroids themselves. Popov et al. (1983) showed that the resistance of echinoderms to their own saponins is due to a particular sterol composition in their cytoplasmic membranes as they have—different from other organisms—a majority of sterols with double bond in position 7 or 9 (Ballantine et al. 1981; Drazen et al. 2008; Goad 1983; Goad et al. 1972; Kicha et al. 2001; Ponomarenko et al. 2001; Shubina et al. 1998; Smith et al. 1973; Stonik et al. 1998). These peculiar sterols show 20–30 % lower interactions with saponins than cholesterol and do not provoke cell lysis (Popov et al. 1983). In the same way as holothuroids and asteroids, their numerous symbionts and parasites do not appear harmed by saponin toxicity (Eeckhaut et al. 2015). Among these symbionts are uncommon fishes from the Carapidae family also known as pearlfishes (Markle and Olney 1990). Although some genera are free-living (*Snyderidia, Pyramodon, Echiodon*) (Trott, 1970; Parmentier 2003), other (*Onuxodon*, *Carapus* and *Encheliophis*) are known for their endosymbiosis with marine invertebrates, such as bivalves (Tyler and Bohlke 1972; Trott and Chan 1972; Machida 1989; Castro-Aguirre et al. 1996; Paredes-Ríos et al. 1999), ascidians (Weber 1913), asteroids (Meyer-Rochow 1977, 1979) and holothurians (Emery, 1880; Smith 1964; Smith and Tyler 1969; Trott and Chan 1972; Trott and Trott 1972; Shen and Yeh 1987; Van Den Spiegel et al. 1972; Jangoux 1984). Species from *Carapus* and *Onuxodon* genera are considered as commensals and *Encheliophis* species as parasites (Trott, 1970; Parmentier et al. 2000; Parmentier 2003; Parmentier and Vandewalle 2005). Recently, we have studied the effects of exposure to saponins from holothurian body wall and from the Cuvierian tubules on the behavior and the gill ultrastructure of pearlfishes and free-living fishes (Eeckhaut et al. 2015). The free-living fishes exhibited a stress response behavior and died about 45 times faster than pearlfishes when exposed to the same quantity of Cuvierian tubules and saponins extracted from holothuroid body wall (Eeckhaut et al. 2015). Histology, scanning and transmission electron microscopy of the gills of the free-living fish *Amphiprion akallopisos* have shown that saponins principally caused damages to the secondary lamellae by inducing epithelia detachment, epithelia edema and pore formation, which leads to the destruction of epithelial cells (Eeckhaut et al. 2015). Carapid gills were also affected, but to a lesser extent.

As the gill anatomy cannot explain the resistance of carapids to saponins, the present work aims at understanding how fishes resist to holothurian saponins through exploring three hypotheses: (1) The quantity of mucus secreted in carapids is higher than in other fishes and its thickness creates a natural barrier against the toxin, (2) the mucus chemical composition in carapids is different from that in other fishes and includes some molecules (e.g., enzymes) that can impede saponin noxious effects and (3) saponins cannot act on carapid gills because of particular sterols in the cytoplasmic membrane of their epithelial cells, mimicking their hosts. This study was realized to improve our global understanding of marine symbioses.

Materials and methods

Models and sampling

Five carapid species (i.e., pearlfishes), five control species (i.e., free-living counterparts) and eight host species (i.e., echinoderms) were considered. Some were collected by snorkeling or purchased from local fishermen around Toliara Reef (Madagascar), mostly in November 2012, whereas others were found by snorkeling in Opunohu Bay of Moorea, French Polynesia, in 2011. In addition, a few control fishes were found in a pet shop in Belgium in 2012 (Table 1).

Pearlfishes were usually found in the general cavity of their holothuroid/asteroid hosts (*B. argus*, *T. ananas*, *H. forskali, C. novaeguinea* and *C. schmideliana)* after their dissection. *O. fowleri* was studied separately, in order to compare it with other carapids, because it was not symbiotic to echinoderms but to various bivalves. All pearlfishes were placed in dark condition in small aquaria (30 × 30 × 20 cm) filled with filtered and air-supplied seawater (28 °C, 35 ‰ salinity, pH 8) that was daily changed. Control fishes were collected directly on the reef by scuba diving and using nets and quinaldine to anesthetize them. Back to the laboratory and after a recovery period of 24 h, below-mentioned experiments were directly performed on fishes.

Table 1 Species collection characteristics

Species	Number	Year	Location	Method of sampling
Carapids				
Carapus boraborensis (Kaup, 1856)	2	2011	Opunohu Bay of Moorea	Snorkeling
Encheliophis homei (Richardson, 1846)	2	2012	Great Reef of Toliara	Scuba diving
Encheliophis homei (Richardson, 1846)	6	2011	Opunohu Bay of Moorea	Snorkeling
Carapus mourlani (Petit, 1934)	10	2011	Opunohu Bay of Moorea	Snorkeling
Encheliophis gracilis (Bleeker, 1856)	11	2012	Great Reef of Toliara	Scuba diving
Encheliophis gracilis (Bleeker, 1856)	3	2011	Opunohu Bay of Moorea	Snorkeling
Onuxodon fowleri (Smith, 1955)	5	2011	Opunohu Bay of Moorea	Snorkeling
Hosts				
Bohadschia argus Jaeger, 1833	3	2012	Great Reef of Toliara	Fishermen
Bohadschia vitiensis (Semper, 1868)	2	2012	Opunohu Bay of Moorea	Snorkeling
Culcita novaeguinea Müller & Troschel, 1842	3	2011	Opunohu Bay of Moorea	Snorkeling
Culcita schmideliana (Retzius, 1805)	2	2012	Great Reef of Toliara	Scuba diving
Holothuria (Metriatyla) lessoni Massin, Uthicke, Purcell, Rowe, Samyn, 2009	1	2011	Great Reef of Toliara	Fishermen
Holothuria (Metriatyla) scabra Jaeger, 1833	2	2012	Great Reef of Toliara	Fishermen
Thelenota ananas (Jaeger, 1833)	3	2011	Opunohu Bay of Moorea	Snorkeling
Thelenota anax Clark, 1921	3	2011	Opunohu Bay of Moorea	Snorkeling
Control fishes				
Amphiprion akallopisos Bleeker, 1853	10	2012	Great Reef of Toliara	Scuba diving
Amphiprion clarkii (Bennett, 1830)	3	2012	Pet shop	Purchase
Amphiprion ocellaris (Cuvier, 1830)	2	2012	Great Reef of Toliara	Scuba diving
Dascyllus aruanus (Linnaeus 1758)	2	2012	Pet shop	Purchase
Dascyllus aruanus (Linnaeus 1758)	9	2012	Great Reef of Toliara	Scuba diving
Dascyllus trimaculatus (Rüppell, 1829)	4	2012	Pet shop	Purchase
Dascyllus trimaculatus (Rüppell, 1829)	4	2012	Great Reef of Toliara	Scuba diving

Mucus recovery and quantification

In order to recover enough mucus to analyze and compare the production, the collection was performed on the whole body and not on the gills. Trott (1970) observed an increase in mucus production when carapids are exposed to Cuvierian tubule extracts, which suggests the implication of the whole body and justifies the approach. The relative mucus quantity secreted by the body of fishes has been measured in two different ways at the IH.SM (Institut d'Halieutique et des sciences marines de Toliara) in Madagascar and at the CRIOBE (Centre de Recherches Insulaires et Observatoire de l'Environnement) in Moorea, French Polynesia.

In the first method, mucus samples were collected on 6 different species (3 carapids and 3 control fishes): *C. boraborensis* ($n = 2$); *C. mourlani*; ($n = 5$); *E. gracilis* ($n = 5$); *A. akallopisos* ($n = 5$); *D. aruanus* ($n = 4$); and *D. trimaculatus* ($n = 4$). Fishes were anesthetized with a solution of tricaine methane sulfonate (MS 222) in seawater (dilution varied according to fish weight, from 2 to 20 mg L^{-1}) and placed in Petri dishes. Body mucus was collected by gliding gently a microscope covering glass slide on the fish skin until total mucus removal. Fishes were then replaced in their aquaria for recovering. The weight of fresh mucus was determined by weighing: The slides and Petri dishes were weighted before and after the samplings.

The second method was conducted on 3 species (1 carapid and 2 control fishes): *E. gracilis* ($n = 3$); *A. akallopisos* ($n = 4$); and *D. aruanus* ($n = 5$). The mucus was collected by placing living organisms in a dry beaker for 5 min. The non-anesthetized fishes spread their mucus on the beaker wall and were then replaced back in their aquarium. The mucus was recovered and lyophilized with Christ Alpha 1–2 LD Freeze Dryer. Dried mucus and fishes were then weighted.

For each method, the mucus absolute weights were standardized by the fish skin areas. As only total fish length was available for some individuals, fish areas were estimated based on this parameter. Different formulas were used for carapids and control fishes according to their morphology (Fig. 1). Prior to area estimations, theoretical ratios of the length parameter to other size parameters (e.g., width and height) were established based on measurements taken on fresh organisms and photographs,

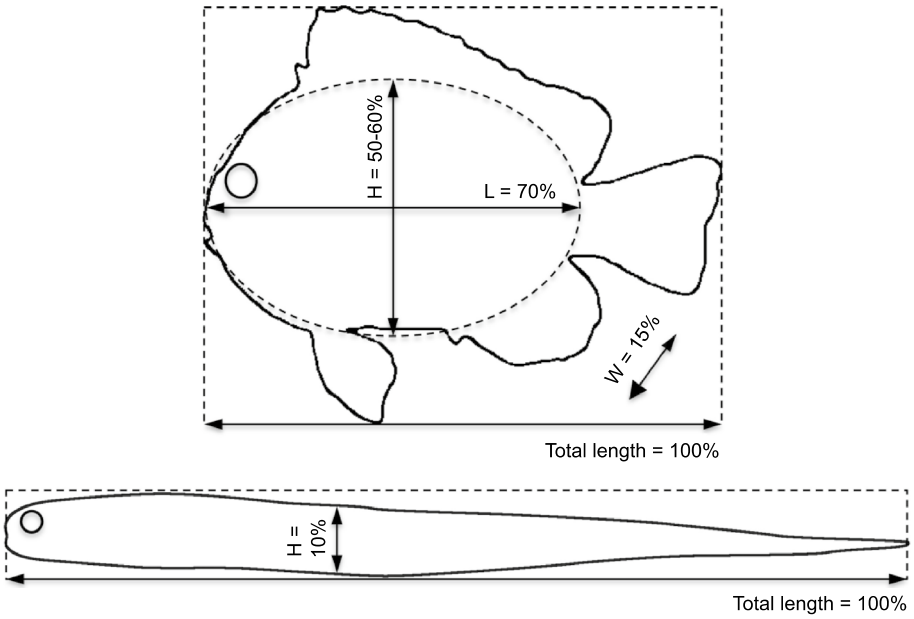

Fig. 1 Estimation of height without dorsal fin (*H*), length without caudal fin (*L*) and width (*W*) for control fishes (at the *top*) and carapids (at the *bottom*)

if they were not directly available from the literature (Ida et al. 1977).

Carapid skin areas were considered as the half surface of a cylinder and measured from the following formula:

$$\text{Carapids skin surface} = \frac{\left[2\pi\frac{H}{2}L\right]}{2},$$

where height (*H*) was valued to 10 % of length (*L*).

Control fish skin areas were estimated from an ellipsoid and based on the formula by Knud Thomsen (Xu et al. 2009).

$$\text{Control fish skin surface} \approx 4\pi\sqrt[p]{\frac{(H^p + L^p W^p + H^p W^p)}{3}},$$

where $p = 1.6075$; height without dorsal fin (*H*) was valued to 50 % of the total length for *A. akallopisos* and to 60 % for the two *Dascyllus* species; width (*W*) was valued to 15 % of the total length, and the length without caudal fin (*L*) was valued to 70 % of the length.

For each method, two-factorial nested ANOVA on ranks was performed to determine whether mucus quantities differed significantly between species or between groups (i.e., control fishes or carapids). A three-factorial nested ANOVA on ranks was also carried out to determine whether methods differed significantly. Statistical analyses were performed using the "R 3.2.2" statistics software (R-package GenABEL). Data visualizations were designed using the "Prism 6" statistic software (GraphPad).

ß-glucosidase detection

Because glucose residues are frequently found in abundance in saponin compositions, the presence of enzymes such as ß-glucosidase in carapid mucus may have an enzymatic action leading to saponin inhibition. Actually ß-glucosidase may protect carapids from saponins by splitting their structures into aglycones on the one hand and glycone groups on the other hand, suppressing their amphiphilic nature and then their cytotoxicity. The ß-glucosidase assay is based on its enzymatic activity, using one of its substrate, namely the p-nitrophenol-α-D-glucopyranoside (PNPG), which is cleaved and produces p-nitrophenol detected at 400 nm. This analysis was performed on mucus from 2 carapid species, namely *C. homei* ($n = 2$) and *E. gracilis* ($n = 2$); and on 3 control species, namely *A. akallopisos* ($n = 2$); *A. ocellaris* ($n = 2$); and *D. aruanus* ($n = 2$).

Assays were performed following the protocol from Calzyme Laboratories (2012) as detailed hereafter. Ten mg-dry mucus were dissolved in 50 µl Tris–HCl buffer (0.05 M, pH 7.8), stabilized with 0.2 % bovine serum albumin (BSA) phosphate buffer (0.01 M, pH 7). Then, 100 µl of acetate buffer (0.01 M, pH 5) and 50 µl of PNPG were added, and the final solution was incubated at 28 °C for 15 min. Reactions were stopped with 200 µl Na_2CO_3 (0.2 M), and optical densities were measured with a Bio-Rad SmartSpec 3000 UV/Vis spectrophotometer at 400 nm. Negative controls were performed with the addition of 200 µl Na_2CO_3 (0.2 M) before incubation. Positive controls were performed with commercial ß-glucosidase from almonds ($\geq$6 U mg^{-1}) (Sigma-Aldrich St. Louis, MO, USA).

Mucus effect on saponins

To estimate degradation of the molecular structures of saponins in the presence of carapid mucus, mass spectrometric

analyses were performed on holothuroid (i.e., *H. scabra*) saponin extracts mixed with mucus from carapids (i.e., *E. gracilis*, $n = 3$) or mucus originating from control fishes (i.e., *A. akallopisos*, $n = 3$).

Saponins were extracted following the protocol of Van Dyck et al. (2009), adapted by Caulier et al. (2013). *H. scabra*'s integument was sampled, homogenized in methanol (70 %) and filtered under vacuum with a Buchner flask on Whatman filter paper 589/2. The recovered methanolic phase was then successively partitioned against n-hexane, dichloromethane and chloroform (v/v). The methanolic phase was evaporated at low pressure at 30 °C, using a rotary evaporator and dissolved in water. It then underwent chromatographic purification on a 20-cm column, using Amberlite XAD-4 as solid phase (Sigma-Aldrich St. Louis, MO, USA). The column was washed with 100 ml water to remove inorganic salts, and saponins were recovered with 30 ml of methanolic elution. Then, the methanolic extract was evaporated and diluted in 5 ml water before it underwent a last liquid–liquid extraction against isobutanol (v/v). The butanolic phase was evaporated again and diluted in 1 ml of sea salts (Sigma-Aldrich St. Louis, MO, USA) (3 %, pH 8) water. Saponin extract (100 µl containing 0.5 mg ml^{-1}) was incubated with 100 µl of rehydrated mucus (25 mg ml^{-1} in Milli-Q water) for 10 min at 28.5 °C (i.e., temperature of the natural ecosystem).

Saponins, mucus and saponin–mucus solutions were analyzed on a Waters Q-ToF Premier mass spectrometer in positive ionization mode, using a MALDI (matrix-assisted laser desorption/ionization) ionization source. The MALDI source was constituted by a Nd-YAG laser, operating at 355 nm with a maximum pulse energy of 104.1 µJ delivered to the sample at 200 Hz repeating rate. All samples were prepared using a mixture of 1 ml of a 100 mg ml^{-1} solution of 2,5-dihydroxybenzoic acid (DHB) in water/acetonitrile (v/v) with 20 µl of *N*,*N*-dimethylaniline as the matrix (Demeyer et al. 2014, 2015). The sandwich method was selected to prepare the sample/matrix co-crystal on the target plate. In this method, the sample analyte is not premixed with the matrix. A sample droplet (1 µl) is applied on top of a fast-evaporated matrix-only bed, followed by the deposition of a second layer of matrix in solvent (1 µl). The sample is then basically sandwiched between the two matrix layers. The sandwich deposit was selected to obtain a more homogeneous co-crystal surface. For the recording of the single-stage MALDI-MS spectra, the quadrupole (rf-only mode) was set to pass ions between *m/z* 250 and 1500, and all ions were transmitted into the pusher region of the time-of-flight analyzer where they were mass-analyzed with a 1-s integration time. For MALDI-MSMS or MALDI-CID (collision-induced dissociation) experiments, the ions of interest were mass-selected by the quadrupole mass filter. The selected ions were then submitted to collision against argon in the T-wave collision cell (pressure estimated at 0.9–1 mbar), and the laboratory frame kinetic energy (E_{lab}), typically at 80 eV, was selected to afford intense enough product ion signals. All the ions coming out of the collision cell, either the product ions or the non-dissociated precursor ions, were finally mass measured with the oa-ToF analyzer. Time-of-flight mass analyses were performed in the reflectron mode at a resolution of about 10,000 (at *m/z* 1000). The MassLynx software from waters was used to parameter the instrument and to analyze the results. Saponin identifications were realized by comparing the MSMS mass spectra of putative saponins with spectra obtained in previous studies (e.g., Caulier et al. 2013). Saponin analyses were performed in the Organic Synthesis and Mass Spectrometry Laboratory, University of Mons.

Sterol extractions and analyses

This analysis was performed on 26 carapid integument samples: *C. boraborensis* ($n = 5$), *C. homei* ($n = 6$), *C. mourlani* ($n = 4$), *E. gracilis* ($n = 6$) and *O. fowleri* ($n = 5$); on 11 control fish integument samples: *A. akallopisos* ($n = 3$), *A. clarkii* ($n = 3$), *A. ocellaris* ($n = 1$), *D. aruanus* ($n = 2$), *D. trimaculatus* ($n = 2$); and on 19 host integument samples: *B. argus* ($n = 3$), *B. vitiensis* ($n = 2$), *C. schmideliana* ($n = 2$), *C. novaeguinea* ($n = 3$), *H. lessoni* ($n = 1$), *H. scabra* (n = 2), *T. ananas* ($n = 3$) and *T. anax* ($n = 3$). Assays were performed according to the method from Vanderplanck et al. (2011).

Integuments were sampled, lyophilized with a Christ Alpha 1–2 LD Freeze Dryer and saponified (±100 mg) with 2.5 ml of 2 M methanolic KOH for 1 h at 80 °C. After cooling, 2.5 ml of Milli-Q water was added. Then, the solutions underwent three liquid–liquid extractions (v/v) with diethyl ether and three washing with Milli-Q water (v/v). The diethyl ether phases were dried over anhydric sodium sulfate, evaporated at low pressure using a rotary evaporator and diluted with 0.5 ml of chloroform. The crude extracts were separated on a thin silicagel layer chromatography [eluent: chloroform/diethylether/ammonia water 28 % (90:10:0.5)]. The sterols were highlighted under UV after spraying an ethanolic 0.2 % 2′,7′-dichlorofluorescein solution. The detected sterols were scraped off and recovered from silica gel with three chloroform washings. Solvent was evaporated at low pressure and under a nitrogen stream. The purified sterol extract was finally derivatized with 50 µl of anhydrous pyridine and 50 µl of silylation reagent (BSTFA + 1 % TMS) at 90 °C for 30 min. The reagents were evaporated under nitrogen, and dry extract was diluted with 200 µl of hexane. Finally, sterol solutions were centrifuged at 10,000 g for 15 min to remove possible solid residues prior to chromatographic analyses. Sterol

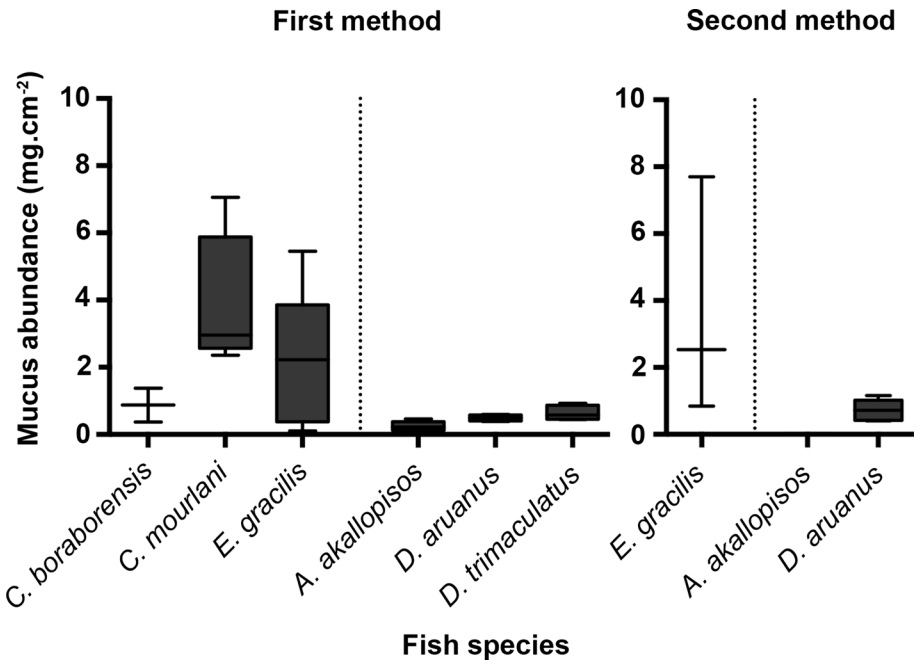

Fig. 2 Box of median abundance of mucus (mg) relative to surface (cm^2) with maxima and minima for carapids (*C. boraborensis, C. mourlani* and *E. gracilis*) and control fishes (*A. akallopisos, D. aruanus and D. trimaculatus*) according to different methods. Mucus production results were displayed in text as mean in mg/cm^2

quantifications were performed with a FISON GC 8000 Gas Chromatograph, coupled to a FID (Flame Ionization Detector). We used a VF-5 ms Capillary displaying the following specifications: 5 %-phenyl-methylpolysiloxane stationary phase; 30 m column length; 0.25 mm inner diameter; and 0.25 mm film thickness. The temperature program started at 60 °C for 1 min, increased then isocratically to 290 °C at 30 °C min^{-1} and was maintained at that temperature for 22 min, followed by a last increase to 325 °C (30 °C min^{-1}) for 7 min. The carrier gas was helium at 1 ml min^{-1}, and injections were performed on-column, using a Thermo Finnigan AS 2000 with split-less mode at 280 °C. The ChromCard software was used to parameter the instruments and to analyze results. Relative quantifications were obtained measuring the area under each sterol peak.

Sterol identification was done with a Finnigan Trace MS Gas chromatograph coupled to a mass spectrometer 5975C inert XL EI/CI LSD with Triple-Axis detector (Agilent Technologies). Injections were carried out with the same capillary column, parameter injection and temperature specification as in GC-FID injections for sterol quantification (see above). The XCalibur software was used to parameter the instrument and to analyze results. Sterols were analyzed at the Unit of Analytical Chemistry, University of Liège—Gembloux Agro-Bio Tech.

A PERMANOVA test—using the Bray–Curtis distance measure and 999 permutations—was performed on groups (hosts, carapids and control fishes) to determine whether carapids have a sterolic composition similar to their hosts. Sterols were grouped into four variables: cholesterol, other Δ^5-sterols, Δ^7-sterols and unknown sterols to increase the analysis relevance. Multiple pairwise comparisons were made when the returned p value was significant ($p < 0.05$). Indicator compound analyses were also carried out to identify a possible group-specific sterol composition. The analyses were visualized by hierarchical cluster analysis, using the average method. All analyses were performed with the "R" software version 2.0.2, using the "vegan" and "labdsv" packages (Vanderplanck et al. 2014).

Results

Mucus quantification

Results obtained with the first extraction method showed that the production of wet mucus differed from one species to another (Fig. 2). With regard to the group of control fishes, *A. akallopisos*, *D. aruanus* and *D. trimaculatus* displayed a mucus production of 0.24 mg cm^{-2}, $n = 5$; 0.49 mg cm^{-2}, $n = 4$; and 0.63 mg cm^{-2}, $n = 4$, respectively. Concerning carapid group, *C. boraborensis, C. mourlani* and *E. gracilis* displayed a mucus production of 0.8 mg cm^{-2}, $n = 2$; 3.97 mg cm^{-2}, $n = 5$; and 2.13 mg cm^{-2}, $n = 5$, respectively. Statistical analyses revealed that the mucus production of the carapid group is significantly different from the mucus production of the control fish group ($p < 0.05$). Moreover, statistical analyses showed a significant difference of mucus production

Table 2 Nested ANOVA on ranks of mucus abundance: (a) species nested within groups (carapids or control fishes) for two different methods (details see text) and (b) species nested within groups and groups nested within methods

	Method 1					Method 2				
	df	SS	MS	*F*	p	df	SS	MS	*F*	p
(a) Mucus abundance comparison										
Groups	1	6.7972	6.7972	12.2924	0.0023	1	4.5530	4.5530	15.417	0.0034**
Groups: Species	4	6.4605	1.6151	2.9209	0.0486	4	3.5792	3.5792	12.120	0.0069*
Residuals	9	2.6579	0.2953			19	10.5063	0.5530		

	df	SS	MS	*F*	p
(b) Method comparison					
Methods	1	1.2736	1.2736	3.3867	0.0759
Methods: Groups	2	14.2032	7.1016	18.8845	5.6e-06***
Groups: Species	4	9.3706	2.3427	6.2295	0.0009***
Residuals	29	10.9056	0.3761		

Significant at $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

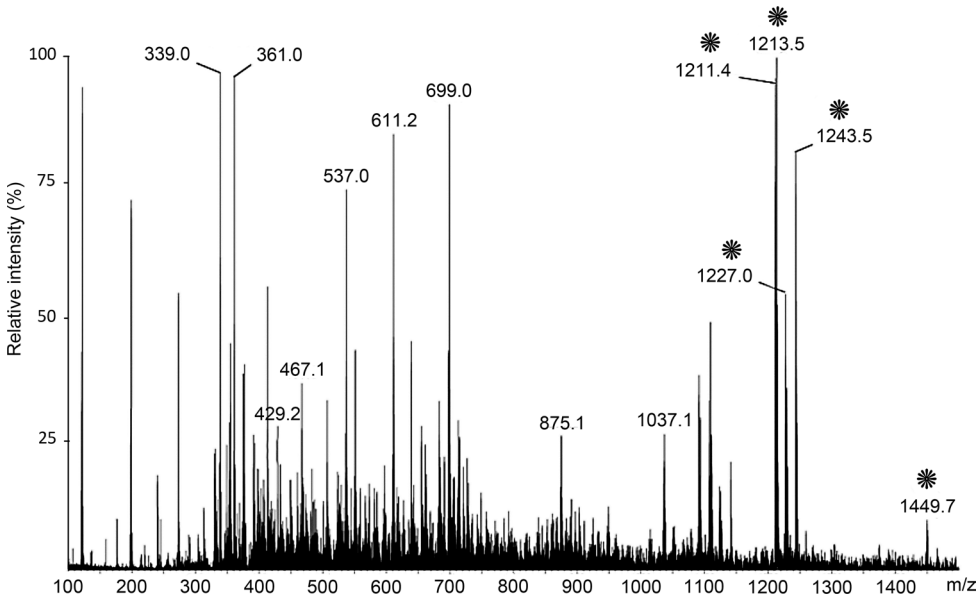

Fig. 3 MALDI-ToF mass spectrum of a synthetic mixture (see text) of mucus extracted from *E. gracilis* and saponins extracted from *H. scabra.* Saponin ions are highlighted with a *star*

between the studied species aside from their group (Table 2a).

According to the second method (Fig. 2), *A. akallopisos* and *D. aruanus* have a mean relative mucus production of 0.01 mg cm^{-2}, $n = 4$ and 0.72 mg cm^{-2}, $n = 5$, respectively, and the carapid species *E. gracilis* had a significantly higher mucus production (3.69 mg cm^{-2}, $n = 3$). As for the first method, statistical analyses show that the mucus production of the carapid group is significantly different from the mucus production of the control fish group ($p < 0.05$), and a significant difference of mucus production between studied species aside from their group was detected (Table 2a).

As mucus production was quantified with two methods, a statistical analysis was conducted to compare their results and showed no significant difference between both methods ($p > 0.05$) (Table 2b).

ß-glucosidase detection and mucus effect on saponins

The minimal detection limit of the applied kit is 2 U L^{-1} that corresponded to an optical density of 0.2 according to our calibration curve with commercial ß-glucosidase ($y = 0.0366x + 0.1487$; $R^2 = 0.98$). Negative and positive controls demonstrated the reliability of the procedure. Different assays on mucus showed an optical density below 0.1 for each individual of every species of carapids and of control fishes. Therefore, no ß-glucosidase activity was detected in the fish mucus.

The MALDI-ToF mass spectrum obtained by analyzing the mixture between *E. gracilis* mucus and *H. scabra* saponins is dominated by $[M + Na]^+$ ions because of the high affinity of saponins for sodium cations (Van Dyck et al. 2009) (Fig. 3). Five ions peaks were identified as saponins

Table 3 Identifications of major sterols according to their ionic peaks (*m/z*) and their presence in each group

No	RT	*m/z*	Compound	Unsat.	Control fishes	Hosts	Carapids
							Δ^7-sterols
1	1.48	485	4-Methylergosta-7,24-dien-3ß-ol	$\Delta^{7,24}$		x	x
2	1.13	485	Stigmasta-7,24(24)-dien-3ß-ol	$\Delta^{7,24}$	x	x	x
3	1.02	442	24-Norcholesta-5,7,22-dien-3ß-ol	$\Delta^{5,7,22}$		x	
4	1.09	471	24-Methylcholesta-7,22-dien-3β-ol	$\Delta^{7,22}$	x	x	x
5	1.01	457	Cholesta-7,22-dien-3ß-ol	$\Delta^{7,22}$	x	x	
6	1.29	487	Stigmasta-7-en-3ß-ol	Δ^{7}		x	
7	1.06	459	Cholesta-7-en-3ß-ol	Δ^{7}	x	x	x
8	1.25	473	Unknown 1	Δ^{7}		x	
							Δ^5-sterols
9	1.22	485	Stigmasta-5,24-dien-3ß-ol	$\Delta^{5,24}$	x	x	x
10	1.15	457	Cholesta-5,24-dien-3ß-ol	$\Delta^{5,24}$	x	x	
11	1.09	472	24-Methylcholesta-5,24(28)-dien-3β-ol	$\Delta^{5,24}$	x		x
12	1.04	471	Ergosta-5,22-dien-3ß-ol	$\Delta^{5,24}$		x	
13	1.00	459	Cholesta-5-en-3ß-ol	Δ^{5}	x	x	x
14	1.20	487	24-Ethylcholesta-5-en-3ß-ol	Δ^{5}	x	x	x
15	1.13	472	24-Methylcholesta-5-en-3β-ol	Δ^{5}		x	
16	1.10	473	Campesta-5-en-3ß-ol	Δ^{5}		x	
							Unknown sterols
17	1.17	466	Unknown 2	–		x	
18	1.04	458	Unknown 3	–		x	
19	1.01	484	Unknown 4	–		x	

No Identification number of compound; *RT* Retention relative time; *Unsat.* Unsaturation position(s)

mainly based on their fragmentation behaviors observed in the MSMS spectra (Fig. 3; *m/z* 1211, *m/z* 1213, *m/z* 1227, *m/z* 1243 and *m/z* 1449). The saponins detected at *m/z* $[M + Na]^+$ 1227 and 1243 are likely to correspond to Scabraside A and Scabraside B, respectively, already described by Han et al. (2009). The saponin detected at *m/z* $[M + Na]^+$ 1449 might correspond to Holothurinoside G described by Van Dyck et al. (2009) and the saponin detected at m/z $[M + Na]^+$ 1213 correspond to Pervicoside C described by Caulier et al. (2013). Fragmentation patterns of saponins detected at *m/z* $[M + Na]^+$ 1211 did not afford a clear-cut evidence of their nature. Finally, a direct comparison between the MALDI-ToF spectra of saponins on the one hand and of mucus on the other hand revealed that other peaks at *m/z* 339, *m/z* 361, *m/z* 429, *m/z* 467, *m/z* 537, *m/z* 611, *m/z* 699, *m/z* 875 and *m/z* 1037 are specific to the mucus. We did not attempt to identify them further.

Sterolic composition analysis

Chromatographic analyses of the sterolic extracts from host, pearlfishes and control fishes showed 63 different peaks. To gain in clarity, only 19 major sterols (>3 % of total sterol content within at least one specie) will be discussed below, including 15 that were tentatively identified according to their fragmentation pattern, their GC–MS relative retention time (RT), their mass-to-charge ratio with TMS group (*m/z*) and their double-bond position(s) (Unsat.) (Table 3). Fragmentation patterns were compared with those from the Wiley275.L Mass Spectral Library and literature data (Brooks et al. 1968; Goad and Akihisa 1997; Knights 1967; Moreau et al. 2002). Some minor sterols were also identified with GC–MS although they were not detected by GC-FID. They have been already described as "trace" in the literature.

Control fishes

The sterol profiles of control fishes are quite simple (Fig. 4). Gas chromatography showed several sterol peaks, including Cholesta-5-en-3ß-ol (13), also known as cholesterol, that was clearly predominant with a relative abundance between 79 and 94 %. 24-Ethylcholesta-5-en-3ß-ol (14) was the only one with Cholesta-5-en-3ß-ol (13) to be present in each control species, but its relative abundance did not exceed 8 %. Stigmasta-5,24-dien-3ß-ol (9) and 24-Methylcholesta-5,24(28)-dien-3β-ol (11) were rather well represented in *A. ocellaris*. Stigmasta-5,24-dien-3ß-ol (9) was also present in small quantities in *D. aruanus*, *A. clarkii* and *A. akallopisos*, whereas 24-Methylcholesta-5,24(28)-dien-3β-ol (11) was only detected in *A. akallopisos*

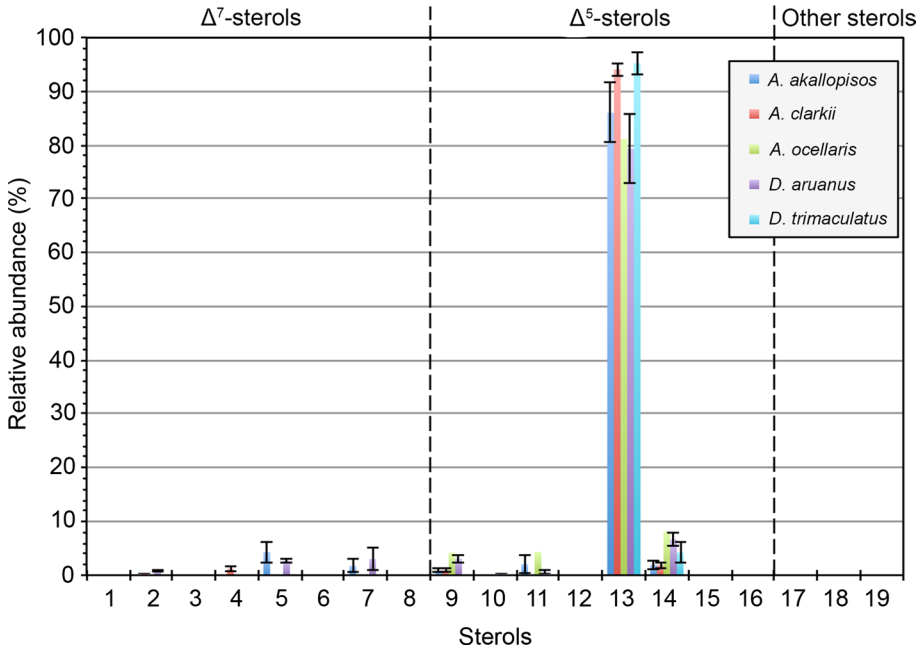

Fig. 4 Relative mean abundance of sterols in control fishes. Identification numbers (Table 2) are used to represent sterols in figures. Δ^7-sterols: on the *left*. Δ^5-sterols: at the center. Non-identified sterols on the *right*. *Error bars* represent standard deviation of the mean

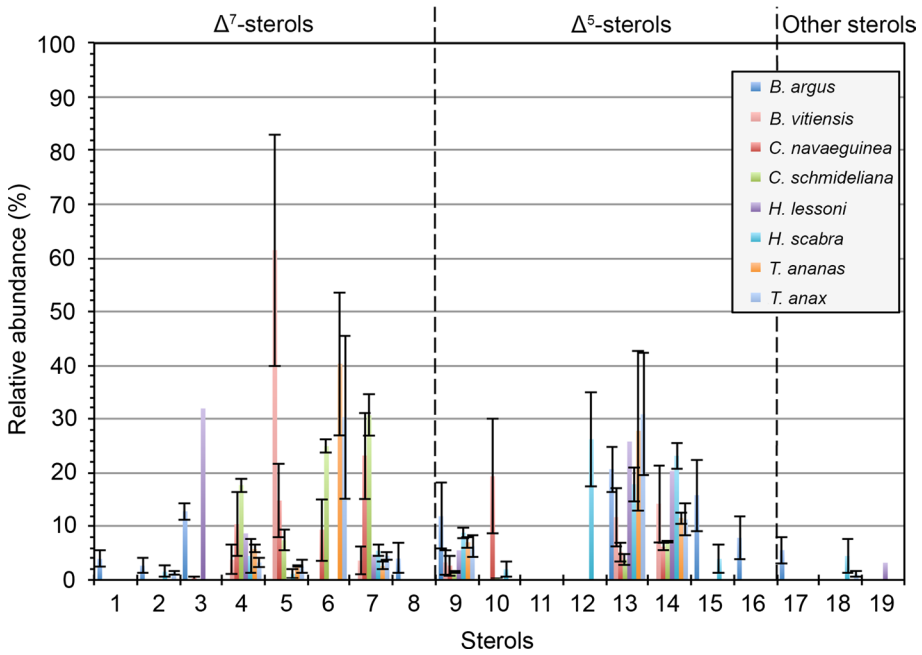

Fig. 5 Relative mean abundance of sterols in hosts. Identification numbers (Table 2) are used to represent sterols in figures. Δ^7-sterols: on the *left*. Δ^5-sterols: at the center. Non-identified sterols on the *right*. *Error bars* represent standard deviation of the mean

and Cholesta-5,24-dien-3ß-ol (10) in *D. aruanus*. *A. akallopisos* and *D. aruanus* were the only species that displayed Δ^7-sterols, namely Cholesta-7,22-dien-3ß-ol (5) and Cholesta-7-en-3ß-ol (7), with a relative abundance higher than 1.75 %. *A. clarkii* also showed a Δ^7-sterol, namely 24-Methylcholesta-7,22-dien-3β-ol (4), but with a relative abundance of 1 %. Stigmasta-7,24(24)-dien-3ß-ol (2) (i.e., Δ^7-sterol) was detected in small quantities in *A. clarkii* and *D. aruanus*, whereas *A. ocellaris* and *D. trimaculatus* did not contain any Δ^7-sterols. However, the sterolic profiles of control fishes were overall dominated by Δ^5-sterols.

Echinoderm hosts

The sterol profiles of hosts are more complex than those previously described (Fig. 5). Gas chromatography showed a lot of sterol peaks, but none exceeded a relative abundance of 65 %. Each species still contained Δ^5-sterols but with lower relative abundances than control fishes. Cholesta-5-en-3ß-ol (13) and Stigmasta-5,24-dien-3ß-ol (9) were the only sterols present in each host species with respective relative abundance surrounding 3–31 and 1–12 %. 24-Ethylcholesta-5-en-3ß-ol (14) was present in all host

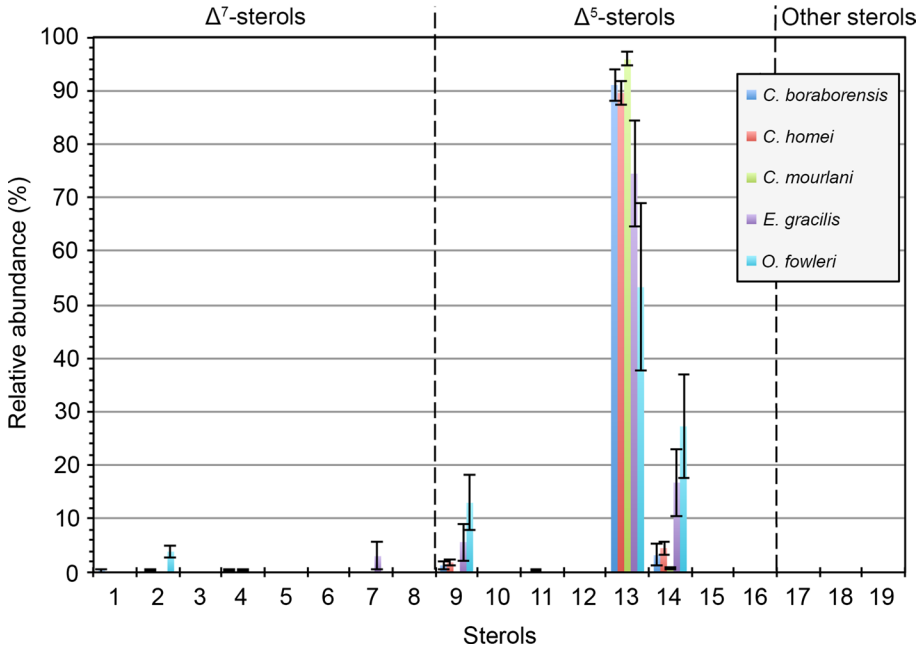

Fig. 6 Relative mean abundance of sterols in carapids. Identification numbers (Table 2) are used to represent sterols in figures. Δ^7-sterols: on the *left*. Δ^5-sterols: at the center. Non-identified sterols on the *right*. *Error bars* represent standard deviation of the mean

species except *B. argus* with a relative abundance surrounding 6–23 %. Cholesta-5,24-dien-3ß-ol (10) was particularly abundant in *C. novaeguinea* and detected in small quantity in *H. scabra* (2 %) and *C. schmideliana* (0.2 %). Ergosta-5,22-dien-3ß-ol (12) and 24-Methylcholesta-5-en-3β-ol (15) were specific to *H. scabra*, while Campest-5-en-3ß-ol was specific to *B. argus*. All host species were characterized by a high relative abundance of Δ^7-sterols. 24-Methylcholesta-7,22-dien-3β-ol (4), Cholesta-7,22-dien-3ß-ol (5) and Cholesta-7-en-3ß-ol (7) were found in all species except *B. argus* with variable relative abundance ranging from 1 to 61 %. Stigmasta-7-en-3ß-ol (6) was only present in *Culcita* and *Thelenota* genera with relative abundance ranging from 9 to 40 %. 4α-Methyl-5α-Ergosta-7,24-dien-3ß-ol (1) and "Unknown 4" (8) were specific of *B. argus*. Stigmasta-7,24(24)-dien-3ß-ol (2) was detected in *B. argus*, *H. scabra* and *T. anax* with relative abundance ranging from 1.5 to 3 %, while 24-Norcholesta-7,22-dien-3ß-ol (3) was present in *B. argus*, *C. novaeguinea* and *H. lessoni* with relative abundance ranging from 0.5 to 32 %. Host species also presented some unknown sterols with undetermined double bond.

Carapids

Similarly to control fishes, the sterol profiles of carapids are quite simple (Fig. 6). Gas chromatography showed several sterol peaks with a wider standard deviation, especially for *E. gracilis* and *O. fowleri*, reflecting a wider individual variability. However, Cholesta-5-en-3ß-ol (13) was clearly predominant with a relative abundance ranging from 53 to 96 %. 24-Ethylcholesta-5-en-3ß-ol (14) was the only one with Cholesta-5-en-3ß-ol (13) to be present in each species of studied control fishes but with lower relative abundances ranging from 0.2 to 27 %. Stigmasta-5,24-dien-3ß-ol (9) was present in all carapids species except *C. mourlani* with relative abundance ranging from 1 to 13 %, while 24-Methylcholesta-5,24(28)-dien-3β-ol (11) was detected in only small quantity in *C. homei*. *C. boraborensis* was the only carapid to present two Δ^7-sterols in low quantities, namely 4-Methylergosta-7,24-dien-3ß-ol (1) and 24-Methylcholesta-7,22-dien-3β-ol (4). 24-Methylcholesta-7,22-dien-3β-ol (4) was also present in *C. mourlani* in low quantity. *E. gracilis* also displayed a Δ^7-sterol, 5α-Cholesta-7-en-3ß-ol (7) but with a relative abundance of 3 %. Stigmasta-7,24(24)-dien-3ß-ol (2) was present in *C. homei* and in *O. fowleri* with respective relative abundance of 0.5 and 4 %.

The PERMANOVA analysis showed a significant difference in the sterolic compositions among groups ($F = 51.982$, $p = 0.001$). Pairwise comparisons only detected significant difference between hosts and other groups: carapids ($p = 0.001$) and control fishes ($p = 0.001$). No difference was detected between carapids and control fishes ($p = 0.305$). Indicator compound analysis showed that cholesterol was significantly associated with the control fish/carapid group and that other Δ^5-sterols, Δ^7-sterols and unknown sterols were significantly associated with the host group. The different group associations were illustrated by the hierarchical cluster analysis (Fig. 7). The figure displays an interlacing between carapid and control fishes groups and an almost exclusive isolation of host group except for three carapid samples that cluster into the host group.

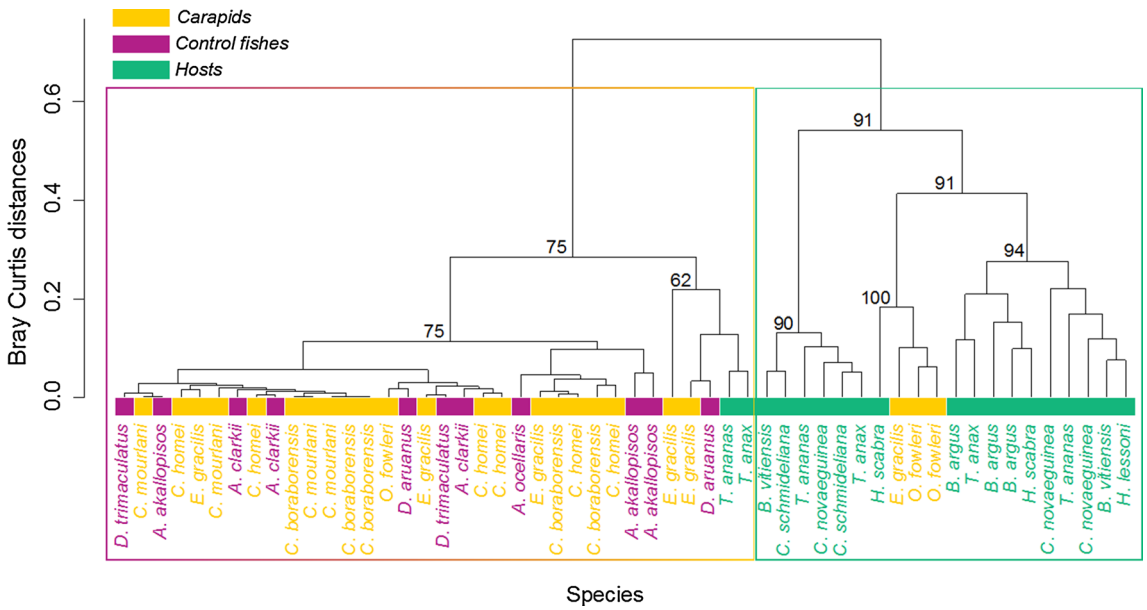

Fig. 7 Hierarchical cluster analysis (HCA) based on categorized sterols: cholesterol, other Δ^5-sterols, Δ^7-sterols and unknown sterols. Bootstrap values are present above nodes

Discussion/conclusion

Mucus quantification

The present study is the first to quantify the amount of mucus produced by fishes and confirms previous observations made on pearlfishes (Trott 1970; Parmentier and Vandewalle 2005). For both methods, mucus quantities collected from different species showed that carapids produced from six (for the first method) to ten times (for the second one) more mucus than control fishes. This difference may be explained by the particular infestation behavior of carapids that infest their hosts through the cloacum (or buccal cavity for *C. mourlani*) and then need to be lubricated for easier intromission (Parmentier and Vandewalle 2003). However, Trott (1970) observed that mucus production increases when carapids are exposed to extracts of Cuvierian tubules, suggesting the implication of mucus in saponin resistance. The large quantity of mucus could also be a factor of resistance by mechanical protection as mucus could form a physical barrier that prevents saponins to interact with cholesterol residues (Cone 2009).

Degradation of saponins by mucus

The enzymatic test showed no ß-glucosidase activity in the mucus, regardless of the fish species. However, the test sensitivity (i.e., 2 U L^{-1}) may be too low to detect enzymes in mucus. Althunibat et al. (2009) have shown that a concentration ranging from 0.003 to 0.015 g L^{-1} of *H. scabra* saponins is needed to lyse 50 % of cells (IC50). Given that ±1 000 g mol^{-1} is the average molar mass for saponins, a concentration ranging from 3 to 15.5 U L^{-1} of saponins is required to lyse 50 % of cells. An enzymatic activity of 3 U L^{-1} would then be the minimum requirement to compensate saponin effects. Therefore, the detection limit of our assay corresponds to the threshold of enzymatic activity expected to validate our hypothesis. Moreover, as the mucus was lyophilized immediately after sampling and stored at 5 °C, potential degradation of enzyme may be ruled out.

Although we used a ß-glucosidase assay, glucose is one of the most common sugars found in holothuroid saponin. Glycone moieties, other enzymes or secondary metabolites could alter saponin molecular structures. For example, lectins could hitch the saccharidic part of saponins to inhibit their amphiphilic character (Tsutsui et al. 2011). However, since mass spectrometry analyses of mucus/saponin mixes did not highlight any saponin degradation, the chemical composition of mucus is probably not involved in saponin resistance for the studied species. Among the saponins detected in the *H. scabra* extract, the peak at *m/z* $[M + Na]^+$ 1211 remains to be identified. As its pattern of fragmentation did not enable us to determine its structure accurately, an extensive study is needed. Another hypothesis is a molecule only present in mucus gills, which can inhibit the saponin activity (Tsutsui et al. 2011).

Sterolic composition analysis

Tissues of control fishes are seemingly constituted with a majority of Δ^5-sterols (especially cholesterol and sitosterol), whereas Δ^7-sterols are present in smaller quantities (<7 %) and are even absent in *A. ocellaris* samples corroborating previous studies (Morris et al. 1982). This sterolic composition of free-living fish tissues explains their sensibility to saponins and their limited survival time.

Conversely, hosts display a lower proportion of Δ^5-sterols (13–56 % except for *H. scabra*) and a larger proportion of Δ^7-sterols (23–81 % except for *H. scabra)*, which is in line with other studies and confirms the involvement of Δ^7-sterols in saponin resistance (Ballantine et al. 1981; Drazen et al. 2008; Goad 1983; Goad et al. 1972; Kicha et al. 2001; Ponomarenko et al. 2001; Shubina et al. 1998; Smith et al. 1973; Stonik et al. 1998). For non-elucidated reasons, the double bond in position 7 decreases interactions with the aglycone part of saponins and avoids cell lysis (Popov et al. 1983). However, the present study suggests that a large proportion of Δ^7-sterols is not essential to resist to saponins since *B. argus* and *H. scabra* have, respectively, only 23 and 13 % of Δ^7-sterols in average. The sterolic profile of the hosts is highly diversified with some unknown sterols, which reflects the complexity of their sterolic tissue composition. Moreover, our results show a high intraspecific variability that influences the average quantities. This variability could be partly due to GC-FID. For example, a variation of 1 ml min^{-1} in the carrier gas flow rate can cause variation of peak intensity between 1 and 5 % (Barwick 1999). Caution has to be paid with regard to sterol identification as it was based on fragmentation patterns. Except of cholesterol, all identifications remain hypothetical. However, the global Δ^7-sterol contents in hosts were always higher than those in control fishes, whereas cholesterol content did not exceed 31 % and was probably replaced with other sterols. This difference among sterols could reflect the adaptation of hosts to resist to their own chemical defenses. No specific Δ^7-sterol was found in all species of echinoderms, but they displayed a large diversity. Moreover, some seemed species specific, like 4α-Methyl-5α-Ergosta-7,24-dien-3ß-ol observed only in *B. argus*, or (22E)-5α-cholesta-7,22-dien-3ß-ol detected with a relative abundance of 61 % in *B. vitiensis.* Like with control fishes, carapid tissues contain a majority of Δ^5-sterols (especially cholesterol) and only little Δ^7-sterols (<4 %).

Statistical analyses confirm the preceding views: PERMANOVA and pairwise comparisons show the similarity between control fishes and carapids and distinguish the host group, which strongly suggests that saponin resistance of carapids is then probably not related to their sterolic composition that is obviously not mimetic to the composition of their hosts. Moreover, the indicator compound analysis allows to explain the vulnerability of control fishes (individualized by cholesterol—the main saponin target) and the resistance of hosts (individualized by other Δ^5-sterols, Δ^7-sterols and unknown sterols) to saponin toxicity. The specificity of cholesterol among carapids allows to rule out the sterolic hypothesis in carapids resistance. The dendrogram obtained by hierarchical cluster analysis illustrates the previous results and analyses (Fig. 7). The presence of *E. gracilis* and two hosts in the wrong group can be explained by the high intraspecific variability, previously discussed, and by the lower content in cholesterol and the higher content in other Δ^5-sterols that other *E. gracilis* replicates. Two out of three *O. fowleri* individuals grouped with hosts, which probably could also be explained by a lower content in cholesterol and a higher content in other Δ^5-sterols. However, *O. fowleri* are not symbiotic to echinoderms, but to bivalves. Therefore, the possible similarity to the host group cannot be related to a hypothetical saponin resistance because these carapids are not naturally exposed to saponins.

In conclusion, this study allows to rule out some hypotheses concerning the resistance of carapids to echinoderm saponins: (1) Carapid mucus has no enzymatic components that act on saponins and (2) the sterolic composition of carapid cytoplasmic membranes is different from that of the echinoderm hosts. This study demonstrates that carapids produce a high quantity of mucus, much more than control fishes. This characteristic is the only difference found between two groups and is probably the only parameter that allows carapids to resist against host saponins.

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References

Althunibat OY, Bin Hashim R, Taher M et al (2009) In vitro antioxidant and antiproliferative activities of three Malaysian sea cucumber species. Eur J Sci Res 37:376–387

Armah CN, Mackie AR, Roy C et al (1999) The Membrane-permeabilizing effect of avenacin A-1 involves the reorganization of bilayer cholesterol. Biophys J 76:281–290. doi:10.1016/S0006-3495(99)77196-1

Augustin JM, Kuzina V, Andersen SB, Bak S (2011) Molecular activities, biosynthesis and evolution of triterpenoid saponins. Phytochemistry 72:435–457. doi:10.1016/j.phytochem.2011.01.015

Bader G, Hiller K (1987) Neue Ergebnisse zur Struktur und Wirkungsweise von Triterpensaponinen. Pharmazie 42:577–597

Bakus GJ (1968) Defensive mechanisms and ecology of some tropical holothurians. Mar Biol 2:23–32. doi:10.1007/BF00351634
Ballantine JA, Lavis A, Morris RJ (1981) Marine sterols. XV. Sterols of some oceanic holothurians. J Exp Mar Bio Ecol 53:89–103. doi:10.1016/0022-0981(81)90086-1
Barwick VJ (1999) Sources of uncertainty in gas chromatography and high-performance liquid chromatography. J Chromatogr A 849:13–33
Benndorf J (1987) Predation. Direct and Indirect Impacts on Aquatic Communities. University Press of New England
Brooks CJW, Horning EC, Young JS (1968) Characterization of sterols by gas chromatography-mass spectrometry of the trimethylsilyl ethers. Lipids 3:391–402. doi:10.1007/BF02531277
Burnell DJ, Apsimon JW (1983) Echinoderm Saponins. In: Marine natural products: chemical and biological perspectives. Academic Press Inc., pp 287–379
Castro-Aguirre JL, Garcia-Dominguez F, Balart EF (1996) Nuevos hospederos y datos morfometricos de Encheliophis dubius (Ophidiiformes: Carapidae) en el Golfo de Californica, Mexico. Rev Biol Trop 44:753–756
Caulier G, Flammang P, Rakotorisoa P et al (2013) Preservation of the bioactive saponins of *Holothuria scabra* through the processing of trepang. Cah Biol Mar 54:685–690
Cimino G, Ghiselin T (2001) Marine natural products chemistry as an evolutionary narrative. In: McClintock JB, Baker BJ (eds) Marine chemical ecology. CRC Press, Boca Raton, pp 115–154
Cone RA (2009) Barrier properties of mucus. Adv Drug Deliv Rev 61:75–85. doi:10.1016/j.addr.2008.09.008
Demeyer M, De Winter J, Caulier G et al (2014) Molecular diversity and body distribution of saponins in the sea star Asterias rubens by mass spectrometry. Comp Biochem Physiol Part B Biochem Mol Biol 168:1–11. doi:10.1016/j.cbpb.2013.10.004
Demeyer M, Wisztorski M, Decroo C et al (2015) Inter- and intra-organ spatial distributions of sea star saponins by MALDI imaging. Anal Bioanal Chem. doi:10.1007/s00216-015-9044-0
Drazen JC, Phleger CF, Guest MA, Nichols PD (2008) Lipid, sterols and fatty acid composition of abyssal holothurians and ophiuroids from the North-East Pacific Ocean: food web implications. Comp Biochem Physiol Part B Biochem Mol Biol 151:79–87. doi:10.1016/j.cbpb.2008.05.013
Eeckhaut I, Caulier G, Brasseur L et al (2015) Effects of holothuroid ichtyotoxic saponins on the gills of free-living fishes and symbiotic pearlfishes. Biol Bull 228:253–265
Emery DC (1880) Fierasfer. Studî intorno alla sistematica, l'anatomia e la biologia delle specie mediterranee di questo digenere. Roma
Flammang P (2002) Biomechanics of adhesion in sea cucumber Cuvierian tubules (Echinodermata, Holothuroidea). Integr Comp Biol 42:1107–1115. doi:10.1093/icb/42.6.1107
Francis G, Kerem Z, Makkar HPS, Becker K (2002) The biological action of saponins in animal systems: a review. Br J Nutr 88:587. doi:10.1079/BJN2002725
Glauert AM, Dingle JT, Lucy JA (1962) Action of saponin on biological cell membranes. Nature 196:953–955. doi:10.1038/196953a0
Goad LJ (1983) Steroid biochemistry of marine invertebrates. Mar Chem 12:225. doi:10.1016/0304-4203(83)90087-7
Goad LJ, Akihisa T (1997) Analysis of sterols, 6th edn. Blackie Academic & Professional, London
Goad LJ, Rubinstein I, Smith AG (1972) The sterols of echinoderms. Proc R Soc B Biol Sci 180:223–246. doi:10.1098/rspb.1972.0016
Ida H, Sano M, Kawashima T, Yasuda F (1977) New records of a pomacentrid fish, *Dascyllus melanurus* and Cirrhitid Fish, *Paracirrhites hemistictus* from Japanese Waters. Jpn J Ichthyol 24:213–217
Jangoux M (1984) Diseases of echinoderms. Helgolander meeresunters 37:207–216
Kalinin VI, Prokofieva NG, Likhatskaya GN et al (1996) Hemolytic activities of triterpene glycosides from the holothurian order dendrochirotida: some trends in the evolution of this group of toxins. Toxicon 34:475–483. doi:10.1016/0041-0101(95)00142-5
Kicha AA, Ivanchina NV, Gorshkova IA et al (2001) The distribution of free sterols, polyhydroxysteroids and steroid glycosides in various body components of the starfish Patiria (= Asterina) pectinifera. Comp Biochem Physiol Part B Biochem Mol Biol 128:43–52. doi:10.1016/S1096-4959(00)00317-1
Knights BA (1967) Identification of plant sterols using combined GLC/Mass spectrometry. J Chromatogr Sci 5:273–282
Kofler L (1927) Die saponine. Springer Vienna, Vienna
Li R, Zhou Y, Wu Z, Ding L (2006) ESI-QqTOF-MS/MS and APCI-IT-MS/MS analysis of steroid saponins from the rhizomes of *Dioscorea panthaica*. J Mass Spectrom 41:1–22. doi:10.1002/jms.988
Machida Y (1989) New distribution records of the pearlfish, *Carapus mourlani*, with notes on its morphometry. Jpn J Ichthyol 36:363–368. doi:10.1007/F02905622
Mackie AM, Singh HT, Owen JM (1977) Studies on the distribution, biosynthesis and function of steroidal saponins in echinoderms. Comp Biochem Physiol Part B Comp Biochem 56:9–14. doi:10.1016/0305-0491(77)90214-0
Maier MS (2008) Biological activities of sulfated glycosides from echinoderms. Bioact Nat Prod 35:311–354. doi:10.1016/S1572-5995(08)80008-7
Markle DF, Olney JE (1990) Systematics of the pearlfishes (Pisces:carapidae). Bull Mar Sci 47:269–410
Meyer-Rochow VB (1977) Comparison between 15 *Carapus mourlani* in a Single Holothurian and 19 C. mourlani from Starfish. Copeia 1977:582. doi:10.2307/1443286
Meyer-Rochow VB (1979) Stomach and gut contents of *Carapus mourlani* from starfish and a holothurian. Ann Zool Fenn 16:287–289
Moreau RA, Whitaker BD, Hicks KB (2002) Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Prog Lipid Res 41:457–500. doi:10.1016/S0163-7827(02)00006-1
Morris RJ, Ballantine JA, Roberts JC, Lavis A (1982) The sterols of some marine teleosts. Comp Biochem Physiol Part B Comp Biochem 73:481–484. doi:10.1016/0305-0491(82)90063-3
Nigrelli RF (1952) The effect of holothurin on fish, and mice with sarcoma 180. Zoologica 37:89–90
Paredes-Ríos GA, Balart EF, Paredes-Rios GA (1999) Corroboration of the Bivalve, Pinna rugosa, as a Host of the Pacific Pearlfish, *Encheliophis dubius* (Ophidiiformes: Carapidae), in the Gulf of California, México. Copeia 1999:521. doi:10.2307/1447503
Parmentier E (2003) Contribution à l'étude des relations entre des poissons de la famille des Carapidae et leurs hôtes invertébrés : une approche multidisciplinaire. University of Liege
Parmentier E, Castillo G, Chardon M, Vandewalle P (2000) Phylogenetic analysis of the pearlfish tribe Carapini (Pisces: Carapidae). Acta Zool 81:293–306
Parmentier E, Vandewalle P (2003) Morphological adaptations of Pearlfish (Carapidae) to their various Habitats. In: Val AL (ed) Fish adaptations. Science Pu, Oxford, pp 261–276
Parmentier E, Vandewalle P (2005) Further insight on carapid holothuroid relationships. Mar Biol 146:455–465. doi:10.1007/s00227-004-1467-7
Ponomarenko LP, Kalinovsky AI, Moiseenko OP, Stonik VA (2001) Free sterols from the holothurians *Synapta maculata*, *Cladolabes bifurcatus* and Cucumaria sp. Comp Biochem Physiol Part B Biochem Mol Biol 128:53–62. doi:10.1016/S1096-4959(00)00318-3
Popov AA, Kalinovskaia NI, Kuznetsova TA et al (1983) Role of sterols in the membranotropic activity of triterpene glycosides. Antibiotiki 28:656–659

Shen SC, Yeh HS (1987) Study on pearlfishes (Ophidiiformes : Carapidae) of Taiwan. J Taiwan Museum 40:45–56

Shubina LK, Fedorov SN, Levina EV et al (1998) Comparative study on polyhydroxylated steroids from echinoderms. Comp Biochem Physiol Part B Biochem Mol Biol 119:505–511. doi:10.1016/S0305-0491(98)00011-X

Smith CL (1964) Some pearlfishes from Guam, with notes on their ecology. Pacific Sci 18:34–40

Smith CL, Tyler JC (1969) Observations on the commensal relationship of the Western Atlantic Pearlfish, *Carapus bermudensis*, and Holothurians. Copeia 1969:206. doi:10.2307/1441726

Smith AG, Rubinstein I, Goad LJ (1973) The sterols of the echinoderm *Asterias rubens*. Biochem J 135:443–455

Stonik VA, Ponomarenko LP, Makarieva TN et al (1998) Free sterol compositions from the sea cucumbers Pseudostichopus trachus, Holothuria (Microtele) nobilis, *Holothuria scabra*, *Trochostoma orientale* and *Bathyplotes natans*. Comp Biochem Physiol Part B Biochem Mol Biol 120:337–347. doi:10.1016/S0305-0491(98)10023-8

Stonik A, Kalinin VI, Avilov SA (1999) Toxins from sea cucumbers (holothuroids): chemical structures, properties, taxonomic distribution, biosynthesis and evolution. J Nat Toxins 8:235–248

Trott LB (1970) Contribution of the biology of carapid fishes (Paracanthopterygian: Gadiformes). Univ Calif Publ Zool 89:1–41

Trott LB, Chan WL (1972) *Carapus homei* commensal in the mantle cavity of Tricadna sp. in the South China Sea. Copeia 872–873

Trott LB, Trott EB (1972) Pearlfishes (Carapidae: Gadiforme) collected from *Puerto Galera*, Minobra, Philippines. Copeia 839–843

Tsutsui S, Komatsu Y, Sugiura T et al (2011) A unique epidermal mucus lectin identified from catfish (*Silurus asotus*): first evidence of intelectin in fish skin slime. J Biochem 150:501–514. doi:10.1093/jb/mvr085

Tyler JC, Bohlke JE (1972) Records of sponge-dwelling fishes, primary of the Caribbean. Bull Mar Sci 22:601–642

Van Dyck S, Gerbaux P, Flammang P (2009) Elucidation of molecular diversity and body distribution of saponins in the sea cucumber *Holothuria forskali* (Echinodermata) by mass spectrometry. Comp Biochem Physiol Part B Biochem Mol Biol 152:124–134. doi:10.1016/j.cbpb.2008.10.011

Vanderplanck M, Michez D, Vancraenenbroeck S, Lognay G (2011) Micro-quantitative method for analysis of sterol levels in honeybees and their pollen loads. Anal Lett 44:1807–1820. doi:10.1080/00032719.2010.526271

Vanderplanck M, Moerman R, Rasmont P et al (2014) How does pollen chemistry impact development and feeding behaviour of polylectic bees? PLoS ONE 9:e86209. doi:10.1371/journal.pone.0086209

Weber M (1913) Die fische der Siboga-expedition. E. J. Brill, Leiden

Xu D, Cui J, Bansal R et al (2009) The ellipsoidal area ratio: an alternative anisotropy index for diffusion tensor imaging. Magn Reson Imaging 27:311–323. doi:10.1016/j.mri.2008.07.018

Yamanouchi T (1955) On the poisonous substance contained in holothurians. Publ Seto Mar Biol Lab 4:183–203