The Journal of Experimental Biology 214, 1347-1356

doi:10.1242/jeb.050930

RESEARCH ARTICLE

The triterpene glycosides of *Holothuria forskali*: usefulness and efficiency as a chemical defense mechanism against predatory fish

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Accepted 10 January 2011

SUMMARY

More than 100 triterpene glycosides (saponins) have been characterized in holothuroids in the past several decades. In particular, *Holothuria forskali* contains 26 saponins in its Cuvierian tubules and 12 in its body wall. This high diversity could be linked to a chemical defense mechanism, the most commonly accepted biological role for these secondary metabolites. We performed an integrated study of the body-wall saponins of *H. forskali.* The saponins are mainly localized in the epidermis and in the mesothelium of the body wall and appear to be released when the holothuroid is stressed. Among the saponins present in the epidermis, one (holothurinoside G) was detected in the seawater surrounding non-stressed holothuroids and three others (holohurinosides C and F, and desholothurin A) were secreted when the animals were stressed. In addition, two new congeners (detected at *m/z* 1301 and 1317) were also present in the immediate surroundings of stressed holothuroids. These new saponins do not originate from the epidermis and could come from an internal organ. Quantities of secreted saponins were very low compared with the body wall and Cuvierian tubules concentrations. At natural concentrations, saponins do not represent a threat to the health of predatory fish. The deterrent effect of saponins seems therefore to act as an aposematic signal, warning potential predators of the unpalatability of the holothuroid tissues.

Key words: Echinodermata, Holothuroidea, saponin, aposematism, chemical ecology.

INTRODUCTION

The body wall of holothuroids has often been investigated for its spectacular changes in stiffness due to its mutable collagenous tissue (Motokawa, 1984; Wilkie, 1996; Wilkie, 2005) or for its content in toxic secondary metabolites, the so-called saponins (Nigrelli, 1952; Yamanouchi, 1955). It seems therefore to have a major importance in the defense of these slow-moving invertebrates. Some authors consider predation on adult holothuroids to be infrequent (Massin and Jangoux, 1976; Da Silva et al., 1986), but a high number of predators have been referenced in the literature (Francour, 1997). Holothuroids have developed a large range of anti-predator mechanisms (Bingham and Braithwaite, 1986; Lawrence, 1987): thickness of the body wall, toxic and noxious skin and organs, body swelling or stiffening, evisceration or autotomy, swimming, nocturnal activity, and cryptic or burrowing behaviors (Francour, 1997). Some species also possess Cuvierian tubules. These tubules are little caeca located in the posterior part of the animal that can be ejected toward a predator in response to an aggression. Expelled tubules lengthen into sticky white threads that serve to entangle the predator (Hamel and Mercier, 2000; Becker and Flammang, 2010). Among all these mechanisms, the toxicity of the body wall and the Cuvierian tubules seem to be the most effective against non-specialist predators (Bakus, 1968; Bingham and Braithwaite, 1986), whereas other mechanisms, such as swimming movements (Margolin, 1976) or shedding a small piece of the body (Kroop, 1982), may thwart predation and permit escape.

Saponins have long been suggested to play a role in the defense of holothuroids as a toxin (Bakus, 1968; Kalyani et al., 1988). Holothuroid saponins are triterpene glycosides. These secondary metabolites exert their effect through selective binding to cell-membrane sterols. This binding induces the formation of saponin–sterol complexes in the membranes, which significantly disturb the selective permeability of the membranes, leading to a decreased viability of cells and their lysis (Popov, 2002). In view of the numerous effects due to this membranolytic action, the defensive role of saponins seems evident but this assumption has never been proved experimentally in holothuroids. However, triterpene glycosides have been presented recently as defense metabolites in other animals. Kubanek et al. reported that the unpalatable nature of extracts from the sponge *Erylus formosus* was due to their triterpene glycoside content and that these molecules could thus protect the sponge from predatory reef fish (Kubanek et al., 2000). In this species, the triterpene glycosides appear to deter predation, microbial attachment, and fouling by invertebrates and algae; in another species, *Ectyoplasia ferox*, they were shown to be anti-predatory and allelopathic (Kubanek et al., 2002). Plasman et al. detected triterpene glycosides in the defensive secretions of chrysomelid leaf beetles (*Platyphora*

ligata, *P. opima* and *Desmogramma subtropica*) (Plasman et al., 2000a; Plasman et al., 2000b). These secretions are produced by glands localized on the elytra and on the pronotum of adult beetles (Plasman et al., 2001).

In holothuroids, there are no data on the mode of action of saponins as a defense mechanism. The aim of the present study was to perform an integrated study on the saponin mixture from the body wall of the holothuroid *H. forskali*. A mass spectrometry imaging approach was designed to localize saponins in the tissue, and a semi-quantitative experiment was performed to evaluate saponin concentrations in the tissues of the holothuroid. In addition, the release of saponins by the animal in its surroundings was investigated, and ecophysiological tests were performed on fish to better understand the role of these molecules in the defense of the holothuroid.

MATERIALS AND METHODS

Sampling

Specimens of *Holothuria forskali* (Delle Chiaje 1823) were collected at a depth ranging from 20 to 30 m by SCUBA diving at the Biological Station of Banyuls-sur-Mer (Pyrénées-Orientales, France). They were transported to the Marine Biology Laboratory of the University of Mons, where they were kept in a marine aquarium with closed circulation (13°C, 33‰ salinity). Individuals used in the different experiments were in one of three physiological states: relaxed, non-stressed or stressed. Relaxed animals were anaesthetized for 2 h in a 0.1% solution of 1-phenoxy-2-propanol (Sigma-Aldrich, Bornem, Belgium) in seawater. Stressed individuals were mechanically stimulated for 4 h by repetitive hitting using a specific device. The device consisted of three loads hanging from a horizontal rotating bar and immersed in the tank containing the animal. Loads were wrapped in parafilm in order to avoid injuries. Non-stressed individuals were resting animals that were neither anaesthetized nor stressed. For the histological approach, both relaxed and stressed animals were used whereas only non-stressed and stressed animals were used for the detection of saponins released in seawater.

Mediterranean fish *Coris julis* (Linnaeus 1758) and *Symphodus ocellatus* Forsskal 1775 were collected using a fishing net at a depth of 3 m by SCUBA diving at the Station de Recherche Sous-marines et Océanographiques (STARESO) near Calvi, Corse, France. They were kept at the station in a marine aquarium with open circulation.

Localization of saponins

Pieces of body wall from relaxed and stressed holothuroids were prepared for light and transmission electronic microscopy and for matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MSI) (Caprioli et al., 1997).

Light and transmission electron microscopy

Pieces ~3–4 mm in width were cut from the body wall and fixed for 3 h at 4°C in a 3% solution of glutaraldehyde in cacodylate buffer (0.1 mol l^{-1}, pH 7.8, adjusted to 1030 mOsm l^{-1} with NaCl). They were washed in the same buffer, post-fixed for 1 h with 1% osmium tetroxide in the cacodylate buffer and then washed again. Pieces were then dehydrated in graded ethanol and embedded in Spurr resin. Semi-thin (1 μm) sections were realized for light microscopy using an Ultramicrotome Reichter Om U2 (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a glass knife and were stained with a 1:1 mixture of 1% aqueous solution of Methylene Blue in 1% sodium tetraborate and 1% aqueous solution of Azur II. Sections were observed with a Zeiss Axio-scope A1 microscope (Carl Zeiss MicroImaging, Göttingen, Germany). Ultra-thin sections (70 nm) were obtained using a diamond knife mounted on an ultramicrotome Leica Ultracut UCT (Leica Microsystems) and contrasted with uranyl acetate and lead citrate. Sections were finally observed with a Zeiss LEO 906E transmission electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany).

Mass spectrometry imaging

Tissue cryosections (12 μm) were obtained from frozen body-wall pieces using a cryostat (Leica Microsystems) and were deposited onto conductive glass slides coated with indium-tin oxide (Bruker Daltonics, Bremen, Germany). α-Cyano-4-hydroxycinnamic acid (HCCA), trifluoroacetic acid, acetonitrile and aniline (ANI) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). A solid ionic HCCA/ANI matrix was used to image the saponins and was prepared following a previously established procedure (Lemaire et al., 2006). Matrix solution was deposited using ImagePrep (Bruker Daltonics). The images were acquired using an UltraFlex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a Smartbeam™ laser. Images data were performed in positive reflectron mode, and MALDI-MS spectra were acquired in the mass/charge (*m/z*) range of 800 to 3000. A total of 300 spectra were acquired at each spot at a laser frequency of 100 Hz. FlexImaging™ 2.1 software (Bruker Daltonics) was used for molecular image reconstruction. Standards for spectral calibration consisted of a mixed solution of peptides ranging between 900 and 3500 Da.

Extraction, purification and analysis of saponins

Extraction from the body wall and Cuvierian tubules

The extraction and purification procedures were described in detail in a previous paper (Van Dyck et al., 2009). They were repeated on several individuals. Briefly, the homogenized tissue was extracted twice with ethanol:water (70:30) followed by filtration. The extract was then evaporated at low pressure in a double boiler at 30°C using a rotary evaporator (Laborota 4001 efficient, Heidolph Instruments GmbH, Schwabach, Germany). The dry extract was diluted in 90% methanol and partitioned against *n*-hexane (v/v). The water content of the hydromethanolic phase was adjusted to 20% (v/v) and then to 40% (v/v), these solutions being partitioned against CH_2Cl_2 and $CHCl_3$, respectively. Finally, the hydromethanolic solution was evaporated and dissolved in water in order to undergo chromatographic purification. The crude aqueous extract was placed on a column of Amberlite XAD-4 (Sigma-Aldrich, St Louis, MO, USA). Washing the column with water removed the inorganic salts and subsequent elution with methanol allowed us to recover saponins. The methanolic phase was then evaporated and the dry extract was diluted in water in order to undergo a last partitioning against isobutanol (v/v). The butanolic fraction contained the purified saponins.

Extraction from seawater

Holothuroids (stressed and non-stressed) were incubated for 4 h in 1 l of artificial seawater (ASW; 445 mmol l^{-1} NaCl, 60 mmol l^{-1} $MgCl_2.6H_2O$, 10 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 2.4 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} Hepes, pH 8). The saponin extracts from the conditioned water were prepared using a shortened extraction and purification protocol. The conditioned ASW was directly run through the column of the Amberlite XAD-4 for the chromatography step. Subsequent steps were identical to those described above.

Mass spectrometry analysis

All MS experiments were performed on a Q-ToF Premier mass spectrometer (Waters, Milford, MA, USA) in the positive ion mode, either using the MALDI or the electrospray ionization (ESI) source. The MALDI source was a nitrogen laser, operating at 337 nm with a maximum output of 500 mW delivered to the sample in 4 ns pulses at 20 Hz repeating rate. All samples were prepared using a 10 mg ml^{-1} solution of α-cyano-4-hydroxycinnamic acid in acetone as the matrix. The matrix solution (1 µl) was spotted onto a stainless steel target and air-dried. Then, 1 µl of each butanolic fraction was applied onto the spots of matrix crystals and air-dried. Finally, 1 µl droplets of a solution of NaI (2 mg ml^{-1} in acetonitrile) were added to the spots on the target plate. Typical ESI conditions were: capillary voltage, 3.1 kV; cone voltage, 50 V; source temperature, 120°C; and desolvation temperature, 300°C. Dry nitrogen was used as the ESI gas.

For the recording of the single-stage MALDI- or ESI-MS spectra, the quadrupole (rf-only mode) was set to pass ions between *m*/*z* 50 and 1500, and all ions were transmitted into the pusher region of the time-of-flight (TOF) analyzer where they were mass-analyzed with a 1 s integration time. For the MALDI- or ESI-MS/MS collision induced dissociation (CID) 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 90–100 Pa) and the laboratory frame kinetic energy was selected to afford intense enough product ion signals. All the ions exiting the collision cell, either the product ions or the non-dissociated precursor ions, were finally mass measured with the orthogonal-acceleration (oa)-TOF analyzer. Time-of-flight mass analyses were performed in the reflectron mode at a resolution of ~10,000.

For the online liquid chromatography (LC)-MS/MS analyses, a Waters Alliance 2695 liquid chromatography apparatus was used. The HPLC device was coupled to the Q-ToF Premier mass spectrometer (Waters) and consisted of a vacuum degasser, a quaternary pump and an autosampler. Sample volumes of 20 µl were injected. Chromatographic separation was performed on a non-polar column (Symmetry C18, 4.6×150 mm, 5 µm, Waters) at 27°C. The mobile phase (1 ml min^{-1}) was a nonlinear gradient programmed from methanol (eluent A) and water (eluent B). The gradient programmed was: 10% of eluent A at start; 0–6 min, 10 to 60% A; 6–13 min, 60 to 95% A; 13–15 min, back to 10% A. The mobile phase flow (1 ml min^{-1}) was splitted prior to injection in the ESI source (200 µl min^{-1}).

Semi-quantitative study

In addition to the weighting of the dry saponin extract, two complementary techniques – measurement of hemolytic activity and estimation of carbohydrate content using the orcinol reaction (Van Dyck et al., 2010a) – were used to estimate the saponin concentration of the various extracts (i.e. from the body wall, from Cuvierian tubules and from conditioned seawater).

Hemolytic activity

The method was adapted from Kalinin et al. (Kalinin et al., 1996) and from Mackie et al. (Mackie et al., 1968). Citrated blood from cows was used in this experiment. Erythrocytes were pelleted by centrifugation at 1000 ***g*** for 15 min, and were washed and centrifuged three times in cold PBS buffer (140 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} KH_2PO_4, 8.1 mmol l^{-1} $Na_2HPO_4 2H_2O$, pH 7.4) until the supernatant was clear and colorless. Two milliliters of packed cells were then diluted to 100 ml with the same buffer. Twenty microliters of each saponin extract was added to 1980 µl of the erythrocyte suspension in a microtube. The suspensions were mixed by inversion, incubated for 1 h at room temperature and then centrifuged. The extinction of the clear red supernatant was then measured at 540 nm using a Labsystems multiscan MS spectrophotometer (Thermo Scientific, Erembodegem, Belgium). A 10 mg ml^{-1} solution of plant saponins (saponins from *Quillaja* bark; S 4521, Sigma-Aldrich) in PBS was serially diluted to make a standard curve.

Orcinol reaction

This method was adapted from Kabat and Mayer (Kabat and Mayer, 1967). Saponin extracts, a 1.6% aqueous orcinol solution and a 60% sulfuric acid solution were mixed in a 1:1:7.5 (v/v) proportion. The resulting solution was incubated in a water bath at 80°C for exactly 15 min and then cooled in tap water to stop the reaction, and its absorbance was measured at 540 nm. A 10 mg ml^{-1} solution of D-xylose (Merck, Darmstadt, Germany) in water was serially diluted to make a standard curve.

Ecophysiology experiments

The ecophysiological experiments were performed using saponin extracts from the body wall of *H. forskali*. Collected fish (*C. julis* and *S. ocellatus*) were kept in the open circulation aquariums at least 12 h before any experiment. None of these species has been described as a holothuroid predator, but both are generalist carnivores, feeding on macroinvertebrate tissues (Froese and Pauly, 2010). Moreover, other Labridae have been reported to be predators of holothuroids (Francour, 1997). For toxicity tests, fish were introduced into 2 l seawater tanks 2 h before the beginning of the test. Two experiments were conducted: (1) small volumes of saponin extract from the body wall were added progressively, every 30 min to the tank (five replicates for each species); and (2) fish were incubated for 2 h (or until their death) in a 2 l tank in which a given quantity of saponin extract from the body wall (0.5–1 ml) was added (four replicates for each species). For both experiments, the reactions of fish were observed and their respiration rate was estimated every 10 min by counting the number of opercular openings per min.

RESULTS

Tissue localization of saponins

MALDI-MSI was used to localize saponins in the body wall of *H. forskali*. The first analyses were performed directly on frozen transverse body wall sections after matrix deposition. Spectra from tissue direct analyses were acquired randomly across the sections to verify the possibility to detect ions from saponins. Fig. 1 shows a comparison between a spectrum obtained after direct analysis of the tissue section and one from a previous work obtained by a classical MALDI analysis after tissue extraction (Van Dyck et al., 2009). The spectrum obtained from the tissue section presents the same profile, with a conservation of the relative abundance of the ions of the eight major saponins of *H. forskali* (Table 1).

Considering these results, MALDI imaging experiments were realized on transverse tissue sections through the body wall. For each experiment, a zone of ~25 mm^2 was scanned with a resolution of 100×100 µm^2 by averaging 300 laser shots. Fig. 2 presents the repartition of the eight saponin ions (in green) detected at *m*/*z* 1125, 1141, 1287, 1303, 1433, 1449, 1463 and 1479, respectively, for a relaxed and a stressed animal. The localization of particular ions at *m*/*z* 1033 (in red in Fig. 2), uniformly distributed and specific to the body wall, was used as a counterstain for a better visualization of

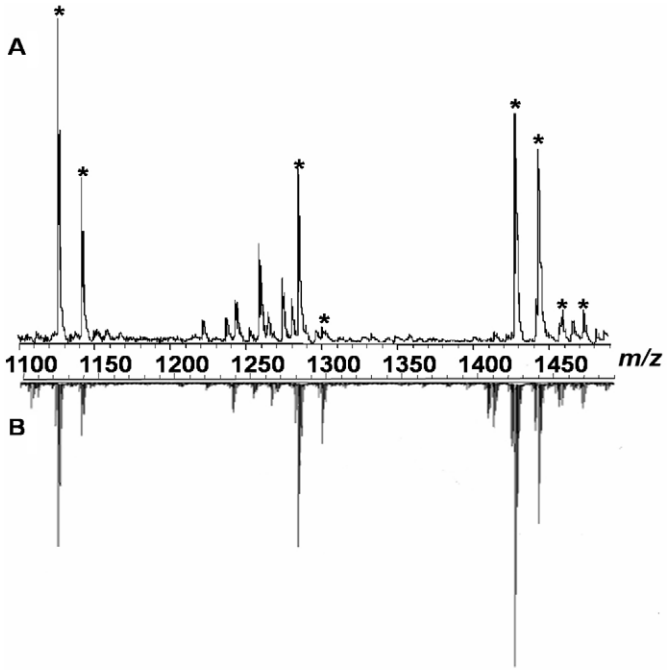

Fig. 1. Comparison of an average mass spectrum of the body wall of *Holothuria forskali* obtained by direct MALDI-MSI analysis (A) and a mass spectrum of saponins after body wall extraction and purification by classical MALDI-MS analysis (B). Saponin ions are marked by an asterisk (see Table 1).

saponins. This counterstain allows us not only to reduce the background noise in the molecular image, but also to achieve a certain 'normalization' for the analyses of the molecular map. In most of the molecular images, a dark zone where no ion was detected was present between the mesothelium and the rest of the dermis. No explanation has yet been found for this unionized zone. An optical image of the tissue section before matrix coating is presented in the first column of Fig. 2. Particular regions are delimited corresponding to the epidermis (upper box), the dermis (middle box) and the mesothelium (lower box). This delimitation, however, is an approximation, as the limits between these different layers are not rectilinear.

Table 1. Complete list of saponins detected by MALDI direct tissue analysis of a body wall tissue section of *Holothuria forskali*

m/z [M+Na$^+$]	Saponin	Molecular formula
1125	Holothurinoside C	$C_{54}H_{86}O_{23}$
1141	Desholothurin A	$C_{54}H_{86}O_{24}$
1287	Holothurinoside E	$C_{60}H_{96}O_{28}$
1303	Holothurinoside A	$C_{60}H_{96}O_{29}$
1433	Holothurinoside F	$C_{66}H_{106}O_{32}$
1449	Holothurinoside G	$C_{66}H_{106}O_{33}$
1463	Holothurinoside H	$C_{67}H_{108}O_{33}$
1479	Holothurinoside I	$C_{67}H_{108}O_{34}$

m/z, mass/charge.

The different saponin ions showed specific localizations within the body wall (Fig. 2). Moreover, their detection also varied with the physiological state of the tissue. In the body wall of relaxed holothuroids, saponins detected at *m/z* 1125, 1433 and 1449 were present only in the epidermis, saponins detected at *m/z* 1303 were present exclusively in the mesothelium and those at *m/z* 1141 were located in both epithelia. The saponin at *m/z* 1287 was observed in the mesothelium as well as in the dermis at very localized points. Finally, saponins detected at *m/z* 1463 and 1479 showed low signals with a lot of noise, but the ions at *m/z* 1463 were mainly observed in the epidermis whereas those at 1479 showed no particular localization. In the body wall of stressed holothuroids, differences in localization were observed for some saponins. Saponin ions detected at *m/z* 1287 and 1303 appeared to be completely absent. The ions detected at *m/z* 1141 were exclusively observed in the epidermis. As for the other epidermal saponins, ions detected at *m/z* 1125 seemed to be less abundant compared with the relaxed tissue whereas ions at *m/z* 1433, 1449 and 1463 seemed to be more abundant. Finally, the saponin at *m/z* 1479 was detected with a poor signal and no specific localization. These results are summarized in Table 2.

To confirm the results obtained by MALDI-MSI, saponins were extracted from the outer part (corresponding mainly to the epidermis and the outer part of the dermis) and from the inner

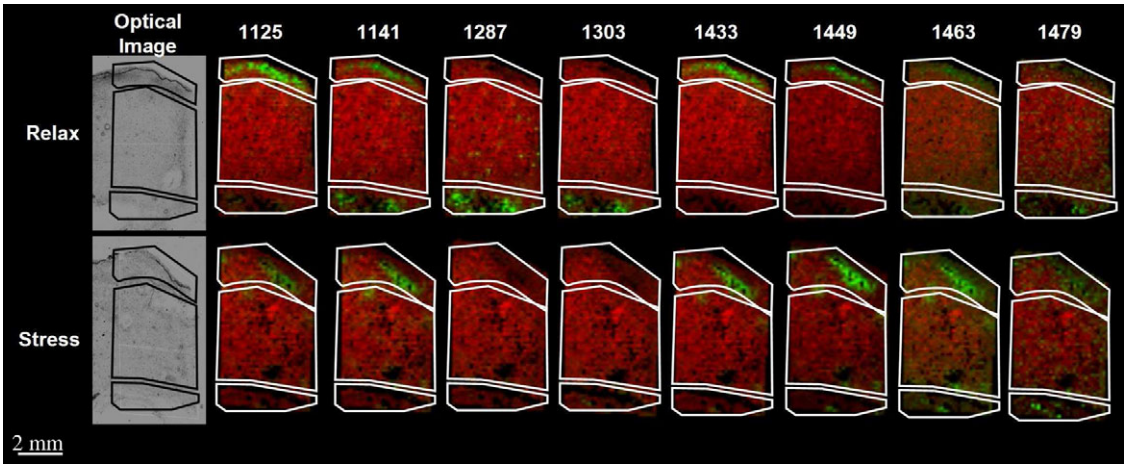

Fig. 2. Molecular images highlighting the localization of saponin ions detected at *m/z* 1125, 1141, 1287, 1303, 1433, 1449, 1463 and 1479, obtained with MALDI-MSI on relaxed and stressed body wall sections of *Holothuria forskali*. Green, saponin ions; red, counterstain. Optical images of the section before matrix deposit are presented in the first column. Tree zones are delimited: epidermis (upper), dermis (middle) and mesothelium (lower).

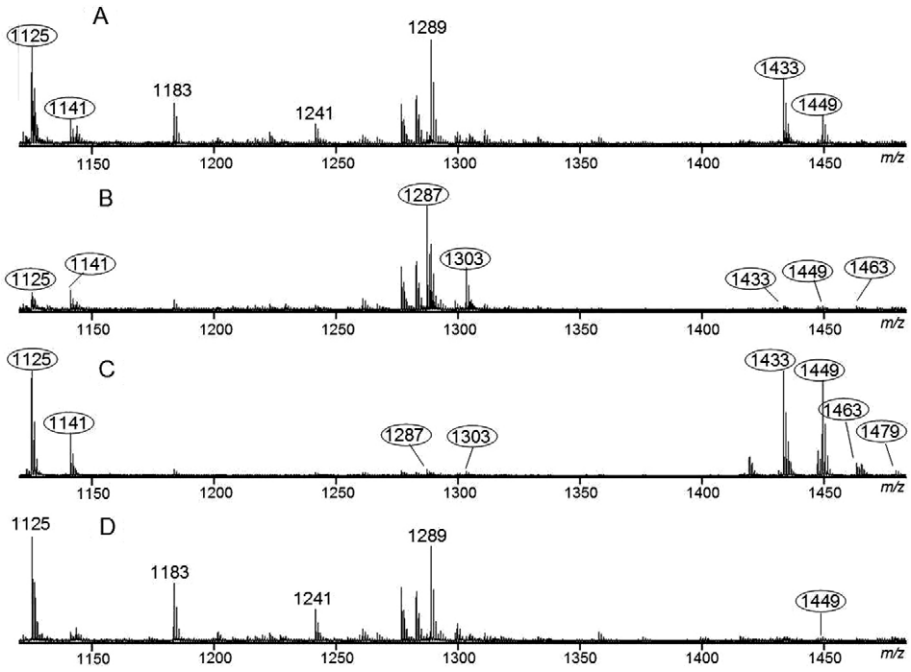

Fig. 3. Full-scan MALDI-TOF mass spectrum analyses of the total saponin mixtures extracted from the outer (A) and inner (B) body wall of a relaxed individual and from the outer (C) and inner (D) body wall of a stressed individual of *Holothuria forskali*. Saponin signals are circled; matrix and polymer contaminant signals are not circled.

part (rest of the dermis and mesothelium) of the body wall for the two conditions. This aim of this experiment was to verify whether the saponin ion distribution showed by MSI was also observable with classical MALDI-TOF mass spectrometry methods. Fig. 3 presents the mass spectra of the different tissue extractions. The results from both techniques are clearly strongly correlated. Indeed, ions that were absent from the molecular image were not detected in the corresponding mass spectrum and *vice versa*. For example, Fig. 3A (outer part of the relaxed body wall) shows the presence of mainly those saponins detected at *m*/*z* 1125, 1141, 1433 and 1449, confirming the MALDI-MSI results (Fig. 2). In the spectrum corresponding to the inner part of the stressed body wall (Fig. 3D), no saponin ions were detected, in agreement with the results shown in Fig. 2, except for the saponins detected at *m*/*z* 1449, which presented a very low signal. Particular attention was paid on the interpretation of the spectra because some contaminant ions, such as the matrix ions detected at *m*/*z* 1289 or the propylene glycol ions at *m*/*z* 1125, 1183 and 1241, were often detected and could be misinterpreted as saponin ions (Fig. 3). As shown in Fig. 4, the ions detected at *m*/*z* 1125 could correspond to the propylene glycol or to the holothurinoside C, but MS/MS analyses allowed verification of the origin of the ions in each spectrum.

Table 2. Localization of saponins in the body wall of relaxed/stressed holothuroids

m/*z* [M+Na$^+$]	Epidermis	Mesothelium
1125	++/+	–/–
1141	+/+	+/–
1287	–/–	+/–
1303	–/–	+/–
1433	+/+	–/–
1449	+/++	–/–
1463	+/+	–/–
1479	–/–	–/–

++, strongly detected; + lightly detected; –, absent.

In order to look more closely at the differences between the body wall of relaxed and stressed individuals, light and transmission electron microscopy (TEM) analyses were performed. Fig. 5 shows TEM pictures of the epidermal zone of a relaxed and a stressed body wall. Both samples present the same epidermal organization, comprising support cells, mucus cells, vacuolar cells and pigment cells [see also Flammang and Jangoux (Flammang and Jangoux, 1992) and VandenSpiegel et al. (VandenSpiegel et al., 1995) for a description of the epidermis in *H. forskali*]. The stressed individual was characterized by the presence of numerous electron-dense granules, measuring ~0.8 μm in diameter, in the cytoplasm of pigment cells. These granules were clearly more numerous in the epidermis of relaxed individuals than in that of stressed animals (no quantification was done, however). In the stressed tissue, the content of these electron-dense granules looked disorganized and many granules even appeared empty (Fig. 5C). This observation

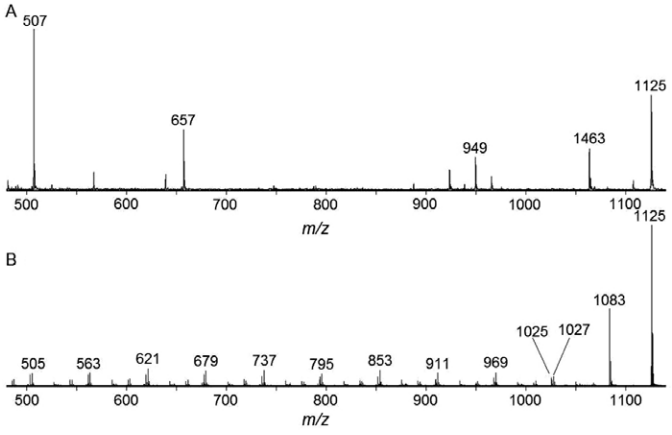

Fig. 4. LC-MS/MS spectra of isomer ions detected at *m*/*z* 1125 in the body wall of *Holothuria forskali*. (A) The saponin holothurinoside C [see Van Dyck et al. (Van Dyck et al., 2009) for molecular structure elucidation]. (B) The propylene glycol polymer.

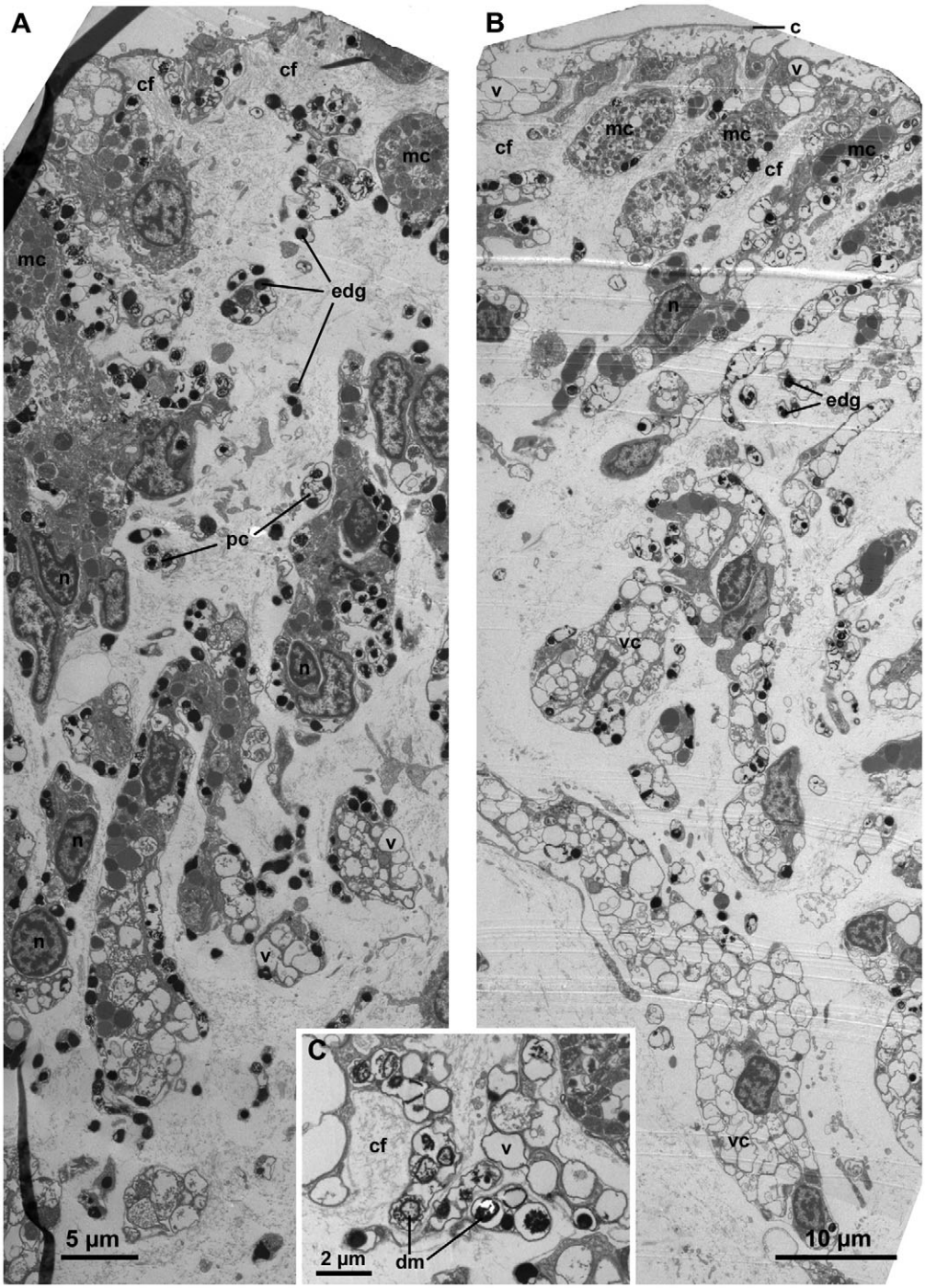

Fig. 5. Transmission electron micrographs of a relaxed (A) and stressed (B,C) body wall of *Holothuria forskali*. A and B correspond to an overall view of the epidermis and C shows details of pigment cell granules. c, cuticle; cf, collagen fibrils; dm, disintegrating material; edg, electron dense granules; mc, mucocyte; n, nucleus; pc, pigment cell; v, vacuole; vc, vacuolar cell.

suggests that the content of these cells was released during the stressing period.

Identification of saponins released in seawater

In order to verify whether saponins were secreted by the holothuroids, saponin extractions were realized directly on ASW conditioned by holothuroids. Two different water samples were extracted: ASW conditioned either by a non-stressed or by a stressed animal. Indeed, starting with the idea that saponins might play a role in the defense of the holothuroid, one might think that saponins could be actively released outside the animal when it is stressed.

Only one saponin, represented by the ions detected at *m*/*z* 1449, was detected in seawater when the animal was not stressed (Fig. 6A). This saponin corresponds to holothurinoside G described in Van Dyck et al. (Van Dyck et al., 2009). When the animal was stressed, it secreted three other known saponins (ions detected at *m*/*z* 1125, 1141 and 1433, corresponding to holothurinoside C, desholothurin A and holothurinoside F, respectively) (Rodrigez et al., 1991; Van Dyck et al., 2009) and two new congeners detected at *m*/*z* 1301 and 1317 (Fig. 6B). MALDI-MS/MS analyses were performed to confirm that the detected ions corresponded to the known saponins and to propose a molecular structure for the two new ones (Fig. 7). These

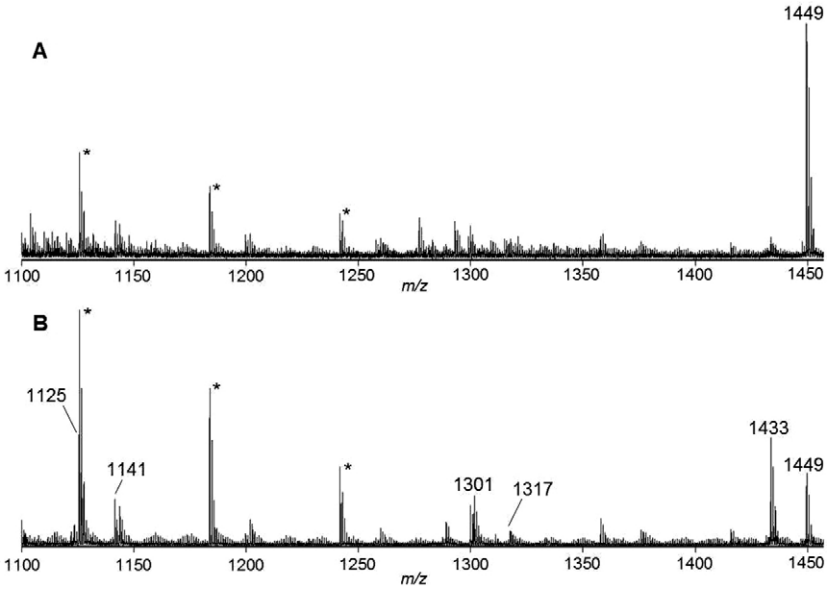

Fig. 6. Full-scan MALDI-TOF mass spectrum analyses of the total saponin mixture extracted from artificial seawater conditioned with non-stressed *Holothuria forskali* (A) and with stressed *H. forskali* (B). Asterisks correspond to the propylene glycol contamination.

saponins have been provisionally named holothurinosides M and L (*m*/*z* 1301 and 1317, respectively) according to the nomenclature of Rodrigez et al. (Rodrigez et al., 1991) and Van Dyck et al. (Van Dyck et al., 2009). An LC-MS analysis has also been performed and confirmed that no saponin isomers were present. The intensity of the peaks in Fig. 6B indicated that saponins are not detected in the same amount in seawater. Holothurinoside C (*m*/*z* 1125) seemed to be the most abundant saponin followed by holothurinosides F and G. Desholothurin A and the two new saponins were secreted in lower quantities.

Saponin quantification

A semi-quantitative analysis was performed on saponin extracts from ASW conditioned by the stressed holothuroids and also on saponin extracts from the body wall and Cuvierian tubules (Table 3). Saponin yield (i.e. ratio of mass of final butanolic extract after drying to tissue wet mass) was calculated for the tissue extracts. In addition, two complementary methods were used based on the structures and properties of saponins. On the one hand, the orcinol reaction evaluated the glycoside content of the different extracts; on the other hand, the measurement of the hemolytic activity of these extracts reflected the effectiveness of the saponin mixtures to lyse the erythrocytes of cow blood. The spectrophotometric measurements obtained with these tests were converted respectively to milligrams of glycoside by gram of tissue and to milligram equivalents of plant saponins by gram of tissue, using the standard curves and the wet masses of the body compartments. For the ASW extracts, only the hemolytic activity was measured as it appeared to be the most sensitive method. The concentration of saponins in the ASW was relatively low compared with that in the tissue extracts, the absorbance measured for the conditioned seawater being close to the detection threshold of the spectrophotometer. Comparison of these data with those measured for body wall saponins indicates that the volume of seawater (1 l) used in the experiments with stressed holothuroids contained ~0.6% of the total saponin content of the body wall. For the tissue extracts, there was high inter-individual variability. It must also be noted that only a limited number of extracts were investigated and the extracts for which the yield was calculated were not the same as those to which the other methods were applied. However, the results presented in Table 3 show different ratios (varying from 6 to 1) between the concentration of saponins in Cuvierian tubules and in the body wall. This suggests that although the saponin content of the Cuvierian tubules seemed to be higher than the one of the body wall, the overall toxicity per gram of tissue would be similar for the two organs.

Effects of saponins on fish

Toxicity tests were performed in order to observe the direct effect of saponins on the fish *C. julis* and *S. ocellatus*. For the first experiment, fish were incubated in increasing quantities of saponins and their respiration rate, estimated by the frequency of opercular openings, was recorded. For both species, a strong but short reaction – the opercular movements increased briefly and the fish swam vigorously and randomly all over the tank – was observed just after the addition of saponins, suggesting that saponins are rapidly detected. Ten minutes after the addition of saponin, however, the fish swam quietly again and the frequency of opercular openings returned to values close to the initial ones (Table 4). After the last saponin addition (cumulative volume of 1000 µl of extract corresponding to ~30% of the total saponin content of the body wall), the reactions changed. Fish seemed to be excited, swimming randomly and showing an increased respiration rate until they died in just a few minutes (*C. julis*, 10.8±2.4 min, *N*=5; *S. ocellatus*, 7.6±2.7 min, *N*=5). For the second experiment, a unique quantity of extract was added to the tank and

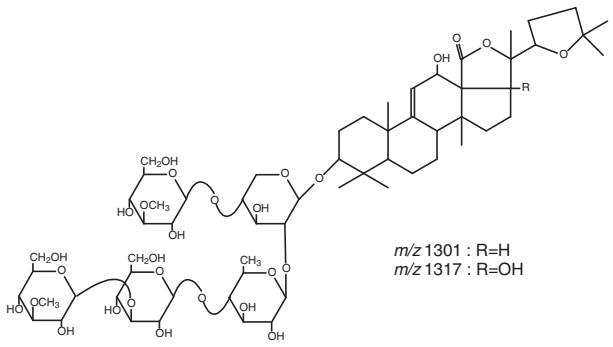

Fig. 7. Molecular structure of the two new saponins detected at *m*/*z* 1301 and 1317, named holothurinosides M and L, respectively.

Table 3. Saponin content of the body wall and Cuvierian tubules of *Holothuria forskali*, and artificial seawater conditioned by a stressed animal

	Extraction yield (mg g^{-1})	Orcinol reaction (mg glycoside g^{-1})	Hemolytic activity (mg equiv. g^{-1})
Body wall	2.667±0.261 (3)	0.011±0.002 (3)	0.649±0.715 (3)
Cuvierian tubules	15.873 (1)	0.024±0.013 (2)	0.604±0.063 (2)
			(mg equiv. l^{-1})
Conditioned seawater	–	–	0.290±0.002 (3)

Values are means ± s.d.; numbers in brackets indicate the number of individuals tested.
Extraction yields are given in dry mass of the final butanol extract per gram of tissue (wet mass).
Hemolytic activity values are given in milligram equivalents of plant saponins per gram of tissue (wet mass) or by liter of seawater.

the reactions and respiration rates of the fish were observed during 2 h. For volumes of saponin extracts ranging from 200 to 900 µl, fish were still alive after 2 h and their opercular opening frequency remained constant during the experiment (data not shown). Increasing the quantity of saponin did not appear to have an influence on the fish respiration rate (Fig. 8). For the highest quantity of saponin extract (1000 µl), fish died rapidly after their placement in the tank.

DISCUSSION

Some recent studies have highlighted the large diversity of triterpene glycosides present in holothuroid tissues (Kobayashi et al., 1991; Kalinin et al., 2008; Maier, 2008). In *H. forskali*, 26 different saponins have been described in the Cuvierian tubules and 12 have been described in the body wall (Rodrigez et al., 1991; Van Dyck et al., 2009). To understand the biological function of this molecular set, we have undertaken an integrated study about their precise localization in the body wall, their respective concentrations in and around the animals, and their effects on potential predatory fish.

The first part of this work aimed at localizing saponins in the body wall of *H. forskali*. Saponin localization on tissue sections by conventional staining or immuno-detection methods is difficult to achieve. During the last decade, MALDI-MSI, a relatively new mass spectrometry-based technique, has emerged as a solution to detect the spatial location of all the components of a tissue, without discrimination and regardless of their abundance. MALDI-MSI allows a label-free molecular mapping of biological samples almost without any treatment and preparation. Applied to holothuroid body wall sections in the present study, this technique demonstrated that saponins present an almost entirely epithelial distribution, with only one or two saponins detected in low amounts in the dermis. Some congeners occurred exclusively in the mesothelium but most were detected in the epidermis. After a prolonged stress, mesothelial saponins were no longer observed on the tissue sections, suggesting they might have been released into the coelomic fluid. For epidermal saponins, however, changes were more difficult to quantify. Holothurinoside C (*m*/*z* 1125) seems to be used or released outside of the epidermis. On the contrary, holothurinoside G (*m*/*z* 1449) appears to be abundantly produced in a state of stress compared with levels in the relaxed condition. In Cuvierian tubules, a direct MALDI analysis of saponins on tissue sections indicated that, in case of a prolonged stress situation, holothurinosides C/C1 (*m*/*z* 1125) are converted to holothurinosides F/F1 and H/H1 (*m*/*z* 1433

Table 4. Effects of cumulative additions of body wall saponins from *Holothuria forskali* on the opercular opening frequency of the fish *Coris julis* and *Symphodus ocellatus*

			Opercular opening frequency (min^{-1})	
Time (min)	Volume of saponin extract added (µl)*	Total volume of saponin extract in the tank (µl)*	*C. julis*	*S. ocellatus*
0			150±7.1	146±12.4
1	200	200		
10			152±16.4	148±10.2
20			153±15.2	144±8.8
30			153±6.8	145±7.2
31	100	300		
40			154±12.9	144±14.5
50			152±7.7	146±9.6
60			154±9.8	143±17.1
61	100	400		
70			153±12.7	142±11.4
80			134±13.4	106±7.8
81	100	500		
90			154±9.6	108±8.1
100			155±10.1	111±12.4
110			157±9.9	112±8.5
120			156±12.4	110±13.6
121	500	1000		
150			188±10.7	194±8.9
157				Death
160			Death	

*100 µl of extract corresponds to ~3% of the total saponin content of the body wall of one individual.
Opercular opening frequencies are means ± s.d.; *N*=5.

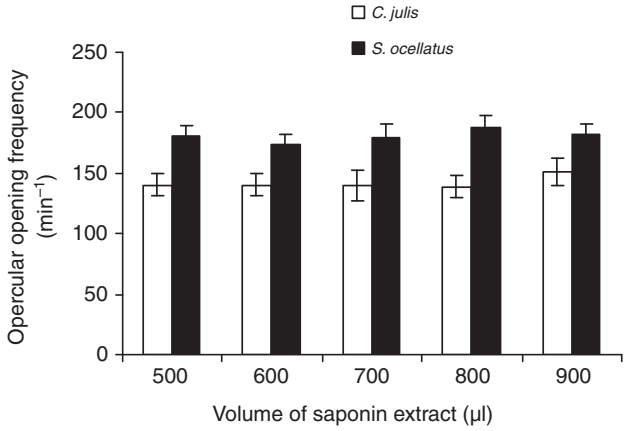

Fig. 8. Respiration rate (mean±s.d. opercular opening frequency, *N*=5) of fish (*Coris julis* and *Symphodus ocellatus*) placed in 2 l tanks after a 2 h exposure to a fixed saponin concentration.

and 1463, respectively), and desholothurins A/A1 (*m*/*z* 1141) to holothurinosides G/G1 and I/I1 (*m*/*z* 1449 and 1479, respectively) (Van Dyck et al., 2010b). In view of the structures of these different saponins (see Van Dyck et al., 2009), this is simply done by the addition of a disaccharide. This modification would make the saponins more hydrophilic (i.e. more soluble in seawater) and more membranolytic (i.e. more toxic) (Kalinin, 2000). From a morphological point of view, it is also at the level of the epidermis that the most spectacular ultrastructural modifications have been observed: pigment cell granules, which were conspicuous in the relaxed body wall, looked emptied in the stressed body wall. These epidermal changes suggest that molecules might be released in the seawater around the holothuroid. However, at this stage, it cannot be ascertained whether these modifications of pigment cells under stressed conditions are related to saponin release.

For the first time, saponins have been detected in the seawater surrounding individuals of *H. forskali*. Kubanek et al. (Kubanek et al., 2002) attempted to find triterpene glycosides in the water surrounding sponges but the detection threshold of their method was not low enough. In the present study, mass spectrometry techniques have allowed the detection of very low concentrations of saponins in conditioned seawater. Only one saponin, holothurinoside G (*m*/*z* 1449), was detected in the surroundings of non-stressed holothuroids. When they were stressed, however, the animals secreted five other saponins including two new ones (holothurinoside C and F, desholothurin A detected at *m*/*z* 1125, 1433 and 1141, respectively; and the provisionally named holothurinosides M and L detected at *m*/*z* 1301 and 1317, respectively). Holothurinoside G could be a background prevention signal whereas the five other saponins could play more important defensive roles. An interesting point is the presence of the two new saponins, which have not been detected in the body wall or in the Cuvierian tubules (Van Dyck et al., 2009). These saponins seem to be stress-specific and could come from another part of the animal, e.g. the respiratory trees. Indeed, when a holothuroid is stimulated, it contracts its body and expels water from the respiratory trees. The saponins coming from inside the animal and those secreted from body wall would then have been extracted and analyzed together in this study. It is likely that the previously described saponins are coming from the epidermis of the body wall because this corresponds to their tissue localization (see molecular images in Fig. 2).

Saponins extracted from the body wall and the Cuvierian tubules of *H. forskali* were quantified by different methods. The results indicate that the Cuvierian tubules contain a proportionally larger quantity of saponins than the body wall; although in terms of hemolytic activity, the two organs appear similar. Elyakov et al. (Elyakov et al., 1973) and Kobayashi et al. (Kobayashi et al., 1991) already reported high concentrations of saponins in the Cuvierian tubules, concentrations that were usually higher than those in the body wall (see also Van Dyck et al., 2010a). In the present study, body wall saponin extracts were tested at different concentrations on the fish *C. julis* and *S. ocellatus* in order to evaluate their overall toxicity. There was clearly a threshold effect: the fish were unharmed by low concentrations of saponins, but when a certain concentration was reached, the effect was brutal and the fish died in a matter of minutes. This fatal saponin concentration corresponds to ~30% of the total saponin content of the body wall diluted in the 2 l tank. This is much more than the amount released by an animal stimulated for as long as 4 h, i.e. 0.6% of the total saponin content of the body wall. Therefore, at natural concentrations, body wall saponins diluted in seawater have no deleterious physiological effects on fish. This was also found to be the case for the species *Holothuria leucospilota*. Although its saponins were ichthyotoxic at high concentrations in closed circulation tanks (Yamanouchi, 1955), they showed no adverse effects on fish in open systems (Hamel and Mercier, 2000) and *a fortiori* in open water in the natural environment.

Then why are saponins released in seawater? Padove Cohen et al. reported recently that the responses of fish to sponge triterpene glycosides (formoside and ectyoplasides A and B) are receptor mediated (Padove Cohen et al., 2008), i.e. fish can smell and/or taste these molecules. Holothuroid triterpene glycosides are structurally close to those of sponges and one might expect that the same kind of receptor-mediated chemodetection exists between fish and holothuroids. This is corroborated by the fact that, in our toxicity tests, the fish readily detected the addition of saponins in their tank even if they were added at low concentrations. Saponins may therefore act as a chemical aposematic signal to warn predators of the noxiousness or, at least, the unpalatability of the holothuroid tissues (Eisner and Grant, 1980; Camazine, 1985).

In the species *H. leucospilota*, Hamel and Mercier reported that Cuvierian tubules could play a dissuasive bait role (Hamel and Mercier, 2000). When the holothuroid was attacked, it released several tubules towards the fish predator. The fish tasted the sticky white threads and probably sensed their unpalatability because it then moved away (Hamel and Mercier, 2000). Moreover, Cuvierian tubule discharge has a preventive effect; fish that had been exposed avoided any contact with holothuroids for several days (Hamel and Mercier, 2000). In *H. forskali*, preliminary palatability tests with the fish *S. ocellatus* showed that food pellets in which saponins were added at the concentration observed in the body wall were eaten, whereas pellets containing saponins at the concentration observed in the Cuvierian tubules were deterrent (S.V.D., M.T. and P.F., unpublished observation). Considering the fact that the Cuvierian tubules and the body wall share several saponins (Van Dyck et al., 2009), a predator detecting the saponins secreted by the body wall in the surroundings of the holothuroid will presumably associate this chemical signal with the unpalatability of the Cuvierian tubules and will avoid biting the holothuroid. Thus, for triterpene glycosides at natural concentrations, palatability may be a more important defensive trait than toxicity for predators capable of learning, such as fish (see also Schulte and Bakus, 1992).

ACKNOWLEDGEMENTS

We thank M. Boulvin, A. Desmons and P. Postiau for technical assistance. S.V.D., G.C. and M.T. benefited from a FRIA doctoral grant (Belgium). P.F. and P.G. are Senior Research Associate and Research Associate, respectively, of the Fund for Scientific Research of Belgium (FRS-FNRS). P.G. thanks the FRS-FNRS for financial support in the acquisition of the Waters Q-ToF Premier Mass Spectrometer and for continuing support. I.F. and M.W. acknowledge funding from Centre National de la Recherche Scientifique (CNRS), Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche, Agence Nationale de la Recherche (ANR PCV to I.F.), Institut du Cancer (INCA to I.F.). Work supported by a FRFC Grant no. 2.4535.10. This study is a contribution of the Centre Interuniversitaire de Biologie Marine (CIBIM).

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