Chemoecology
https://doi.org/10.1007/s00049-022-00368-6

CHEMOECOLOGY

ORIGINAL ARTICLE

Crinoid anthraquinones as kairomones allowing host selection for the symbiotic snapping shrimp *Synalpheus stimpsonii*

Guillaume Caulier[1,2] · Alexia Lourtie[1,3] · Lola Brasseur[1] · Jerome Mallefet[3] · Pascal Gerbaux[4] · Patrick Flammang[1] · Igor Eeckhaut[1,2]

Received: 12 May 2021 / Accepted: 20 January 2022

Abstract

Quinones are one of the major pigment groups that provide such bright colors to feather stars (Echinodermata, Crinoidea). These secondary metabolites also act as defensive molecules rendering crinoids unpalatable and repellent to other organisms. However, feather stars are usually associated with numerous symbiotic organisms, amongst which the ectocommensal snapping shrimp *Synalpheus stimpsonii*. We investigated the chemical stimulus allowing host selection in *S. stimpsonii* through the combination of behavioral tests, chemical extractions, and mass spectrometry analyses. The individuals of *S. stimpsonii* used in the experiments were sampled around the Great Reef of Toliara (Madagascar) where they are found in association with two crinoid species: *Comanthus wahlbergii* and *Phanogenia distincta*. The chemical attractiveness of the two crinoid hosts and a non-host species, *Cenometra bella*, was tested in an olfactometer. The three crinoids produced attractive kairomones allowing the snapping shrimp to recognize them. Mass spectrometry analyses on purified extracts of *P. distincta* revealed the presence of three different anthraquinones (rhodoptilometrin, comantherin, and a new crinoid anthraquinone). Compared to the existing literature, this anthraquinonic cocktail is specific to *P. distincta*. When these extracts were injected in the olfactometer, they triggered similar attracting behavior suggesting that crinoid anthraquinones are kairomones allowing host selection for *S. stimpsonii*. This hypothesis is also supported by the fact that shrimps were chemically attracted by pure commercial anthraquinones. In addition to their traditional defensive role (allomones), anthraquinones would, therefore, also function as kairomones, maintaining the symbiosis between *S. stimpsonii* and its crinoid hosts.

Keywords Marine chemical ecology · Echinoderm · Crustacean · Ectocommensal · Y-tube · Olfactometer

Introduction

Quinones represent a major family of polyketides widely distributed in the biosphere. More than 1200 quinones have been described so far. They are composed of diones that form diverse aromatic structures differing by the number of aromatic cycles and their associated radicals (El-Najjar et al. 2011). Quinones are essential to many biological processes: some derivatives of quinones serve as electron acceptors in electron transport chains playing a role in photosynthesis and aerobic respiration (Elling et al. 2016; Lubitz 2003; Nohl et al. 1986); they play a role in the cellular membrane stabilization (Rao et al. 2008) and in the formation of vitamins and antioxidants (Beattie et al. 2007; Gong et al. 2008). Quinone-related compounds are of high interest for human health. For example, they constitute a component of nearly every modern drug to cure cancer (Asche 2005; Lu et al. 2013). Quinones are found in a wide

Communicated by Patrick Fink.

✉ Guillaume Caulier
guillaume.caulier@umons.ac.be

1 University of Mons-UMONS, Research Institute for Biosciences, Biology of Marine Organisms and Biomimetics Unit, 23 Place du Parc, B-7000 Mons, Belgium

2 Belaza Marine Station (IH.SM-UMONS-ULIEGE), Toliara, Madagascar

3 University Catholic of Louvain–UCLouvain, Earth and Life Institute, Marine Biology Laboratory, 3 Place Croix du Sud, bt 4, B-1348 Louvain-la-Neuve, Belgium

4 University of Mons–UMONS, Research Institute for Biosciences, Organic Synthesis and Mass Spectrometry Laboratory, 23 Place du Parc, B-7000 Mons, Belgium

Published online: 08 March 2022

variety of plants, bacteria, and fungi (El-Najjar et al. 2011; Sunassee and Davies-Coleman 2012). Except for the ubiquitous ubiquinones, these molecules are rare in the animal kingdom and only found in small concentrations in marine sponges, cnidarians, mollusks, arthropods, tunicates, and echinoderms (Gordaliza 2010; Singh et al. 1967; Sunassee and Davies-Coleman 2012; Thomson 2012). Concerning echinoderms, naphthoquinones are produced exclusively by echinoids while anthraquinones are specifics to crinoids (*i.e.* feather stars) (Kornprobst 2005; Shikov et al. 2018).

In crinoids, neither the biogenesis nor the function of quinones are well documented (Feng et al. 2017). Previous literature has highlighted the presence of anthraquinones in different organs, from the skeleton to the viscera of feather stars (Bartolini et al. 1973; Ghaly et al. 2009; Kornprobst 2005; Thomson 2012). Also, numerous studies underline their importance as natural pigments, partially causing the colorful appearance of most species (Baker and Sutherland 1968; Feng et al. 2017; Kent et al. 1970; Powell and Sutherland 1967; Rideout and Sutherland 1981, 1985; Singh et al. 1967; Smith and Sutherland 1971; Sutherland and Wells 1967). Quinonic pigments were even detected in the macrofossils crinoids *Liliocrinus* sp. dated from the Trias-Jurassic period (Wolkenstein et al. 2006). It has also been shown that polyketides such as anthraquinonic pigments, especially when sulfated, have a feeding-deterrent activity on fishes and could play the role of chemical defense to deter predators and parasites (Kasumyan et al. 2020; Rideout et al. 1979; Takahashi et al. 2002). Several studies also detail the chemical defense mechanism provided by the secretion of quinones in insects (Berenbaum 1995; Kittredge et al. 1974; Pasteels et al. 1988). Quinones cytotoxicity is mainly attributed to their ease of reduction and therefore to their ability to act as oxidizing or dehydrogenating agents (El-Najjar et al. 2011).

Although they produce repellent chemicals (Tinkova et al. 2014), comatulids usually host various symbiotic metazoans like crustaceans, fishes, gastropods, myzostomids, nematodes, ophiuroids, and polychaetes (Britayev and Mekhova 2011; Deheyn et al. 2006; Lanterbecq et al. 2010; Zmarzly 1984). Amongst them, the snapping shrimp *Synalpheus stimpsonii* (Alpheidae) is an obligatory ectocommensal regularly found alone or in a heterosexual pair under the calix of their crinoid host (Fig. 1a) where they are protected from

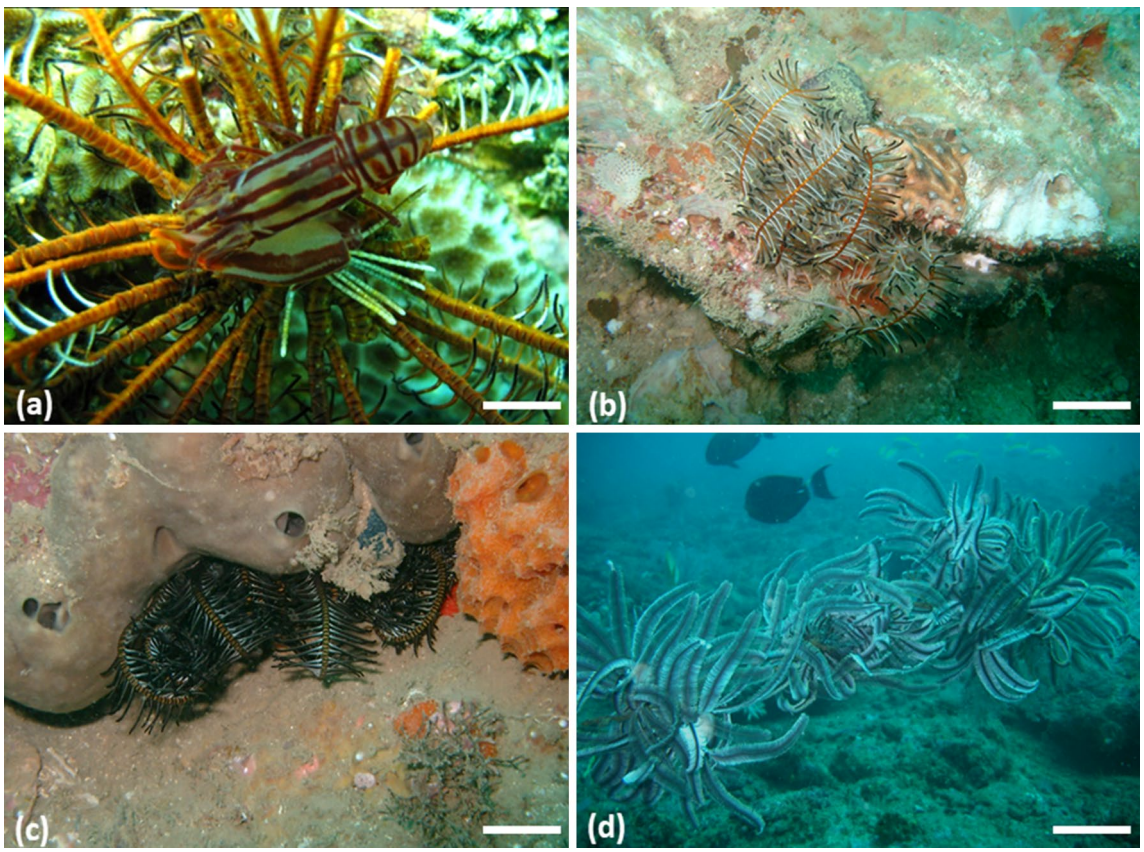

Fig.1 **a** The snapping shrimp *Synalpheus stimpsonii* under the calyx of a crinoid host *Phanogenia distincta*. **b** The crinoid *Phanogenia distincta* partially hidden in rock anfractuosity. **c** The crinoid *Comanthus wahlbergii* partially hidden in rock anfractuosity. **d** The crinoids *Cenometra bella* attached to a whip black coral. The scale bar represents 6 mm in (**a**); 26 mm in (**b**); 28 mm in (**c**) and 53 mm in (**d**)

predators (VandenSpiegel et al. 1998). Shrimps sometimes leave their host to reproduce with a mate located on another host. This host-switching behavior has been well explored in ectosymbiotic crustaceans (Baeza and Thiel 2007; De Bruyn et al. 2016) and highlights the existence of a specific sensory system allowing the symbiont to select its host. Our team already performed host selection experiments testing the visual and chemical recognition of crinoids by the snapping shrimp *S. stimpsonii* in Hansa Bay, Papua New Guinea (VandenSpiegel et al. 1998). The results suggested that the shrimp uses both visual and chemical information to recognize its host at a distance. Chemical communication was much more accurate than the visual one. However, to date, no study has ever identified the kairomone (*i.e.* chemical signal emitted by hosts when they elicit a commensal or a parasitic symbiosis) released by crinoids that influence the shrimp behavior.

In a previous study, we highlighted that the repellent, holothuroid-specific saponins were the attractive kairomones allowing host selection by the holothuroid associate Harlequin crab *Lissocarcinus orbicularis* (Caulier et al. 2013). Recently, we also demonstrated that spinochromes (naphthoquinones) produced by the sea urchins *Echinometra mathaei* are recognized by its shrimp symbiont *Tuleariocaris holthuisi* (Brasseur et al. 2018b). We hypothesize that many other symbiotic organisms have diverted a natural chemical defense to their advantage: these defensive molecules would be used by symbionts to identify their hosts and to protect themselves against their predators. The present study aims to investigate whether crinoid anthraquinones could be the kairomones attracting the snapping shrimp *S. stimpsonii*. For this purpose, we carried out olfactometry behavioral experiments, anthraquinone chemical extractions, and mass spectrometry analyses.

Methods and materials

Samplings, chemical extractions, and behavioral experiments were conducted during five scientific field missions in the Fishery and Marine Science Institute of the University of Toliara, southwest of Madagascar (January–February 2011 and 2012; November–December 2013, 2014 and 2019). Chemical purification and mass spectrometry analyses were performed at the University of Mons (Belgium).

Sampling procedure

Crinoids and their symbiotic shrimps were hand-collected by scuba diving between 18 and 32 m depth on the northern external slope of the Great Reef of Toliara (23° 20′ 59" S; 43°36′ 45" E). Three crinoid species were commonly found in this area: *Phanogenia distincta* (Carpenter 1888) (Fig. 1b); *Comanthus wahlbergii* (Müller 1843) (Fig. 1c) and *Cenometra bella* (Hartlaub 1890) (Fig. 1d) (Marshall and Rowe 1981). We noticed a modification in comatulid distribution in the sampling area from one year to another, with a rarefaction of *C. bella* and *C. wahlbergii* along with an increase of the *P. distincta* population. Anthraquinonic extractions (see here below) were then only performed on *P. distincta*. Each sampled crinoid was carefully stored in hermetic plastic bags to prevent the loss of symbiotic shrimps. Once in the laboratory, the crinoid and symbiont species were identified and the number of *Synalpheus stimpsonii* (de Man 1888) recorded. Hosts and symbionts were then kept separately in two semi-open water aquaria for at least 24 h before the behavioral experiments. The seawater used in this study was 1 µm filtered natural seawater, 27 °C and 35 ‰ salinity, pumped directly from the sea. Animals used in our experiments were maintained and treated in compliance with the guidelines specified by the Belgian Ministry of Trade and Agriculture.

Behavioral experiments

Because host fidelity implies host choice preferences by olfactory imprinting (Hasler et al. 1978; VandenSpiegel et al. 1998), we only used shrimps collected on *P. distincta* for the behavioral experiments. The chemical communication between symbiotic shrimps and comatulids was tested in a Y-tube olfactometer system (Davenport 1950), following the methodology used in Brasseur et al. (2018b) and Caulier et al. (2013). The system consisted of a 20 cm long unpaired branch and two 10 cm paired branches that were each connected to a 5-L aquarium. The tube diameter was 3 cm. One of the aquaria (aquarium A) contained test seawater and the other (aquarium B) was filled with control seawater (Fig. SI1). Test water consisted of seawater conditioned either with living crinoids (*i.e.* conditioned with 3 individuals in a 5-L aquarium for 20 min) or with purified anthraquinones. Water flowed from these two aquaria through the olfactometer and was evacuated at the base of the unpaired branch of the Y-tube where the shrimp was introduced at the beginning of each test. Flow laminarity inside the olfactometer was controlled using fluorescein salt before the experiments and the water flux was regulated to a speed of 2–3 cm s^{-1}.

An attractive chemical communication was recorded when a significant proportion of shrimps moved up the flow and orientated towards the olfactory stimulus source. During a typical trial, one snapping shrimp *S. stimpsonii* was introduced at the base of the unpaired branch of the Y tube. If it was not stimulated, the shrimp remained at the base of the branch without moving and the run was aborted and considered as null after 10 min. If the shrimp was stimulated, it moved into the unpaired branch up to the junction of the two-paired branches where it usually

stopped moving, tested the water fluxes, and entered one of the paired branches. Two types of behaviors were therefore recorded in the Y tube: the motion behavior, when shrimps moved for at least 10 cm into the unpaired branch towards the stimulation source, and the orientation behavior, when shrimps entered one of the two paired branches and reached the corresponding aquarium (Brasseur et al. 2018b; Caulier et al. 2013). Videos SI1 and SI2 illustrate the motion and the orientation behaviors of the symbiont *S. stimpsonii* in the Y-tube during the end of a test. Those videos were recorded for illustration only as typical tests were not filmed to avoid any bias (*i.e.* the shrimp could visually detect the camera or the motion of the scientist filming).

At least twenty different shrimps were tested for each experiment and aquaria A and B were exchanged every five runs. Each shrimp individual was only tested once to avoid any potential habituation effect. The entire device was washed after each trial (i.e. after each shrimp). To test the significance of the motion behavior, the number of times shrimps started to move under a chemical stimulation (i.e. chemotaxis) was compared by a Pearson's chi-squared test with Yates continuity correction ($\chi^2 = 0.05$, df = 1) to the number of times shrimps were moving when control seawater filled both aquaria. The orientation behavior was assessed using a binomial test by comparing the percentage of shrimps that entered aquarium A to a random distribution (Probability of success = 0.5). All statistical tests were performed with the R software (R project, ×64 3.2.3; R Core Team (2015)).

Shrimp behaviors were first recorded when only seawater filled the olfactometer (negative control). Then, chemically mediated host selection was tested with water conditioned by each species of crinoid. Finally, anthraquinones extracted from the host *P. distincta* and pure commercial anthraquinones (Alfa Aesar; CAS 84-65-1) were tested for their potential attractiveness to *S. stimpsonii*. The chemical structure of this commercial compound can be found in supplementary material (Fig. SI2). All extracts were diluted to 0.05 g L^{-1} in artificial seawater (made by dissolving 26 g of NaCl, 6 g of $MgCl_2$, 0.7 g of KCl, 1.47 g of $CaCl_2$ and 0.2 g of $NaHCO_3$ in 1 L ultrapure MilliQ water) and introduced into one of the two aquaria of the olfactometer at a flow rate of 10 ml min^{-1} using a peristaltic pump (Amersham P-1). The natural concentration of anthraquinones released in surrounding water by crinoids is still unknown. Therefore, different anthraquinones concentration used in this study were tested while setting up the protocol of this experiment and the most efficient dose to induce the positive chemotaxis of the symbiotic shrimp *S. stimpsonii* was 0.05 g L^{-1} (data not shown due to low replication). A second negative control was realized with seawater injected by the peristaltic pump.

Anthraquinone purification

Twenty-six entire *P. distincta* were used for anthraquinone extraction. Each crinoid was briefly rinsed with distilled water in order to remove the salt excess on their integument and was then crushed with a mortar and macerated in 50 ml of acetone for 6 h. The homogenate was then filtrated under vacuum with a Buchner flask and the extract was evaporated at low pressure at 60 °C (Zhu et al. 2008) using a rotary evaporator (Laborota 4001 efficient, Heidolph) or an oven. The dried crude extract was dissolved in 50 ml milliQ water and was then partitioned against diethyl ether (v/v; 1:1). The aqueous phase was recovered and dried. Anthraquinone concentrations were then calculated by dividing the dry weight of the anthraquinone extracts by the wet weight of crinoids.

Twenty extracts from twenty different individuals *P. distincta* were combined, dissolved in artificial seawater and tested in the Y-tube olfactometer. Combining the extracts resulted in a homogenous chemical stimulus to be tested in the olfactometer to potentially attract the shrimps. The remaining six extracts were kept separately for mass spectrometry analyses: the aqueous phases were recovered, evaporated to less than 2 ml and centrifuged at 10,000 g for 10 min to remove potential solid residues. Finally, each supernatant was evaporated to dryness using a Speed Vac (RC 10.22, VWR international) and was re-dissolved in 80% methanol.

Mass spectrometry analyses

For the LC–MS analyses, a Waters Alliance 2695 liquid chromatography device (HPLC) was used. The system comprises a quaternary pump, a vacuum degasser, and an autosampler. The chromatography was carried out on a reversed-phase column (Kinetex® 5 µm Biphenyl 100 Å, 50 × 4.6 mm, Phenomenex) at 30 °C and with a sample volume injected of 10 µl and a constant flow (1.25 ml min^{-1}) of a gradient of eluent A (water, 0.1% formic acid) and eluent B (acetonitrile) (Table SI1).

Mass spectra of the purified extracts were first obtained on a Waters Quattro Ultima in the negative ionization mode by scanning between m/z 100 and 1,500 using Electrospray ionization (ESI) source. The ESI conditions were capillary voltage 3.1 kV; cone voltage 40 V; source temperature 120 °C; desolvation temperature 300 °C. Dry nitrogen was used as the ESI gas with a flow rate of 70 L h^{-1} for the gas cone and 600 L h^{-1} for the desolvation gas. Accurate mass measurements were then performed on a Waters Q-Tof Premier using ESI source, in the negative ionization mode by scanning between m/z 50 and 600 with scan durations of 1 s and an interscan time of 0.1 s. The ESI conditions were capillary voltage 3.1 kV; cone voltage 40 V; source temperature 120 °C; desolvation temperature 300 °C. Dry

nitrogen was used as the ESI gas with a flow rate of 50 L h^{-1} for the gas cone and 600 L h^{-1} for the desolvation gas. The mass spectrometer was equipped with a lockspray source to obtain high mass accuracy of anthraquinones. Sodium iodide ($[I\text{-}Na]^- = 126.9045$), was used as a lock mass, being continuously sprayed into a second ESI source and sampled every 10 s.

Mass spectrum analyses were performed on MassLynx 4.1. mass spectrometry software (Waters). Chemical structures were determined from accurate mass databases and from existing literature. Compositions were validated only under a mass error of 10 ppm. Anthraquinone structures were finally drawn using BKChem software.

Results

Occurrence of shrimps on crinoids

Table 1 describes the number of collected individuals for each species of crinoids and the occurrence of the snapping shrimps *Synalpheus stimpsonii*. In total, 245 crinoids from three species, *Cenometra bella*, *Comanthus wahlbergii,* and *Phanogenia distincta* were collected. They were associated with 247 *S. stimpsonii*. Only the two species *C. wahlbergii* and *P. distincta* hosted this shrimp species with the prevalence of 84 and 68%, respectively. No snapping shrimp was found on *C. bella*. Shrimps presented mimetic colors corresponding to their hosts; individuals collected on *C. wahlbergii* were darker than the individuals collected on *P. distincta*. Sometimes, after several days in an aquarium without hosts, the symbiotic shrimps lost their colors, becoming transparent. Comatulids never hosted more than two snapping shrimps but other symbionts such as myzostomids, eulimids, clingfishes *Discotrema crinophilum*, squat lobsters *Allogalathea elegans* and shrimps *Laomenes* sp. and *Periclimenes* sp. were regularly found on the same host. Most organisms were finally released in different diving sites near the coral reef after behavioral experiments.

Table 1 Occurrence of *Synalpheus stimpsonii* on three comatulid species in Toliara Bay, Madagascar

Crinoids			Shrimps *S. stimpsonii*		
Species	Observed specimens	Colonized specimens	Hetero-sexual pairs	Single	Total
Cenometra bella	38	0	/	/	0
Comanthus wahlbergii	36	30	24	6	54
Phanogenia distincta	171	116	77	39	193
Total	245	146	101	45	247

Host selection behavioral experiments

Most *S. stimpsonii* did not move in the olfactometer only filled with seawater ($P > 0.05$) (Fig. 2_1). Therefore, there is no attractive chemical or rheological stimulus in regular seawater. On the contrary, water conditioned by *C. wahlbergii* or *P. distincta* triggered specific crustacean chemodetection behaviors (*i.e.* flicking of the antennae and the antennules) that subsequently initiated movements of the shrimps towards their host by chemotaxis. Shrimps significantly ($P < 0.01$) performed the "motion behavior" (*i.e.* they moved up-flow in the unpaired branch of the olfactometer) and 80–90% of them significantly ($P < 0.01$) performed the "orientation behavior" (*i.e.* they chose the branch corresponding to their host conditioned water) (Fig. 2_2–3; Video SI1). The mean time (± SD) required to enter the conditioned aquarium was 1.8 (± 1.1) min. Similar results were obtained with the non-host species *C. bella* (Fig. 2_4) with a mean time of 2.3 (± 0.8) min. When given the choice between *P. distincta* and *C. bella*, 18 shrimps out of 20 preferred the water conditioned by their host (Fig. 2_5) in 2.1

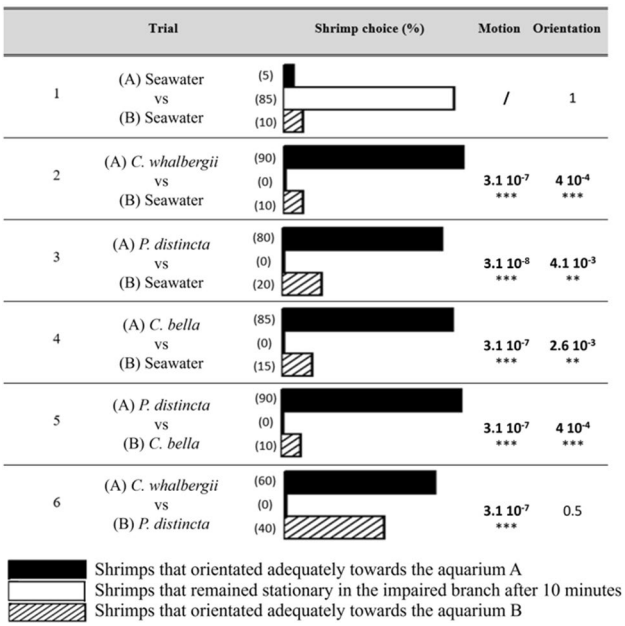

Fig.2 Results of the behavioral experiments conducted with a Y-tube olfactometer on the symbiotic shrimp *Synalpheus stimpsonii* with crinoid conditioned water. The horizontal bar charts represent the percentage of shrimps (value in brackets) that remained stationary in the impaired branch after 10 min (white bars), that orientated adequately towards the aquarium A (black bars), or orientated towards the control aquarium B (striped bars). The *P* value of the statistic results for motion (*Pearson's chi-squared test* with *Yates continuity correction*, $\chi^2 = 0.05$, df = 1) and orientation (*binomial test*, 0.5 proportion) behaviors can be found in the corresponding columns. Significant results are in bold and characterized by asterisks (*for $p < 0.05$; ** for $p < 0.01$ or *** for $p < 0.001$). $N = 20$ replicates for each test except for test $n°$ 3 that was realized on 25 replicates. "vs" stands for versus

Table 2 Anthraquinones extracted from the crinoid *Phanogenia distincta*

Anthraquinone identification	Retention time (min)	Elemental composition (Neutral)	Theoretical mass $[M-H]^-$ (m/z)	Measured mass $[M-H]^-$ (m/z)	Error (ppm)
(a) 1,3,8-Trihydroxy-6-[(1S)-1-hydroxypropyl] anthracene-9,10-dione also known as rhodoptilometrin	2.7–3.5	$C_{17}H_{14}O_6$	313.0712	313.0700	−3.8
(b) 4-Hydroxy-5,6 dimethoxy-2-methylbenzo[g]chromen-8-one also known as comantherin	8.3–9.0	$C_{16}H_{14}O_5$	285.0763	285.0742	−7.4
(c) Unknown 1	6.0–6.7	$C_{20}H_{20}O_2$	291.1385	291.1366	−6.5

Corresponding chemical structures for (a) and (b) can be found in Fig. 3. Chemical names are provided following the International Union of Pure and Applied Chemistry (IUPAC) nomenclature standard

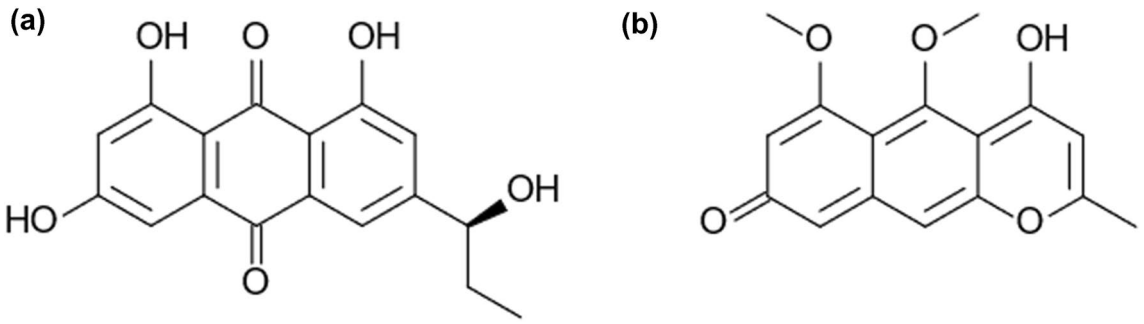

Fig.3 Presumptive molecular structures of the anthraquinones detected in the crinoid *Phanogenia distincta*. **a** 1,3,8-Trihydroxy-6-[(1S)-1-hydroxypropyl] anthracene-9,10-dione or rhodoptilometrin and **b** 4-hydroxy-5,6 dimethoxy-2-methylbenzo[g]chromen-8-one or comantherin. The chemical structure of the new anthraquinone **c** is not shown as it cannot be confirmed only by mass spectrometry

(±0.4) min. Finally, when confronted with odors issued from the two host species *C. wahlbergii* and *P. distincta*, shrimps were attracted by the conditioned water ($P<0.01$) (no symbiont remained at the origin of the olfactometer) but they did not orientate significantly towards one aquarium ($P>0.05$) (Fig. 2–6). In this last test, the mean time of an assay was 3.9 (±1.6) min.

Anthraquinone mass spectrometry analyses

Mass spectrometry analyses and accurate mass measurements on the final extracts revealed a cocktail of three anthraquinones (Table 2). Two of them had already been described in crinoids (Bartolini et al. 1973): 1,3,8-trihydroxy-6-[(1S)-1-hydroxypropyl] anthracene-9,10-dione (Fig. 3a)—also known as rhodoptilometrin-, and 4-hydroxy-5,6 dimethoxy-2-methylbenzo[g]chromen-8-one (Fig. 3b)—also known as comantherin. The third one is a new anthraquinone congener bearing one or a few alkyl side chains with a total of 6 carbons. The distribution of the relative intensity of the three ion peaks is well conserved across all replicates ($n=6$) with rhodoptilometrin as the major ion peak (around 50 times more intense than the other anthraquinone ion peaks) (Fig. 4). Other ion

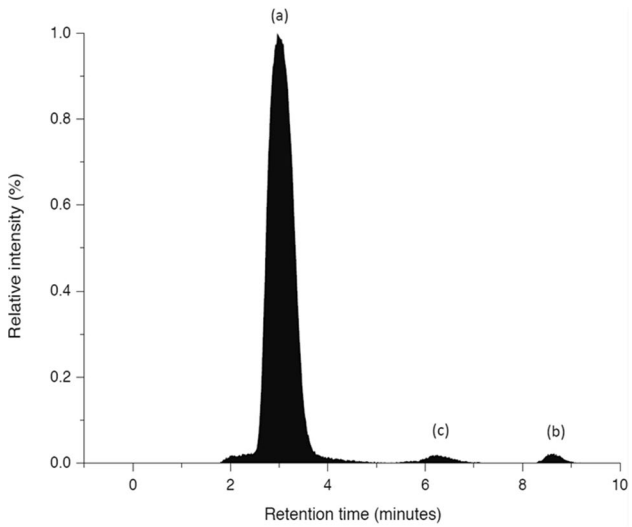

Fig.4 LC–MS consensus chromatogram of *Phanogenia distincta* anthraquinone extracts from all individuals. **a** 1,3,8-Trihydroxy-6-[(1S)-1-hydroxypropyl] anthracene-9,10-dione or rhodoptilometrin; **b** 4-hydroxy-5,6 dimethoxy-2-methylbenzo[g]chromen-8-one or comantherin; **c** unknown 1. Corresponding MS results can be seen in Table 2 and corresponding chemical structures for (**a**) and (**b**) in Fig. 3

peaks were observed in the spectra but their composition, derived from their accurate masses, suggests they are not anthraquinone derivatives. Even if the anthraquinonic extracts are not 100% pure, we provide a first attempt to quantify the concentration of these molecules in crinoids. Concentration was calculated as the ratio of the extract dry weight (11.36 (± 3.33) mg) to the entire crinoid wet weight (4.95 (± 1.34) g). The mean concentration of the extract was therefore 3.63 (± 1.2) mg g^{-1}.

Snapping shrimp chemotaxis towards anthraquinone-conditioned water

The use of the peristaltic pump in the olfactometer had no impact on the behavior of *S. stimpsonii* ($P > 0.05$) (negative control; Fig. 5_1). Purified anthraquinone extracts from *P. distincta* triggered the same behavior pattern as seawater conditioned by live comatulids. Several shrimp individuals were even found closely in contact with the distal tube of the peristaltic pump. Seventeen individuals out of 20 were chemically attracted by the purified anthraquinones (Fig. 5_2; Video SI2). Commercial anthraquinones were also attractive to the symbiotic shrimps (Fig. 5_3). The mean time shrimps required to enter the conditioned aquarium was 2.3 (± 0.8) min with *P. distincta* extracts and 3.5 (± 1.7) min for synthetic anthraquinones.

Discussion

In addition to the present work, two other studies, those of VandenSpiegel et al. (1998) and Eeckhaut et al. (2001) focused on the chemical attractiveness of crinoids on symbionts. The Papuan *Synalpheus stimpsonii* studied by VandenSpiegel et al. (1998) were observed on 4 crinoid species out of the 25 recorded in the research area (Hansa Bay) (Deheyn et al. 2006). The crab *Harrovia longipes*, also studied in Papua New Guinea by Eeckhaut et al. (2001), was observed on 9 crinoid species out of 25 (Deheyn et al. 2006). In the two cases, olfactory experiments highlighted some general rules about the chemoattraction exerted by crinoids on crustacean symbionts: (*i*) symbionts are chemically attracted by their crinoid hosts, (*ii*) they also are chemically attracted by non-host crinoids and (*iii*) they can chemically distinguish host crinoids from non-host crinoids. Eeckhaut et al. (2001) also demonstrated that the crabs *H. longipes* are not attracted by other echinoderms (holothuroids and echinoids). From an ecological point of view, the ability to detect the proper host is an advantage that ensures the obligatory and specific symbiont to live at the safest place: they can hide on crinoids and be protected by their repellent semiochemicals. In nature, the crinoid semiochemicals allow shrimps to discriminate hosts from non-hosts with a significant preference for the hosts although the specificity of the observed associations is due to multiple factors. In Madagascar, the

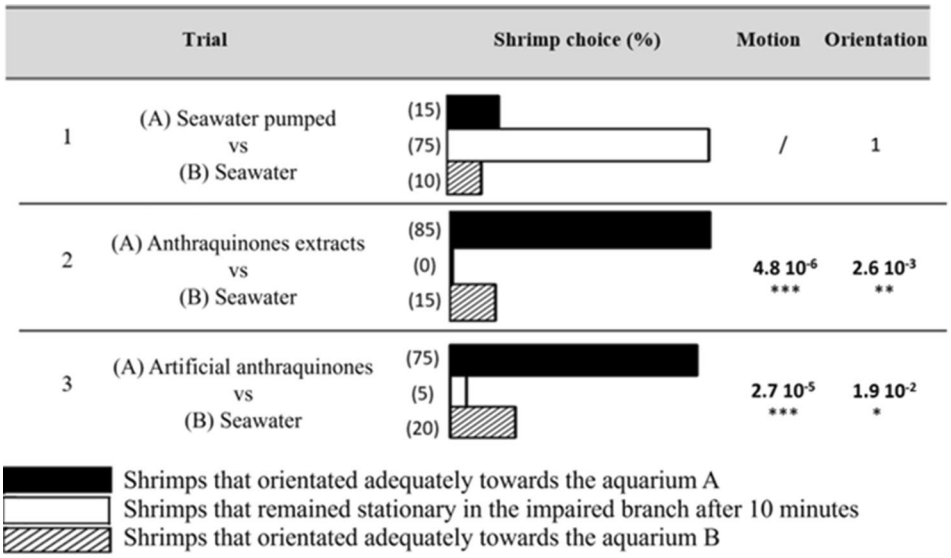

Fig.5 Results of the behavioral experiments conducted with a Y-tube olfactometer on the symbiotic shrimp *Synalpheus stimpsonii* with water conditioned by anthraquinones extracted from *Phanogenia distincta* or by commercial anthraquinones. The horizontal bar charts represent the percentage of shrimps (values in brackets) that remained stationary in the impaired branch after 10 min (white bars), orientated adequately towards aquarium **A** (black bars) or orientated towards the control aquarium **B** (striped bars). The *P* value of the statistic results for motion (*Pearson's chi-squared test* with *Yates continuity correction*, $\chi^2 = 0.05$, df = 1) and orientation (*binomial test*, 0.5 proportion) behaviors can be found in the corresponding columns. Significant results are in bold and characterized by asterisks (*for $p < 0.05$; ** for $p < 0.01$ or *** for $p < 0.001$). $N = 20$ replicates for each test. "vs" stands for versus

crinoid specificity shown by *S. stimpsonii*, recorded on the crinoids *Phanogenia distincta* and *Comanthus wahlbergii* but not on *Cenometra bella*, can be explained by ecological factors. The way of life of the first two species is very different from the one of the last species: *C. bella* lives mainly tied on the top of black coral branches, always exposing their whole body to water current, whereas the two hosts are usually hidden in crevices of the coral reef (Tinkova et al. 2014). The shrimps settling on *C. bella* would be much less protected than those on the hosts and would potentially be eliminated through predation.

The anthraquinonic content of crinoids has been reported for several species (Bartolini et al. 1973; Feng et al. 2017; Kemami Wangun et al. 2010; Kent et al. 1970; Lee and Kim 1995; Rideout et al. 1979; Thomson 2012; Tseng et al. 2019; Wolkenstein et al. 2018, 2009). As in other studies, only a few different anthraquinones molecular structures were detected in *P. distincta*. Indeed, in general, only two or three congeners have been found in each species investigated, and even though Bartolini et al. (1973) found five different pigments in *Comanthus bennetti*, four were very close and only differed in their degree of oxidation. However, one anthraquinonic pigment, rhodoptilometrin, was found across all species studied until now. This particular molecule could play an important metabolic/ecological role or could have a major function in the biosynthesis of other anthraquinones.

As an obligatory symbiont, the snapping shrimp could have adapted its chemosensory system to detect preferentially anthraquinones specific to crinoids. However, since they are also attracted by synthetic anthraquinones, a broad range of anthraquinones may attract *S. stimpsonii*. It can also discriminate the chemical signals between a host and non-host feather star what could be explained by different anthraquinonic cocktails or the presence of other semiochemicals than anthraquinones acting as attracting kairomones. In vivo, host selection by snapping shrimps is not only performed by chemical detection of anthraquinones but also by visual stimuli perception (Huang et al. 2005; VandenSpiegel et al. 1998). Therefore, visual communication probably also plays a role to avoid non-hidden or colorful feather stars like *C. bella* (Huang et al. 2005; VandenSpiegel et al. 1998).

Mimetic colors of the shrimps could be caused by the accumulation of anthraquinonic pigments in the symbiont tissues (see Brasseur et al. 2018b). Comparison of our results with those of the literature shows that *P. distincta* has its own cocktail of three different anthraquinone pigments, giving it a proper chemical signature. Two of these anthraquinones are well known in the literature (Feng et al. 2017; Kent et al. 1970; Rideout et al. 1979) and the third one is a potentially new anthraquinone. Although mass spectrometry can only provide accurate mass information and so potential composition, we can estimate that this new molecule is organized as an anthraquinone derivative with one or a few alkyl sidechains (with 6 carbons in total). Future works may concentrate on the anthraquinone extraction method detailed in this study to obtain enough pure dry extract to conduct NMR-spectroscopy analyses that could reveal the precise chemical structure of this new anthraquinone and its name. The relative intensity of ion peaks showed a large overabundance of rhodoptilometrin compared to the other anthraquinones. This result can be directly related to the molecule abundance in the crinoid extract but maybe also to its higher ionization potential. Only additional analyses with internal referents could allow quantifying the relative proportion of each congener in the anthraquinone cocktail. The natural concentration of anthraquinones contained in the tissues of crinoids is not currently known. A similar question concerns the amount of kairomones released in surrounding water by crinoids.

So far, only three host chemical factors involved in marine symbiotic associations have been identified in comparison to the thousands of molecules highlighted in plant–insect interactions, which underlines the significant lack of knowledge existing in marine chemical ecology. The first group of molecules is the amphikuemins (Murata et al. 1986) which acts in the symbiosis between sea anemones and anemonefishes (Konno et al. 1990). The second involves the saponins (Caulier et al. 2013) which mediates partner-recognition between the Harlequin crabs *Lissocarcinus orbicularis* and their holothuroid hosts. The kairomonal function of holothuroid saponins has also been confirmed in another symbiotic interaction, these molecules being chemoattractant for the symbiotic pearlfish *Encheliophis vermicularis* (Miyazaki et al. 2014). The third group of molecules is spinochromes (naphthoquinones) (Brasseur et al. 2017, 2018a) which are produced by the sea urchins *Echinometra mathaei* and recognized by its shrimp symbiont *Tuleariocaris holtuisi* (Brasseur et al. 2018b). We here demonstrate that crinoid anthraquinones are attractive to a symbiotic shrimp. In addition to their traditional defensive role (repellent allomones), quinones, therefore, would also function as attracting kairomones, maintaining the symbiosis between *S. stimpsonii* and its crinoid hosts.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00049-022-00368-6.

Acknowledgements This work is a contribution of the "Marine Station of Belaza" and part of AL's doctoral thesis supported by a FRIA (F.R.S.-FNRS) grant. JM and PF are Research associate and Research Director of the Fund for Scientific Research of Belgium (F.R.S.-FNRS), respectively. PG thanks the FRS-FNRS for financial support in the acquisition of the Waters QToF Premier Mass Spectrometer and for continuing support. All the authors thank Corentin Decroo for his valuable help. The authors thank the Fishery and Marine Science Institute of the University of Toliara for collaboration.

Author contributions GC and LB realized the behavioral experiments in Madagascar and chemical extractions and analyses in Belgium with an equal collaboration of the other co-authors. Each author contributed equally to the analyses of the results and the redaction of this manuscript.

Funding This work was supported by the F.R.S.-FNRS (GC was a FRIA PhD student, LB was funded by the FRFC grant n°14603427 and AL is a FRIA PhD student). GC and LB thank the Leopold III Fund, the Franeau Fund (UMONS) and the Agathon de Potter Fund (Académie Royale de Belgique) for their important financial support for field trips.

Availability of data and material The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Animals used in our experiments were maintained and treated in compliance with the guidelines specified by the Belgian Ministry of Trade and Agriculture.

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability Not applicable.

References

Asche C (2005) Antitumour quinones. Mini Rev Med Chem 5:449–467

Baeza JA, Thiel M (2007) The mating system of symbiotic crustaceans. A conceptual model based on optimality and ecological constraints. Evolutionary ecology of social and sexual systems: crustaceans as model organisms: 250–267

Baker JT, Sutherland MD (1968) Pigments of marine animals: VIII. Precursors of 6, 6′-dibromoindigotin (tyrian purple) from the mollusc *Dicathais orbita* Gmelin. Tetrahedron Lett 9:43–46

Bartolini GL, Erdman TR, Scheuer PJ (1973) Anthraquinone pigments from the crinoid *Comanthus bennetti*. Tetrahedron 29:3699–3702

Beattie JR, Maguire C, Gilchrist S, Barrett LJ, Cross CE, Possmayer F, Ennis M, Elborn JS, Curry WJ, McGarvey JJ (2007) The use of Raman microscopy to determine and localize vitamin E in biological samples. FASEB J 21:766–776

Berenbaum MR (1995) The chemistry of defense: theory and practice. Proc Natl Acad Sci 92:2–8

Brasseur L, Hennebert E, Fievez L, Caulier G, Bureau F, Tafforeau L, Flammang P, Gerbaux P, Eeckhaut I (2017) The roles of spinochromes in four shallow water tropical sea urchins and their potential as bioactive pharmacological agents. Mar Drugs. https://doi.org/10.3390/md15060179

Brasseur L, Caulier G, Lepoint G, Gerbaux P, Eeckhaut I (2018b) *Echinometra mathaei* and its ectocommensal shrimps: the role of sea urchin spinochrome pigments in the symbiotic association. Sci Rep. https://doi.org/10.1038/s41598-018-36079-8

Brasseur L, Caulier G, Flammang P, Gerbaux P, Eeckhaut I (2018a) Mapping of Spinochromes in the Body of Three Tropical Shallow Water Sea Urchins. Nat Prod Commun 13: 1934578X1801301222

Britayev TA, Mekhova ES (2011) Assessment of hidden diversity of crinoids and their symbionts in the Bay of Nhatrang, Vietnam. Org Divers Evol 11:275–285

Caulier G, Flammang P, Gerbaux P, Eeckhaut I (2013) When a repellent becomes an attractant: harmful saponins are kairomones attracting the symbiotic Harlequin crab. Sci Rep. https://doi.org/10.1038/srep02639

Davenport D (1950) Studies in the physiology of commensalism: 1. The polynoid genus arctonoe. Biol Bull 98:81–93

De Bruyn C, David B, Motreuil S, Caulier G, Jossart Q, Rigaud T, De Ridder C (2016) Should I stay or should I go? Causes and dynamics of host desertion by a parasitic crab living on echinoids. Mar Ecol Prog Ser 546:163–171

Deheyn DD, Lyskin S, Eeckhaut I (2006) Assemblages of symbionts in tropical shallow-water crinoids and assessment of symbionts' host-specificity. Symbiosis 42:161–168

Eeckhaut I, VandenSpiegel D, Michel A, Jangoux M (2001) Host chemodetection by the crinoid associate, *Harrovia longipes* (Crustacea: Brachyura: Eumedonidae) and a physical characterization of a crinoid-released attractant. Asian Mar Biol 17:111–123

Elling FJ, Becker KW, Könneke M, Schröder JM, Kellermann MY, Thomm M, Hinrichs KU (2016) Respiratory quinones in Archaea: phylogenetic distribution and application as biomarkers in the marine environment. Environ Microbiol 18:692–707

El-Najjar N, Gali-Muhtasib H, Ketola RA, Vuorela P, Urtti A, Vuorela H (2011) The chemical and biological activities of quinones: overview and implications in analytical detection. Phytochem Rev 10:353–370

Feng Y, Khokhar S, Davis RA (2017) Crinoids: ancient organisms, modern chemistry. Nat Prod Rep 34:571–584

Ghaly NS, Melek FR, Mabry TJ (2009) Angular naphthopyrones with DPPH scavenging activity from the crinoid *Oligometra serripinna*. Rev Latinoam Quim 37:252–261

Gong X, Gutala R, Jaiswal AK (2008) Quinone oxidoreductases and vitamin K metabolism. Vitam Horm 78:85–101

Gordaliza M (2010) Cytotoxic terpene quinones from marine sponges. Mar Drugs 8:2849–2870

Hasler AD, Scholz AT, Horrall RM (1978) Olfactory imprinting and homing in Salmon: recent experiments in which salmon have been artificially imprinted to a synthetic chemical verify the olfactory hypothesis for salmon homing. Am Sci 66:347–355

Huang H-D, Rittschof D, Jeng M-S (2005) Visual orientation of the symbiotic snapping shrimp *Synalpheus demani*. J Exp Mar Biol Ecol 326:56–66

Kasumyan A, Isaeva O, Dgebuadze P, Mekhova E, Britayev T (2020) Comatulids (Crinoidea, Comatulida) chemically defend against coral fish by themselves, without assistance from their symbionts. Sci Rep 10:1–13

Kemami Wangun HV, Wood A, Fiorilla C, Reed JK, McCarthy PJ, Wright AE (2010) Gymnochromes E and F, cytotoxic phenanthroperylenequinones from a deep-water crinoid, *Holopus rangii*. J Nat Prod 73:712–715

Kent RA, Smith IR, Sutherland MD (1970) Pigments of marine animals. X. Substituted naphthopyrones from the crinoid *Comantheria perplexa*. Aust J Chem 23:2325–2335

Kittredge JS, Takahashi FT, Lindsey J, Lasker R (1974) Chemical signals in the sea: marine allelochemics and evolution. Fish Bull 72:1–11

Konno K, Qin G-W, Nakanishi K, Murata M, Naya Y (1990) Synthesis of amphikuemin and analogs: a synomone that mediates partner-recognition between anemonefish and sea anemone. Heterocycles (sendai) 30:247–251

Kornprobst J-M (2005) Substances naturelles d'origine marine: chimiodiversité, pharmacodiversité, biotechnologies. Lavoisier Tec & Doc

Lanterbecq D, Rouse GW, Eeckhaut I (2010) Evidence for cospeciation events in the host–symbiont system involving crinoids (Echinodermata) and their obligate associates, the myzostomids (Myzostomida, Annelida). Mol Phylogenet Evol 54:357–371

Lee NK, Kim YH (1995) New cytotoxic anthraquinones from the crinoid Ptilometra: 1'-deoxyrhodoptilometrin-6-O-sulfate and rhodoptilometrin-6-O-sulfate. Bull Korean Chem Soc 16:1011–1013

Lu J-J, Bao J-L, Wu G-S, Xu W-S, Huang M-Q, Chen X-P, Wang Y-T (2013) Quinones derived from plant secondary metabolites as anti-cancer agents. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents) 13: 456–463

Lubitz W (2003) Photochemical processes in photosynthesis studied by advanced electron paramagnetic resonance techniques. Pure Appl Chem 75:1021–1030

Marshall JI, Rowe FWE (1981) The crinoids of Madagascar

Miyazaki S, Ichiba T, Reimer JD, Tanaka J (2014) Chemoattraction of the pearlfish *Encheliophis vermicularis* to the sea cucumber *Holothuria leucospilota*. Chemoecology 24:121–126

Murata M, Miyagawa-Kohshima K, Nakanishi K, Naya Y (1986) Characterization of compounds that induce symbiosis between sea anemone and anemone fish. Science 234:585–587

Nohl H, Jordan W, Youngman RJ (1986) Quinones in biology: functions in electron transfer and oxygen activation. Adv Free Rad Biol Med 2:211–279

Pasteels JM, Braekman J-C, Daloze D (1988) Chemical defense in the Chrysomelidae. In: Jolivet P, Petitpierre E, Hsiao TH (eds) Biology of chrysomelidae. Springer, Netherlands, pp 233–252

Powell VH, Sutherland MD (1967) Pigments of marine animals. VI. Anthraquinoid pigments of the crinoids *Ptilometra australis* Wilton and *Tropiometra afra* Hartlaub. Aust J Chem 20:541–553

R Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Rao RN, Talluri MVNK, Shinde DD (2008) Simultaneous separation and determination of coenzyme Q10 and its process related impurities by NARP-HPLC and atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). J Pharm Biomed Anal 47:230–237

Rideout JA, Sutherland MD (1981) Pigments of marine animals. XIV. Polyketide sulfates from the crinoid *Comatula pectinata*. Aust J Chem 34:2385–2392

Rideout JA, Sutherland MD (1985) Pigments of marine animals. XV. Bianthrones and related polyketides from *Lamprometra palmata* gyges and other species of crinoids. Aust J Chem 38:793–808

Rideout JA, Smith NB, Sutherland MD (1979) Chemical defense of crinoids by polyketide sulphates. Experientia 35:1273–1274

Shikov AN, Pozharitskaya ON, Krishtopina AS, Makarov VG (2018) Naphthoquinone pigments from sea urchins: chemistry and pharmacology. Phytochem Rev 17:509–534

Singh H, Moore RE, Scheuer PJ (1967) The distribution of quinone pigments in echinoderms. Experientia 23:624–626

Smith IR, Sutherland MD (1971) Pigments of marine animals. XI. Angular naphthopyrones from the crinoid *Comanthus parvicirrus timorensis*. Aust J Chem 24:1487–1499

Sunassee SN, Davies-Coleman MT (2012) Cytotoxic and antioxidant marine prenylated quinones and hydroquinones. Nat Prod Rep 29:513–535

Sutherland MD, Wells JW (1967) Pigments of marine animals. IV. The anthraquinoid pigments of the crinoids, *Comatula pectinata* L. and *C. cratera* AH Clark. Aust J Chem 20:515–533

Takahashi D, Maoka T, Tsushima M, Fujitani K, Kozuka M, Matsuno T, Shingu T (2002) New quinone sulfates from the crinoids *Tropiometra afra macrodiscus* and *Oxycomanthus japonicus*. Chem Pharm Bull (tokyo) 50:1609–1612

Thomson RH (2012) Naturally occurring quinones. Elsevier

Tinkova TV, Kasumyan AO, Dgebuadze PY, Oanh K, Britaev TA (2014) Deterrence of feather stars (Crinoidea, Comatulida) from Southern Vietnam for the Indo-Pacific sergeant-fish *Abudefduf vaigiensis*. Dokl Biol Sci 456:195–198

Tseng C-C, Lai Y-C, Kuo T-J, Su J-H, Sung P-J, Feng C-W, Lin Y-Y, Chen P-C, Tai M-H, Cheng S-Y (2019) Rhodoptilometrin, a crinoid-derived anthraquinone, induces cell regeneration by promoting wound healing and oxidative phosphorylation in human gingival fibroblast cells. Mar Drugs 17:138

VandenSpiegel D, Eeckhaut I, Jangoux M (1998) Host selection by *Synalpheus stimpsoni* (De Man), an ectosymbiotic shrimp of comatulid crinoids, inferred by a field survey and laboratory experiments. J Exp Mar Biol Ecol 225:185–196. https://doi.org/10.1016/S0022-0981(97)00222-0

Wolkenstein K, Gross JH, Falk H, Schöler HF (2006) Preservation of hypericin and related polycyclic quinone pigments in fossil crinoids. Proc Royal Soc B Biol Sci 273:451–456

Wolkenstein K, Schoefberger W, Müller N, Oji T (2009) Proisocrinins A– F, Brominated Anthraquinone Pigments from the Stalked Crinoid *Proisocrinus ruberrimus*. J Nat Prod 72:2036–2039

Wolkenstein K, Fuentes-Monteverde JC, Nath N, Oji T, Griesinger C (2018) Hypalocrinins, Taurine-Conjugated Anthraquinone and Biaryl Pigments from the Deep Sea Crinoid *Hypalocrinus naresianus*. J Nat Prod 82:163–167

Zhu L, Yu S, Zeng X, Fu X, Zhao M (2008) Preparative separation and purification of five anthraquinones from *Cassia tora* L. by high-speed counter-current chromatography. Sep Purif Technol 63:665–669

Zmarzly DL (1984) Distribution and ecology of shallow-water crinoids at Enewetak Atoll, Marshall Islands, with an annotated checklist of their symbionts