Volatile secondary metabolites as aposematic olfactory signals and defensive weapons in aquatic environments

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Olfaction is considered a distance sense; hence, aquatic olfaction is thought to be mediated only by molecules dissolved in water. Here, we challenge this view by showing that shrimp and fish can recognize the presence of hydrophobic olfactory cues by a "tactile" form of chemoreception. We found that odiferous furanosesquiterpenes protect both the Mediterranean octocoral *Maasella edwardsi* and its specialist predator, the nudibranch gastropod *Tritonia striata*, from potential predators. Food treated with the terpenes elicited avoidance responses in the cooccurring shrimp *Palaemon elegans*. Rejection was also induced in the shrimp by the memory recall of postingestive aversive effects (vomiting), evoked by repeatedly touching the food with chemosensory mouthparts. Consistent with their emetic properties once ingested, the compounds were highly toxic to brine shrimp. Further experiments on the zebrafish showed that this vertebrate aquatic model also avoids food treated with one of the terpenes, after having experienced gastrointestinal malaise. The fish refused the food after repeatedly touching it with their mouths. The compounds studied thus act simultaneously as (*i*) toxins, (*ii*) avoidance-learning inducers, and (*iii*) aposematic odorant cues. Although they produce a characteristic smell when exposed to air, the compounds are detected by direct contact with the emitter in aquatic environments and are perceived at high doses that are not compatible with their transport in water. The mouthparts of both the shrimp and the fish have thus been shown to act as "aquatic noses," supporting a substantial revision of the current definition of the chemical senses based upon spatial criteria.

marine chemical ecology | chemical defense | olfactory aposematism | avoidance learning | volatile terpenes

Traditionally, the sense of smell has been regarded as a distance sense (like vision), whereas the sense of taste has been treated as a contact sense (like touch). This classification of the chemical senses (olfaction and gustation) based on their spatial range persists in contemporary literature. Accordingly, biomolecules smaller than ~300 Da, which can be transported through air, and which finally bind to odorant receptors expressed in olfactory neurons, are generally considered odorant molecules. Conversely, the molecules sensed by taste have to be in solution and in contact with the receptor (1). However, the claim that "only olfaction can provide information on the identity of the water mass encountered" by marine organisms (2) implies that water-soluble compounds, which are typically "tasted" by the tongues of terrestrial animals, act as olfactory cues in aquatic environments. Consequently, it may be asked how typical odorant molecules such as small terpenes from plants and marine sponges that combine high volatility in air with insolubility in water can produce olfactory sensations in marine organisms, thereby giving protection from predators and cues to finding food and mates.

The distinction between olfaction and gustation based upon spatial criteria is prevalent in the literature on aquatic chemical communication (3). However, this differentiation has been reevaluated in a recent perspective article (4). Therein, a substantial revision of the traditional classification of the chemical senses has been proposed based on the cues actually involved in chemical sensations rather than upon spatial criteria. In the present study, we provide empirical support for this thesis by challenging the notion that the aquatic sense of smell cannot be mediated by hydrophobic and airborne olfactory molecules.

We report the finding of furanosesquiterpenes isofuranodiene (**1**), (−)-atractylon (**2**), and (−)-isoatractylon (**3**) (Fig. 1) in *Maasella edwardsi* (de Lacaze-Duthiers, 1888) (Fig. 2 *A* and *C*), a Mediterranean octocoral (Cnidaria: Anthozoa: Alcyonacea) from which the related sesquiterpene lactones **4–6** (Fig. 1) had previously been reported (5). The same compounds were also found in the nudibranch *Tritonia striata* Haefelfinger, 1963 (Fig. 2 *B–D*), a mollusk (Mollusca: Gastropoda: Nudibranchia) that usually seeks refuge among the pedicels of *M. edwardsi*. Compounds **1–3** act as typical olfactory cues, showing characteristic odors when exposed to air. It is noteworthy that isofuranodiene (**1**), often misleadingly called furanodiene (6), also occurs in marine organisms (7–12), but it is better known for contributing to the smell of several terrestrial flowering plants, including myrrh and white turmeric, some of which are commonly used as food additives and fragrance ingredients (6). Furthermore, atractylon (**2**), which has also been found in marine animals (10), is a major odor-active compound from

Significance

This report gives empirical evidence indicating that chemoreception of volatile/odorant lipophilic compounds, almost insoluble in water, can occur in aquatic environments, by means of "tactile" forms of olfaction. This thesis has been proved by exploring the defensive role of terpenes isolated from benthic invertebrates. The isolated metabolites were found to act both as defensive toxic weapons and as olfactory signals. In addition, the most abundant compound induced avoidance learning in crustaceans and fish that experienced postingestive negative effects.
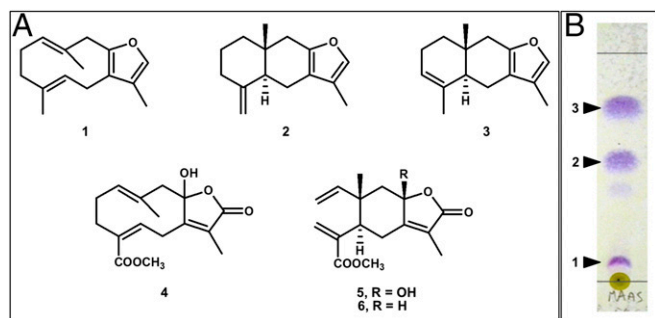
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Fig. 1. Metabolites from *M. edwardsi*. (*A*) Chemical structures of isofuranodiene (**1**), atractylon (**2**), isoatractylon (**3**), edwardsiolide A (**4**), edwardsiolide B (**5**), and edwardsiolide C (**6**). (*B*) Detail of the analytical AgNO$_3$-treated TLC plate revealing the occurrence of bands corresponding to furanosesquiterpenes **1–3** in the crude diethyl ether extract of *M. edwardsi* (MAAS), after spraying with Ehrlich's reagent. Eluent is *n*-hexane/diethylether 95:5.

plants (13, 14). In contrast, the volatile compound **3** has only been reported from the marine cnidarian *Dasystenella acanthina* (Wright & Studer, 1889) as the (+)-enantiomer (7). Isofuranodiene and atractylon are also known for their pharmacological properties. In particular, isofuranodiene shows anticancer and antiangiogenic activity (15–22), as well as analgesic properties when acting on brain opioid receptors (23). Atractylon exhibits antiinflammatory activity (24), cytotoxicity against various human cancer cell lines (25, 26), acaricidal activity (27), and inhibition of Na$^+$, K$^+$-ATPase activity (28). However, previous attempts to explore the natural function of compound **1** in the Antarctic gorgonian *D. acanthina* have failed to recognize its feeding deterrent properties against the goldfish *Carassius auratus* (Linnaeus, 1758), although the compound showed toxicity to the mosquitofish *Gambusia affinis* (Baird & Girard, 1853) (7). Similar results have been obtained for the (+) enantiomer of compound **3**, supporting the idea that these volatile compounds do not induce olfactory-mediated recognition and avoidance in fish, although they are icthyotoxic. This point is a critical one in marine chemical ecology because previous studies have not clearly shown that volatile compounds can deter predation in marine environments (29, 30).

In this report, recognition and avoidance of hydrophobic odors in water has been studied by evaluating the ecological role of the compounds detected in *M. edwardsi* and *T. striata*. The finding of the nudibranch's egg masses in spirals on the surface of coral colonies in the field (Fig. 2*C*) suggested a trophic relationship between the two marine benthic invertebrates, and a possible defensive role of compounds **1–3**. Nudibranchs are known to lay their egg masses on or near the prey they feed on (mainly sponges and cnidarians) and from which they obtain defensive compounds. A specifically designed aquarium system (Fig. S1*A*) allowed us (*i*) to follow the life cycle of *M. edwardsi* colonies collected in the archipelago of Li Galli in Southern Italy (Fig. S1*B*), (*ii*) to observe directly the nudibranch feeding on the octocoral tissues and larvae, and (*iii*) to photograph the larvae (veligers) of *T. striata* inside an egg mass laid in captivity (Fig. 2*D*). The feeding deterrence activity of compounds **1–3** was evaluated against the cooccurring generalist shrimp *Palaemon elegans* Rathke, 1837, following an approach already used to assess the palatability of other bioactive metabolites (31), including marine furanosesquiterpenes (32, 33). Like other palaemonid species (34, 35), *P. elegans* is suitable for evaluations of this kind, which require a good view of the ingested food in the digestive system (31). Given that decapod crustaceans exhibit complex learning ability (36, 37), the possibility that the shrimp learn to avoid the compounds after experiencing aversive olfactory stimuli was also considered. Subsequently, we conducted additional behavioral assays on the zebrafish (*Danio rerio* Hamilton, 1822). Although this animal model is not of direct ecological relevance to

the system under investigation, it provided additional information about the possible effects of compound **3** on aquatic lower vertebrates. General toxicity of the compounds was estimated by using the brine shrimp *Artemia salina* (Linnaeus, 1758), allowing us to provide information about the level of toxicity within a recognized scale of toxicity (38).

Results

Purification, Identification, and Quantification of the Furanosesquiterpenes. Isofuranodiene (**1**), (−)-atractylon (**2**), and (−)-isoatractylon (**3**) were extracted and isolated from *M. edwardsi*, and identified by comparison of spectroscopic data with the literature (7, 10, 13). Compounds **2** and **3** displayed negative optical rotation values (**2**: $[\alpha]_D = -78$, c 0.15, hexane; **3**: $[\alpha]_D = -128$, c 0.22, hexane), opposite in sign to those previously reported (7, 13). The characteristic spicy and woody odors of the three isolated furanoterpenes, which can be distinguished by their slightly different olfactory nuances, were clearly detected during purification, revealing the presence of the compounds in the chromatographic fractions even before visualizing them on TLC plates (Fig. 1*B*). A quantification of the volatile metabolites in the crude extracts of three individuals of both *M. edwardsi* and *T. striata* was carried out by GC-MS. Although, under the conditions used, we were not able to discriminate between isomeric compounds **2** and **3**, owing to their similar chromatographic behavior, the total amount of the three compounds was apparently higher in the mollusk than in its prey (Fig. 3*A*). The mixture of furanosesquiterpenes **1–3** was further detected by TLC in a sample of the mucus secreted by *M. edwardsi* following sampling with a cotton swab on the surface of a living colony. Furthermore, the presence of isofuranodiene (**1**) was ascertained by ^{1}H NMR in the extract from a few planulae of *M. edwardsi*, although the low amount of extracted material and the volatility of the furanosesquiterpenes prevented the identification of all of the other metabolites present in the extract and their realistic quantification. Subsequently, before proceeding with the evaluation of the biological activity of the compounds, we evaluated the natural volumetric concentrations (in milligrams per milliliter) of each compound in three whole small colonies of *M. edwardsi*, and in three individuals of *T. striata* by quantitative ^{1}H NMR. This analysis showed that (−)-isoatractylon (**3**) was the major sesquiterpene in both animals (Fig. 3*B*).

Feeding Deterrence Activity. Compounds **1–3** were then tested for their activity as feeding deterrents against *P. elegans*. The presence of a red spot visible by transparency in the gastric mill and the stomach of the shrimp was considered as acceptance of food (Movie S1), and the absence of the spot gave a rejection response
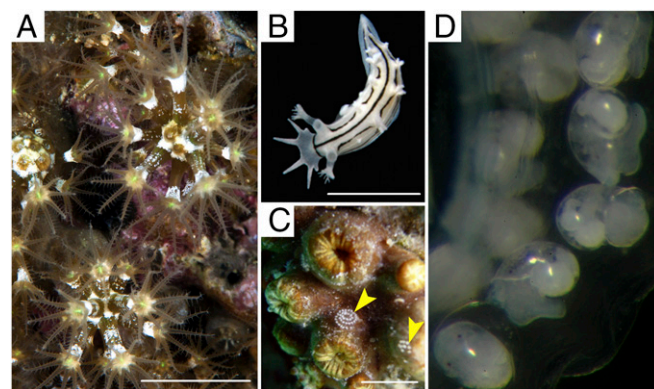

Fig. 2. Studied animals. (*A*) *M. edwardsi*; (*B*) *T. striata*; (*C*) yellow arrows indicate egg masses of *T. striata* on *M. edwarsdi* (in retracted state). (Scale bars, 5 mm.) (*D*) *T. striata* larvae inside its egg mass.
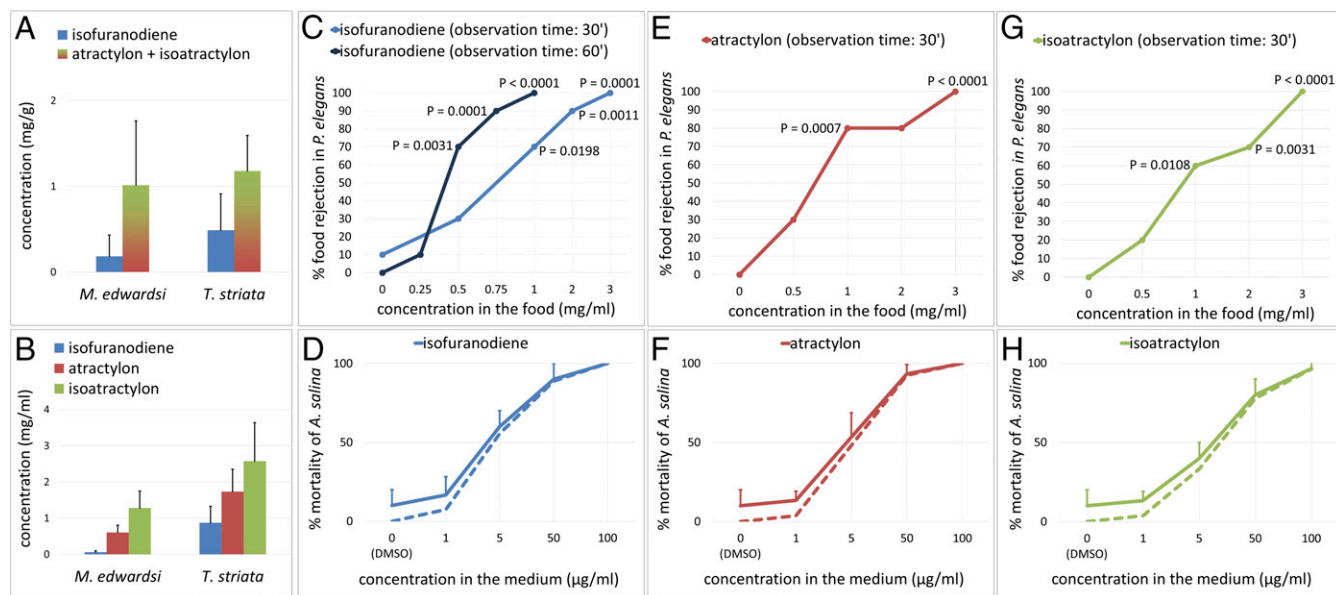
Fig. 3. Quantification of the metabolites and evaluation of their biological activity. (*A* and *B*) Concentration of compounds **1**–**3** quantified by (*A*) GC-MS and (*B*) ^{1}H NMR. Values shown are means and SDs from triplicate extractions from different colonies/individuals. (*C*, *E*, and *G*) Feeding deterrence dose–response curves against *P. elegans*. The significant differences were evaluated using the two-tailed Fisher's exact test ($n = 10$ for each tested concentration, $\alpha = 0.05$). (*D*, *F*, and *H*) Dose–response curves of toxicity to *A. salina*. The results are shown as the means and SDs of triplicate wells. Because deaths also occurred in the control (vehicle DMSO only), percentages of deaths were corrected by using Abbott's formula (dashed lines).

(Movie S2). As summarized in Fig. 3 *C*, *E*, and *G*, the purified compounds were all significantly active, starting from a minimum effective dose of 1.0 mg/mL. It is noteworthy that, in some cases, the treated food induced vomiting in the shrimp when ingested; vomiting was always preceded by spasmodic contractions of the stomach, which were easily observable because of the transparency of the animals (Movie S3). The regurgitations occurring within an observation time of 30 min were treated as cases of food rejection, contributing to the dose–response curves, whereas emetic events were not noticed in shrimp that had accepted the control food, even upon prolonging the observation time to 1 h. Conversely, the incidence of vomiting increased with the observation time, occurring after a delay varying from 5 min to 50 min. Accordingly, assays carried out on isofuranodiene (**1**), and with the observation time extended to 60 min, revealed significant food rejection at a concentration as low as 0.5 mg/mL, due to the occurrence of delayed regurgitation events, which had not been observed using observation intervals of 30 min in the previous experiment (Fig. 3*C*).

Avoidance Learning. The role of regurgitation events in avoidance learning was next evaluated by offering food containing 4 mg/mL of (−)-isoatractylon (**3**) to a larger sample of shrimp after 2 d fasting ($n = 15$) (Fig. 4*A*). In the first treatment, 10 individuals did not accept the food, whereas the remaining 5 individuals ingested the food but then vomited it within 30 min (Fig. 4*A*, step I). In the next administration (Fig. 4*A*, step II), none of the shrimp accepted the treated food. Finally, untreated control food was significantly ($P < 0.0001$) accepted by the shrimp during the last step (Fig. 4*A*, step III). It is noteworthy that all shrimp that had previously experienced emetic events significantly ($P = 0.0079$) rejected the treated food during a subsequent administration, having touched it, while significantly ($P = 0.0476$) accepting nontreated food during the last experimental step (Fig. 4*B*). Different responses were observed using the zebrafish as a vertebrate model (Fig. 5). When food treated with (−)-isoatractylon (**3**) (4.0 mg/mL) was offered to the fish, after 1 d of fasting, the food pellets were accepted during three subsequent administrations at 15-min intervals, but apparently undigested food was found at the bottom of

most of the tanks within 5 h after the beginning of the experiment. Analysis of recorded videos in slow motion led us to observe that such material was not regurgitated/vomited food, but instead was a sort of undigested fecal bolus (different from the excrement normally produced by zebrafish). The fish completely rejected the treated food in a subsequent administration (Fig. 5*A*, time 5 h 15 min). The memory recall was induced after the fish repeatedly took the food into their oral cavity before definitely refusing it (Movie S4). However, fish also avoided control plain food offered 15 min later; but just 2 min later, they accepted the commercial pellets (Fig. 5*A*, time 5 h 32 min).

Brine Shrimp Toxicity Assay. The assay revealed high toxicity in vivo of compounds **1**–**3** against *A. salina*, with $LC_{50} < 50$ µg/mL (Fig. 3 *D*, *F*, and *H*), whereas a dose-dependent gradual attenuation of
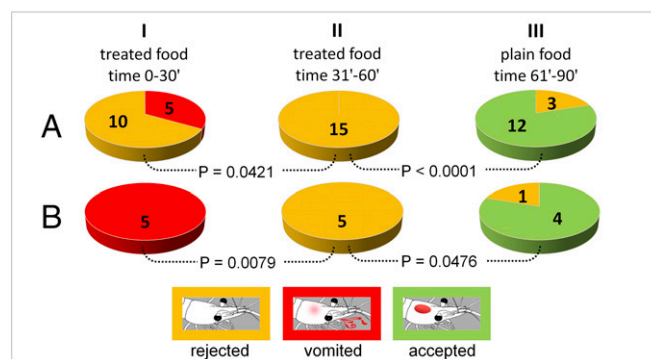

Fig. 4. Avoidance learning assay on *P. elegans*. Recorded responses: green, food acceptance (as shown in Movie S1); yellow, food rejection after touching (as shown in Movie S2); and red, vomiting (as shown in Movie S3). (*A*) Response from the whole sample ($n = 15$) to food containing (−)-isoatractylon at a concentration of 4.0 mg/mL (step I, step II) and untreated plain food (step III). (*B*) Response from the subgroup of the vomiting shrimp ($n = 5$). *P* values are calculated using the two-tailed Fisher's exact test ($\alpha = 0.05$).
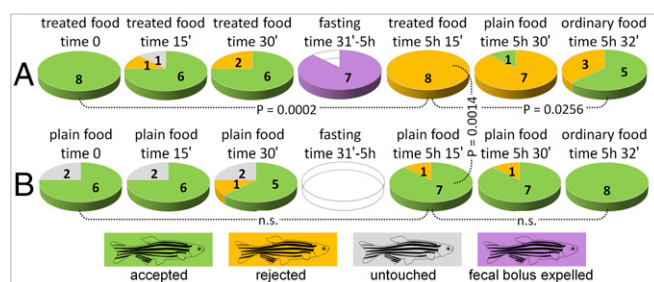
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Fig. 5. Avoidance learning in the zebrafish. Recorded responses: green, food acceptance; yellow, food rejection after touching (as shown in Movie S4); gray, rejection without touching; and violet, evacuation of fecal bolus. (*A*) Time-dependent responses to food containing (−)-isoatractylon (**3**) followed by responses to nontreated plain food, and to commercial granular pellets (ordinary food). (*B*) Responses to plain food. *P* values are calculated using the two-tailed Fisher's exact test (n.s. = not significant; *n* = 8; α = 0.05).

the brine shrimp movements was also noticed under the action of the assayed compounds.

Discussion

It is believed that the chemosensory world of marine organisms is limited to water-soluble, and does not include volatile, chemical stimuli (39, 40). The present report contradicts this preconceived idea by showing that aquatic animals also sense volatile biomolecules when they face the vital problem of determining food palatability.

The first phase of the study focused on the isolation of the metabolites and on the problem of a possible interference by chemical transformations that can confound assay readouts when working with labile compounds. The odorant compounds iso-furanodiene (**1**), (−)-atractylon (**2**), and (−)-isoatractylon (**3**) have been found in adult colonies of *M. edwardsi* collected along Italian coasts, whereas germacrane and elemane class sesquiterpenoid lactones, previously isolated from *M. edwardsi* from Tunisia (5) (Fig. 2*A*), were not detected. This discrepancy could reflect either geographical variations in the chemical composition of the octocoral or a degradation of the furanosesquiterpenes **1–3**, possibly due to postcollection oxidation or to the use of methanol as the extraction solvent. Furans are known to be susceptible to oxidative processes that can lead to five-membered lactones. They undergo photo-oxygenation reactions under natural or fluorescent light and in so-lution in methanol (41, 42). In the present work, we also avoided the use of chloroform, which induces rapid transformations of the compounds. Instead, acetone and benzene were used for the ex-traction, and deuterated benzene (benzene-d_6) was used to record the NMR spectra. Once the compounds were identified, GC-MS and ^{1}H NMR studies proved, chemically, the prey–predator trophic relationship between the octocoral and the mollusk. Compounds **1–3** were detected in both the prey and the predator, and the presence of isofuranodiene (**1**) was also ascertained by ^{1}H NMR in the extract from the larvae (planulae) of *M. edwardsi* that the mollusk actively eats after the octocoral spawns (Movie S5). The higher concentra-tion of compounds **1–3** in the mollusk than in the octocoral supports bioaccumulation farther up the food chain. Moreover, the most abundant furanoterpene in both organisms was compound **3**, which also was particularly abundant in *T. striata*.

Among a wide range of potential predators of both *M. edwardsi* and *T. striata*, we selected the cooccurring generalist shrimp *P. elegans* for the chemoecological assays. The transparency of this shrimp allows an easy visualization of the food once ingested (Movie S1). This crustacean can be maintained for a long time in a small volume of seawater, allowing us easily to perform series of individual replicates in a chemical laboratory, where it is actually possible also to control the state of the compounds immediately

before the experiments. Dose–response curves provided information about the thresholds of activity of compounds **1–3** independently from the levels of the metabolites measured in the samples, thus describing the change in effect on shrimp caused by differing levels of exposure to compounds **1–3**, after a certain exposure time. By setting an observation interval of 30 min, and considering emetic events as cases of food rejection, all compounds were significantly active as feeding deterrents starting from a concentration of 1 mg/mL, and reaching 100% rejection at a concentration of 3 mg/mL. Although the natural concentrations of these unstable, volatile compounds may have been underestimated, and despite the fact that a small loss of compounds was likely to occur during artificial food preparation, our measurements indicated that the most abundant compound **3** was present in both studied animals in suf-ficient amounts to induce food rejection and/or vomiting by po-tential predators (Fig. 3 *A* and *B*). However, in the case of compound **1**, extension of the observation interval to 60 min allowed the detection of delayed regurgitation events, revealing sig-nificant rejection even at concentrations as low as 0.5 mg/mL (Fig. 3*C*). Further experiments carried out on (−)-isoatractylon (**3**) led us to observe that the shrimp that swallowed the treated food, and then vomited it, completely avoided the treated food in a subsequent administration, but only after having repeatedly touched it. Consequently, the protective effect seems to have been achieved both through the induction of a basic olfactory aversion to compounds **3** and by the olfactory recall of postingestive toxic effects. General toxicity measurements carried out by *A. salina* lethality assays revealed high toxicity for compounds **1–3** (Fig. 3 *D*, *F*, and *H*). However, the toxic doses of compounds **1–3** against brine shrimp were significantly lower than the doses required to deter *P. elegans* from feeding, or to induce vomiting. This finding can be explained by taking into account that the exposure route, as well as the physicochemical properties of the involved compounds, is extremely important in determining toxicity. In the tests with *A. salina*, compounds **1–3** were conveyed in water using DMSO. Under such conditions, the compounds act like waterborne mole-cules and can also directly reach the respiratory system of the brine shrimp. Conversely, in the feeding assays on *P. elegans*, the lipo-philic molecules have a very low propensity to diffuse in water, due to hydrophobic interactions. Consequently, chemoreception takes place by touching the food with the chemosensory mouthparts (see Movie S2), whereas uptake of the products can occur only after ingestion (see Movie S1). Subsequently, the compounds can be poorly absorbed or immediately detoxified by the digestive system, and vomiting also helps to avoid food poisoning (see Movie S3). However, the toxicity of compounds **1–3** to brine shrimp is further suggestive of an involvement in mechanisms of predation as part of the alimentary strategies of *M. edwardsi* in nature, helping to cap-ture dietary zooplanktonic crustaceans (e.g., copepods) that come into contact with the mucus layer covering the octocoral, and which lose their ability to escape, owing to intoxication. Obviously *M. edwardsi* does not use DMSO as a cosolvent to dissolve the li-pophilic compounds in water, but when small planktonic organisms come into contact with the octocoral, they actually remain em-bedded in the secretions that cover the octocoral, like flies on flypaper. An attenuation of the movements has been also noticed in *A. salina* under the action of the assayed compounds, and the furanosesquiterpenes have also been detected in the mucus that covers the surface of *M. edwardsi* and first comes in contact with its zooplanktonic prey. Again, this finding implies that the relative insolubility of the compounds in water contributes to ensuring adequate local concentrations, allowing their effectiveness in both predation and defense (Fig. 6). The furanosesquiterpenes of *M. edwardsi* and *T. striata* thus convey different messages depending on the different receiver organisms. They can act as both defensive warning messages and toxic chemical weapons if directed to po-tential predators or prey, but they evidently do not deter *T. striata* (the specialist predator) from feeding. On the contrary, in the
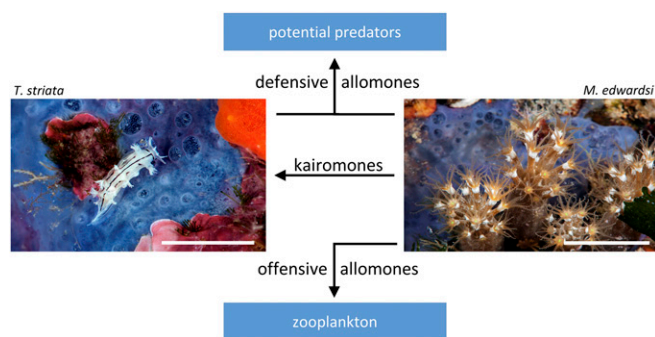
Fig. 6. Proposed multiple ecological roles of furanosesquiterpenes **1–3**. (Scale bars, 10 mm.)

latter case, the compounds possibly indicate a food source for the receiver (acting as kairomones), which is able to handle and reuse them in self-defense. Our observation of *T. striata* actively feeding on *M. edwardsi* and its larvae in aquarium strongly supports this hypothesis. Moreover, the chemical message is stronger in *T. striata*, which accumulates higher levels of the dietary compounds **1–3**, and it is reinforced by a conspicuous coloration (white color striped with black lines) that is reminiscent of the aposematic pattern of striped skunks and their famous defensive scent. According to the model developed by Gohli and Högstedt (43), the visual aposematic signal of *T. striata*, which is easily detectable by potential predators with well-developed visual systems, could have evolved in the mollusk, favored by the strongest message conveyed by high levels of protective olfactory chemicals.

Aposematism describes the association between easily detectable conspicuous signals (visual, olfactory, and acoustic) and unprofitability (33, 44). However, as is true for plants that simultaneously use visual and olfactory aposematic signals for animal attraction (45), *T. striata* uses double signaling, but for defensive purposes, whereas *M. edwardsi* relies on olfactory aposematism, which may provide protection from a wider range of predators, including those lacking efficient visual systems (35). It is worth underlining here that compounds **1–3** induce both toxic and olfactory-guided food avoidance effects and, differently from the cases considered by Eisner and Grant (44), they function as conditioned olfactory stimuli for indicating their own intrinsic toxicity and unprofitability. However, odors that themselves are noxious have already been described in insects (46). Although the experiments on the zebrafish failed to recognize vomiting events in this vertebrate animal model, food treated with compound **3**, once ingested, induced a sort of gastrointestinal malaise in the fish, which led to the evacuation of an apparently undigested fecal bolus, and to subsequent avoidance learning. Differently from the shrimp, however, the zebrafish seemed to learn from a wider range of sensory signals associated with the negative postingestive toxic experience, possibly including not only visual and chemical senses but also the perception of the consistency of the food. In *P. elegans*, instead, olfaction seems to be the main way to avoid dangerous foods. However, in both animals, the memory recall and the consequent food rejection were always induced after touching the food (as shown in Movies S2 and S4). The need to repeatedly touch the food, before deciding whether to swallow or refuse it, is consistent with the extremely short range of action of insoluble olfactory cues in the aquatic environment. Accordingly, the observations made in this work call for some reflections on the range of effectiveness of volatile and hydrophobic olfactory cues in terrestrial and marine environments. In particular, to say that the odiferous isofuranodiene (**1**), a major constituent of the extract of terrestrial plants, can act in long-distance signaling on land is an evident truism. Conversely, our results on *P. elegans* show that the minimum concentrations at which compounds **1–3** produce significant effects on alimentary behavior are significantly higher

than those allowing terrestrial animals to recognize and avoid an unpleasant food. This fact, combined with the extremely low number of genes encoding for odorant receptors in marine fish compared with terrestrial tetrapods (47), and the very low solubility in water of compounds **1**, **2**, and **3** [estimated to be about 0.0001, 0.0004, and 0.0005 mg/mL, respectively (48)], makes the feeding deterrent action of the compounds at a distance in the marine environment implausible. Although solubility in saline water should even be lower than the estimated solubility in fresh water, the estimated concentrations of saturated solutions with compounds **1–3** are three orders of magnitude lower than the experimentally determined minimum concentrations in artificially prepared food able to deter *P. elegans* from feeding. Accordingly, during the experiments, the shrimp were hungry and actively searching for food, repeatedly "smelling" the potential sources of food by touching it with their mouthpart chemosensors. Even after having experienced the negative experience of vomiting, the shrimp needed to touch the food before deciding whether to accept or reject it, evidently because they were unable to detect the unpleasant smell dissolved in water. These aquatic animals are thus provided with a sense of smell that can be mediated by stimulatory molecules that are not in solution. Olfactory biomolecules, such as compounds **1–3**, which cannot be transported at effective doses in an aquatic medium, can instead be perceived at high doses by direct contact with the emitter (as shown in Movies S2 and S4). This is a fact that we must no longer ignore, even if it will require a substantial revision of the current definition of the chemical senses based on their spatial range.

Materials and Methods

Animals. Colonies of *M. edwardsi* and 10 individuals of *T. striata* were sampled by scuba diving along the coast of the Campania region (southern Italy) in shallow rocky sites at Secca di Vatolla and in the archipelago of Li Galli, with the permission of the Marine Protected Areas of Santa Maria di Castellabate and Punta Campanella, respectively (Fig. S1*B*). The samples were transported to the laboratory in cooled seawater and then frozen (–20 °C) to prevent degradation of metabolites. Further colonies/individuals of both animals were reared in aquarium (Fig. S1*A*) as described in *SI Materials and Methods*, to observe the spawned eggs (Fig. S2*A*), the fully developed swimming/crawling planulae (Fig. S2 *B* and *C*), and *T. striata* engulfing the planulae (Movie S5).

Chemical Study. Extraction, purification, and identification of compounds **1–3** from *M. edwardsi* were carried out as detailed in *SI Materials and Methods*. The acetone extract of the mucus secreted by *M. edwardsi*, collected with a cotton swab on the surface of a living colony in aquarium, was analyzed by AgNO$_3$-SiO$_2$ TLC, and 20 larvae (planulae) of *M. edwardsi* collected in the aquarium 7 d to 10 d after spawning were directly extracted with benzene-d_6 and analyzed by ^{1}H NMR.

Quantification of the Metabolites. Natural concentrations of metabolites **1–3** in *T. striata* and *M. edwardsi* were estimated by quantitative GC-MS and ^{1}H NMR analysis by using dimethylfumarate as an internal standard, as reported in *SI Materials and Methods*.

Feeding Deterrence Assay. The compounds were tested for their feeding deterrence activity against a shrimp, *P. elegans*, which is widely distributed in the Mediterranean Sea and along the European coast of the Atlantic (49). Artificial food was prepared as detailed in *SI Materials and Methods*. The shrimp were collected along the coast of Pozzuoli, Italy, and habituated to the control food in captivity for a week before experiments. After 2 d of fasting, 10 randomly picked shrimp were assayed as a series of individual replicates for each concentration and the control (*n* = 10 for each series). Shrimp were placed individually into 500-mL plastic beakers filled with 300 mL of seawater. A colored food strip was given to each shrimp, and shrimp were not reused. For each tested compound, control and treatments were carried out in parallel. The presence of a red spot, visible by transparency in the gastric mill and the stomach of the shrimp, was considered as acceptance of food, and the absence of the spot gave a rejection response. The regurgitation events that occurred within the observation time were considered as cases of food rejection. Statistical analysis between treatments and controls was performed using the two-tailed Fisher's exact test, with $\alpha = 0.05$.

Avoidance Learning Assays. Fifteen shrimp (*P. elegans*) habituated to the control food in captivity were fasted for 2 d. Subsequently, food pellets treated with compound **3** (4 mg/mL) were offered to the shrimp in a series of individual replicates. After 30 min, food residuals were removed from the bottom of the beakers, and food treated with compound **3** was given to all shrimp again. Finally, 30 min later, control food was given to all shrimp. Percentages of rejection, acceptance, and vomiting were recorded for each step, and differences between treatment and control were evaluated using the two-tailed Fisher's exact test, with $\alpha = 0.05$. Compound **3** was also tested for its palatability at a concentration of 4 mg/mL against the zebrafish (*D. rerio*), after 1 d of fasting. The experiments were conducted in the animal facility of the Department of Biology, Federico II University of Napoli (Italy), under animal handling and experimental procedures approved by the University ethics committee on animal experiments (ethical permit protocol 2013/0096061). The artificial food was prepared as detailed in *SI Materials and Methods*. Eight randomly picked zebrafish were assayed as a series of individual replicates. Fish were placed individually into 1,000-mL plastic containers filled with 600 mL of water. Treated food was offered to each fish in three subsequent administrations. Subsequently, after an interval of 5 h, during which the majority of individuals had expelled a fecal bolus, treated food was offered again to each individual. Finally, nontreated pellets and commercial granular food were given to the fish. The time-dependent response to nontreated control food was evaluated on a further series of eight fish.

Brine Shrimp Toxicity Assay. The toxicity of compounds **1–3**, dissolved in DMSO, was evaluated using the brine shrimp (*A. salina*) lethality test, by following the reported toxicity scale (50, 51). Given that deaths also occurred in the solvent/vehicle controls, the percentage of deaths for each treatment was corrected using Abbott's equation (52). For details, see *SI Materials and Methods*.

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