Original Article

MARINE BIOTECHNOLOGY

Induction of Attachment of the Mussel *Perna perna* by Natural Products from the Brown Seaweed *Stypopodium zonale*

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Abstract

Marine invertebrates settle, attach, and/or metamorphose in response to signals from several sources, including seaweeds. In response to the aquaculture challenge of producing constant numbers of juveniles from cultured species, natural inducers have been screened for their ability to improve those processes. However, few chemical inducers of attachment of invertebrates have been identified, and even less of these were secondary metabolites. The goal of this work was to isolate the natural products responsible for induction activity using bioassay-guided fractionation of the organic extract of the brown seaweed *Stypopodium zonale* and the attachment of juveniles of the common brown mussel, *Perna perna*, as a model. The meroditerpene epitaondiol, identified by comparison of spectral data with the literature, promoted as much as 4.7 times more mussel attachment compared to controls at the natural concentration found in this alga (0.041% of the crude extract or 0.012% of algal dry weight). This is the first report showing that a seaweed produces terpenoid compounds as cues for invertebrate attachment, and future studies evaluating this action on settlement of mussels in the field are expected to improve aquaculture technology by increasing mussel spat production.

Keywords: attachment — brown mussels — chemical signals — induction — *Perna perna* — *Stypopodium zonale*

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Introduction

A number of chemical inducers of settlement have been postulated from observations of specific settlement on distinct natural substrata, but only a small number of natural inducers have been isolated and chemically characterized (Steinberg et al. 2001; Paul et al. 2006). These inducers originate from or are associated with various sources and have great ecological importance, belonging to three main types: (1) conspecific individuals, e.g., the adult habitat such as the gregarious settlement in some barnacles (Wright and Boxshall 1999), polychaetes (Pawlik and Faulkner 1986; Okamoto et al. 1998) and molluscs (Slattery 1992); (2) bacterial or diatom microbial films (Wieczorek and Todd, 1997; Lau and Qian, 2001; Gallardo and Buen, 2003; Zhao et al. 2003; Dobretsov and Qian 2004); and (3) prey species (Morse 1990; Hadfield and Paul 2001). Although there are several evidences of the existence of specific chemical inducers, some species may respond to a wide range of compounds and physical cues (Hardege et al. 1998).

Along the Brazilian littoral, natural banks of the brown mussel *Perna perna* have been exploited for food for decades. Since ca. 15 years ago, mussel farming in Brazil has increased significantly, reaching 10 metric tons a year only in the state of Santa Catarina (southern Brazil) in 2000, and an increase rate of 20% per year is expected (Roczanski et al. 2000; da Silva et al. 2002). Undoubtedly, the remarkable success in mussel cultivation has evidently resulted in major social and economic benefits, but these are menaced by the lack of a steady production of mussel spat. In this way, juvenile-adult retention may be an important aspect to issue in Brazil's aquaculture. The brown mussel *Perna perna* forms dense natural banks at the

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inter- and subtidal zones on rocky shores of southeastern Brazil. On the other hand, this species has great economic importance and represents a protein source for significantly disadvantaged coastal populations (Abessa et al. 2005; Marenzi and Branco 2005). Moreover, mussel farms are quickly spreading along the Brazilian coast, constituting an additional economic activity for many coastal cities (Abessa et al. 2005). In fact, mussel cultivation also possesses a worldwide economic importance (Smaal 2002).

Several mussel species are known to settle on seaweed surfaces (Petersen 1984; Eyster and Pechenik 1988; Davis and Moreno 1995; Lasiak and Barnard 1995; Alfaro et al. 2004), but only one work investigated the role of algal chemical cues in this context (Davis and Moreno 1995). These authors, however, found no evidence that chemical signaling played an important role in attracting the mussel *Choromytilus chorus*. The brown seaweed *Stypopodium zonale* (Lamouroux) Papenfuss is abundant along the Brazilian coast and produces mixed biosynthesis compounds known as meroditerpenes, several of which display diverse and useful ecological (e.g., Pereira et al. 2004) and pharmacological properties (e.g., Wessels et al. 1999). *Stypopodium zonale* is usually found partially covered by fouling-algae and invertebrates, including juveniles of the brown mussel, *P. perna* (da Gama, *pers. observation*), and previous investigations suggested that chemical cues from *S. zonale* induce the attachment of fouling organisms in field experiments (da Gama et al. 2002) and in laboratory assays (da Gama et al. 2003). In general, mussel culture requires knowledge of the factors that regulate natural recruitment, and of coastal zone regulation and management. Here, we present further evidence supporting these findings and go one step further. Our objectives were to determine: (1) Is the crude extract of the *S. zonale* really able of promoting the attachment of juveniles of the mussel *P. perna*? (2) What compound is responsible for the induction of mussel attachment?

Materials and Methods

Seaweed Collection. Specimens of the brown seaweed *S. zonale* were collected off the Brazilian coast- Forno inlet, Búzios, Rio de Janeiro State (22°45′S, 41°52′W), at depths ranging from 5 to 10 m in February, 2001. The seaweed specimens were transported to laboratory in ice cases, and then gently washed in seawater to eliminate macroscopic epibiota associated, as well as microorganisms loosely attached on thalli.

Collection of Experimental Animals. Specimens of the mussel *Perna perna* were collected during low tides from the rocky coastal area of Itaipu (Niterói city, Rio de Janeiro, Brazil) and kept in a 230 l recirculating laboratory aquarium (equipped with biological filtering, protein skimming and activated carbon) at constant temperature (20°C), salinity (ca. 35 psu), and aeration for a 12-h period. Juvenile individuals were then disaggregated by carefully cutting byssus threads and maintained in a plastic tray with seawater until the moment of use.

Attachment of* P. perna *Laboratory Assays. The method for quantification of juvenile mussel attachment was fully described by da Gama et al. (2003). *Perna perna* attachment bioassays were performed as follows: in the bottom of sterile polystyrene Petri dishes were placed entire water-resistant filter papers cut into 9-cm diameter circles (soaked in dichloromethane [DCM]-control filter, internal or within-treatment control) and over this, another 9-cm diameter set of filter paper (treatment filter) cut in a chessboard pattern (1-cm squares) and soaked in the natural concentration of extract, of the fractions (determined according to the yield of each step of fractionation) or of the pure metabolite, diluted in DCM. For each set of experiments, a set of Petri dishes in which the superior filter paper was treated with solvent only served as an external control. Each dish was filled with 80 ml of seawater and three juvenile mussel specimens (2.0 to 3.0 cm length) were added. A total of 10 to 12 replicates of each treatment were used. Experiments were allowed to run for 12 h.

Juvenile mussel activity was recorded immediately after the beginning of the experiment, after 2 h (data not shown), and then after 12 h. The activities recorded were substrate exploring behavior, and number of byssal threads attached to each substrate (control or treated filter paper, data shown; shell of another mussel or border of Petri dish, data not shown). After the 12-h period, all records of attachment were checked, mussels were placed in plastic mesh bags tagged according to treatment, and suspended into a sea aquarium for 24 h to check for possible mortality due to exposure to test substances. Experiments with the purified compound were performed twice to further confirm results.

All the experiments were realized using the natural concentration of the crude extract, of each fraction obtained in the bioassay-guided purification of the crude extract or of the pure compound, according to the yield calculated at each step. After the trials, treated filter papers (containing *S. zonale* extract, fractions or the pure compound) were taken from dishes and allowed to air dry. Filter papers were then reextracted, the solvents evaporated, and

the remaining applied on a thin-layer chromatography (TLC) plate for comparison with the original crude extract, the fractions, or the active pure compound.

Extraction and Separation. Air-dried specimens of *S. zonale* (69.00 g, dry weight [DW]) were successively extracted with DCM, at room temperature (±25°C), in an ultrasound apparatus (3×2l for 20 min) for 3 weeks. The solvent was evaporated under reduced pressure yielding a dark green residue (10.15 g).

Bioassay-guided fractionation and purification were performed as follows: The crude extract (10.15 g) was initially fractionated on a silica gel (Merck, Kiesselgel 60, 70–230 mesh) vacuum liquid chromatography (Coll and Bowden 1986) and eluted with pure *n*-hexane (150 ml), *n*-hexane-CH_2Cl_2 (1:1, 300 ml; 4:6, 300 ml), pure CH_2Cl_2 (600 ml), CH_2Cl_2-EtOAc (9:1, 300 ml; 8:2, 300 ml), pure EtOAc (200 ml), EtOAc-MeOH (8:2, 200 ml), and pure MeOH (150 ml) to give eight fractions (Fr. A to H). Among these fractions, Fr. A, Fr. C, and Fr. E displayed significant differences in their effect on the attachment of mussels, relative to other fractions.

Fraction A (*n*-hexane-CH_2Cl_2, 1:1) was subjected to silica gel column chromatography (φ 22 mm×200 mm) and eluted with CH_2Cl_2-EtOAc mixtures of increasing polarity (going from 100% CH_2Cl_2 to 100% EtOAc, in steps of 10%, 50 ml) to give seven fractions, Fr.A1 to Fr.A7. Byssal attachment in response to Fr. A1, Fr. A4, and Fr. A7 was significantly higher than in other fractions. Therefore, Fr. A1 was fractionated by silica gel column chromatography (22.0 mm×150.0 mm) and eluted with a stepwise gradient of pure *n*-hexane (100 ml), *n*-hexane-CH_2Cl_2 (7:3, 150 ml; 1:1, 150 ml), pure DCM (200 ml), CH_2Cl_2-EtOAc (9:1, 200 ml; 8:2, 150 ml), and pure EtOAc (100 ml) to yield eight fractions, Fr. A1a to Fr. A1h. Further purification of Fr. A4 was not possible due to the small amount that remained after the bioassays. Fraction Fr. A7 showed a single spot on TLC (eluted with chloroform-ethyl acetate 7:3) under UV (254 nm), which was visualized as a pink-colored spot by spraying a solution 2% of $Ce(SO_4)_2$ in H_2SO_4 followed by heating at 80°C.

The fraction Fr. C (pure CH_2Cl_2) was fractionated by preparative thin-layer chromatography (PTLC), which was conducted on silica-gel pre-coated plates (Merck Kiesegel 60 F-254) with DCM:ethyl acetate (8:2) to yield five fractions (Fr.C1 to Fr.C5). Among these fractions, only the fraction Fr. C2 promoted the attachment of *P. perna*. This fraction was purified by recrystallization under low temperature with DCM to yield the pure compound.

The fraction Fr. E (CH_2Cl_2-EtOAc 8:2) was also fractionated by PTLC with DCM: ethyl acetate (8:2) mixture to yield seven fractions (Fr. E1 to Fr. E7).

The crude extract and all the fractions obtained were analyzed by ^{1}H-nuclear magnetic resonance (1H-NMR) spectroscopy ($CDCl_3$, 300 MHz) and analytical TLC.

All the processes of fractionation of the crude extract and purification of active compound were performed at room temperature (±25°C) and protected from light and heat by using dark glass vials covered with aluminum foil. These cares were taken to warrant that artifacts were not generated through thermal or photodegradation of natural products.

Characterization of the Inducer. The chemical inductor of attachment of the mussel *P. perna* was analyzed and identified by infrared (IR), ultraviolet (UV), ^{1}H-NMR ($CDCl_3$, 300 MHz), ^{13}C-NMR ($CDCl_3$, 75 MHz), COSY, HMQC, HMBC, and mass spectroscopy.

Statistical Analysis. The data from the bioassays were analyzed as number of attached byssal threads using the Wilcoxon paired-sample test, a nonparametric alternative to the *t*-test for dependent samples (attachment of mussels to treated substrata is assumed to be dependent on attachment to control substrata within each experimental unit) because rarely variables were normally distributed. Differences were considered significant whenever $P<0.05$.

Results

***Attachment of* P. perna.** Mussel attachment was variable among sets of experiments. As shown in Figure 1A, the natural concentration of the *S. zonale* extract (17% DW) significantly promoted the attachment of the mussel *P. perna* (6.5 times more attachment than in controls, $n=10$, $P< 0.02$). From the first fractionation of the extract from *S. zonale* that yielded eight fractions, fractions Fr. A and Fr. C exhibited the highest induction activity to the attachment of *P. perna* (26 and 30 times more attachment than in controls, respectively; $n=10$, $P<0.01$; Figure 1B). Fraction Fr. E also induced mussel attachment, but it was significantly lower (8-fold more attachment than in controls; $n=10$, $P<0.04$; Figure 1B). Three active fractions were obtained from the fractionation of the active fraction Fr. A, and all fractions obtained significantly induced mussel attachment (Fr. A1, 5.3 times and Fr. A4, 19 times, with $n=10$, $P<0.05$ and Fr. A7, 12.5 times more

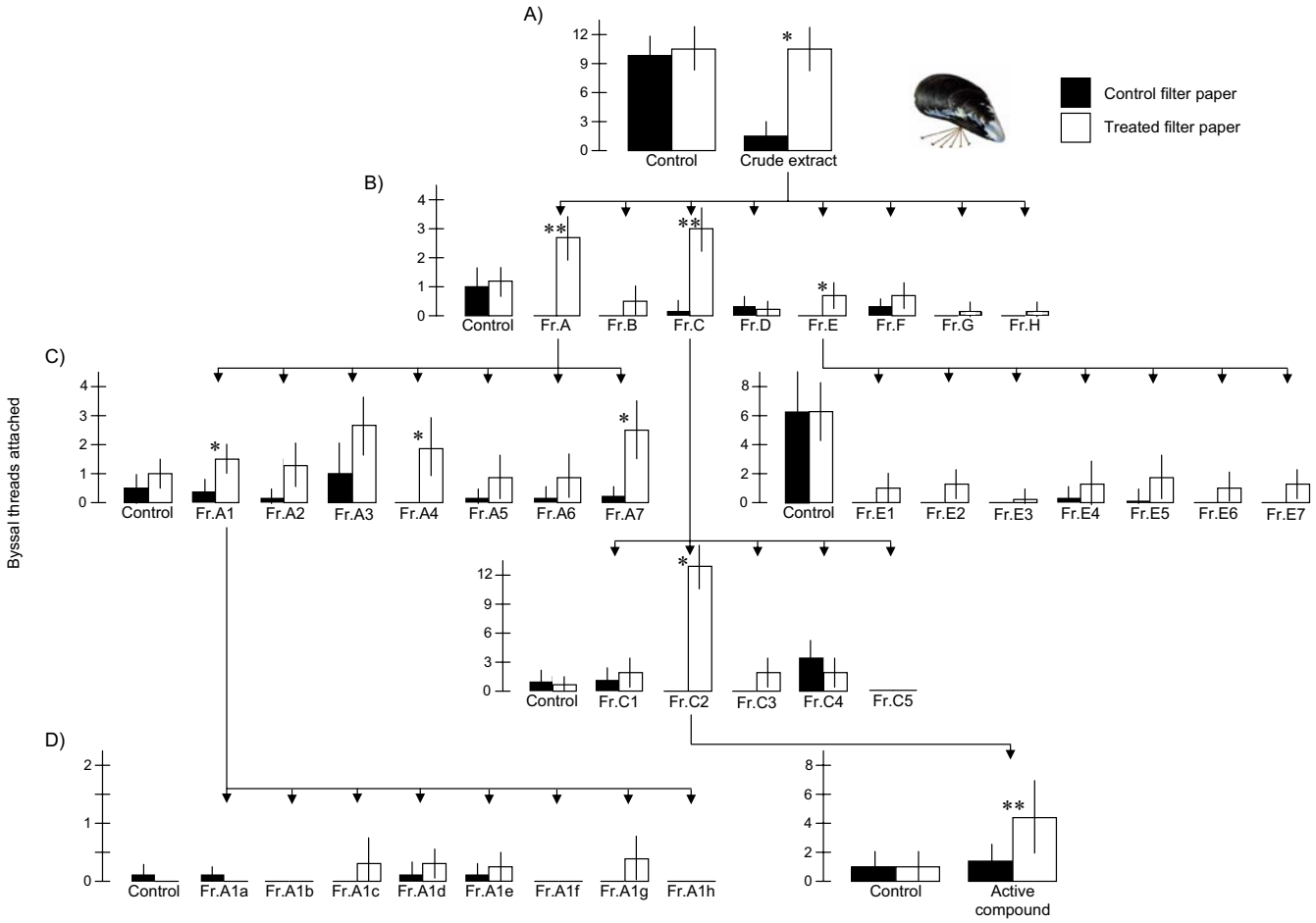

Figure 1. Bioassay-guided fractionation of *Stypopodium zonale* extract exposed to mussel attachment. Data are presented as mean number (± standard deviation) of byssal threads of *Perna perna* attached to the indicated substrata (control or treatment filter paper) after 12 h to each set of bioassays. Assays were run at natural concentrations of the (**A**) crude extract, (**B**) fractions Fr. A to Fr. H, (**C**) fractions derived from the active fractions of (**B**), and (**D**) fractions derived from active fraction Fr. A1 and assay with pure compound originated from Fr. C2. Each bioassay set had its own control experiment in which the treated filter paper was soaked in solvent only. N=10 replicates per treatment except for Fr. C1 to C5, which had 12 replicates. Significant differences at * P<0.05 and ** P<0.01. Note that each set of experiments has an external control, as well as control filter paper within each treatment.

attachment than controls; n=10, P<0.03; Figure 1C). The refractionation of the active fraction Fr. A1, an attempt to isolate and identify the active compounds, did not yield any active fraction (Figure 1D), probably due to the loss of mass or degradation of compounds throughout the fractionation steps. Fraction A4 was not further purified owing to its extremely reduced mass. Fraction Fr. A7 was obtained in small amounts and thus it was impossible to identify the active compound by spectroscopic analyses. The active Fr. C was fractionated again and yielded one active fraction that induced 13-fold more byssal attachment than controls (n=12, P<0.01; Figure 1D), Fr. C2, which was purified again to yield one pure compound. The natural concentration (0.041% of the crude extract and 0.012% DW) of this pure compound significantly induced the attachment of *P. perna* in two new bioassays (3.8 to 4.7 times more attachment, n =10, P<0.01; Figure 1D). Finally, Fr. E was fractionated and did not yield any active fraction (Figure 1). At 24 h after the end of experiments, there was not a single mortality due to exposure to test compounds.

TLC analysis of the treatment filter papers extracted after the end of the experiments revealed that many spots containing polar substances were solubilized in seawater, although the substances present in the active fractions and the identified active compound were still present.

Separation and Characterization of the Inducer. Elution in dichloromethane (100%) yielded epitaondiol (Figure 2) as a semipure solid that on repeated CC

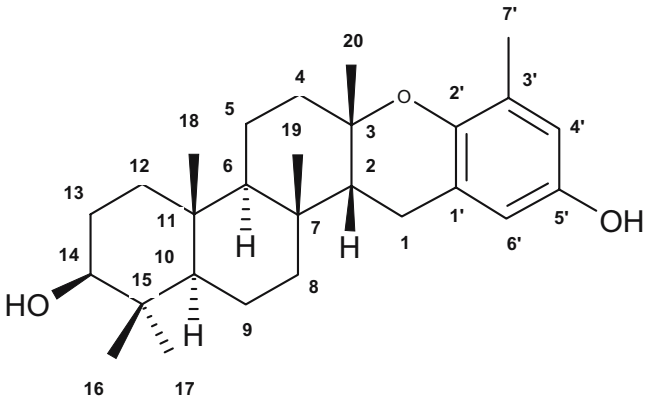

Figure 2. Molecular structure of the inducer of mussel attachment isolated from the brown seaweed *Stypopodium zonale*.

appeared as a pure white solid (71 mg). The spectroscopic data obtained are in conformity with data from previously isolated epitaondiol from *Stypopodium zonale* (Gerwick and Fenical 1981) and *S. flabelliforme* (Sánchez-Ferrando and San-Martín 1995), and are reported here: **UV** λ_{max} **($CHCl_3$)** 243.0, 297.5, 350.5 nm**; IR ($CHCl_3$)** υ_{max} 3367, 2933, 1608, 1470, 1379, 1225, 1131, 1021, 665 cm^{-1}; **^{1}H-NMR ($CDCl_3$, 300** MHz): δ 0.80 (3H, s, H-19),0.93 (3H, s, H-18), 1,00 (3H, s, H-20), 1.19 (3H, s, H-16), 1.23 (3H, s, H-17), 1.28 (1H, m, H-6), 1.34 (1H, dd, J=5.4, 7.5 Hz, H-10), 1.40 (2H, m, H-8), 1.45 (1H, dd, J=3.0, 9.3, H-12), 1.48 (2H, m, H-5), 1.54 (1H, dd, J=3.0, 9.3 Hz, H-12), 1.68 (1H, dd, J=4.8, 13.8 Hz, H-3), 1.72 (1H, m, H-9), 1.75 (1H, d, 12.9 Hz, H-10), 1.75 (2H, m, H-13), 1.88 1H, m, H-4), 2.08 (3H, s, 7′), 2.14 (1H, m, H-4), 2.49 (1H, dd, J=4.8, 16.8 Hz, H-2), 2.69 (1H, dd, J=13.8, 16.8 Hz, H-1), 3.27 (1H, dd, J=5.1, 11.4 Hz, H-14), 4. 36 (1H, br s, OH), 6.45 (1H, d, J=2.7 Hz, H-4′), 6.37 (1H, d, J=2.7, H-6′); **^{13}C-NMR ($CDCl_3$, 75 ;MHz)**: 23.1 (C-1), 48.3 (C-2), 76.5 (C-3), 40.1 (C-4), 16.9 (C-5), 47.9 (C-6), 34.8 (C-7), 31.4 (C-8), 17.0 (C-9), 45.1 (C-10), 36.9 (C-11), 31.7 (C-12), 29.0 (C-13), 79.4 (C-14), 39.0 (C-15), 24.8 (C-16), 28.0 (C-17), 21.6 (C-18), 15.9 (C-19), 29.1 (C-20), 122.3 (C-1′), 145.1 (C-2′), 127.0 (C-3′), 115.3 (C-4′), 147.7 (C-5), 112.5 (C-6′), 16.0 (C-7′). The NMR data are based on standard 1D and 2D techniques (COSY, HMBC, and HSQC).

Discussion

In the present work, it was verified that the lipophilic extract of the brown seaweed *Stypopodium zonale* promoted the attachment of the brown mussel *Perna perna.* In addition, the bioassay-guided fractionation of this active extract demonstrated that one chemical cue responsible for this activity is a lipophilic secondary metabolite identified as epitaondiol, a known compound previously isolated from different populations of *S. zonale* (Gerwick and Fenical 1981; Gerwick et al. 1985) as well as from other species of this genus, such as *S. flabelliforme* (e.g., Rovirosa et al. 1992). This meroditerpene is known to exhibit biological activity as an antiinflamatory (Gil et al. 1995), antibiotic (Rovirosa and San-Martín 1997), and insecticide agent (Rovirosa et al. 1992).

In general, chemical cues are often surface-associated molecules and generally water-soluble (polar) primary metabolites such as carbohydrates or peptides (e.g., Decho et al. 1998; Fleck and Fitt 1999; Hadfield and Paul 2001; Steinberg et al. 2001; Fusetani 2004). However, chemical induction of invertebrate settlement does not appear to be limited only to the action of hydrophilic molecules. For example, Daume et al. (1999) encountered evidence that the settlement inducers of abalone larvae (*Haliotis laevigata*) were insoluble in water (nonpolar). In cnidarians, Leitz and Wagner (1993) isolated a lipophilic compound originating from the marine bacterium *Alteromonas espejiana* growing on gastropod shells occupied by hermit crabs that induce larvae of the hydrozoan *Hydractinia echinata* to settle. Our results thus seem to broaden the effects of lipophilic compounds as substratum cues in the marine environment.

Interestingly enough, epitaondiol is a compound structurally related to the epoxid of δ-tocotrienol, isolated from the brown alga *Sargassum tortile*, which induced larval settlement of the epibiotic hydroid *Coryne uchidai* (Kato et al. 1975). Besides promoting *P. perna* attachment, previous field investigations demonstrated that chemical cues to fouling settlement are also produced by *S. zonale* (da Gama et al. 2002, 2003). In fact, *S. zonale* in nature is frequently overgrown by fouling organisms, such as crustose and articulated coralline algae, polychaetes, gastropod egg cases, or juvenile mussels, suggesting that chemical cues from *S. zonale* could induce settlement of a broad number of organisms. Epitaondiol acts as an attachment-promoter for the mussel *P. perna* at the natural concentration, but the environmental conditions under which this substance would be secreted or released by the alga in seawater are currently unknown, and therefore it is uncertain whether the mussel would respond to the metabolite in the field. Further work is needed to determine the concentration of this metabolite at the algal surface, or even if it is present there. However, it is noteworthy that juvenile mussels have already been found settled on *S. zonale* (da Gama, *pers. obs.*).

During mussel aquaculture, a reliable source of mussel spat is crucial to a sustained grow out operation (Hoagland et al. 2003), as the availability

of mussel spat can severely limit aquaculture productivity, and mussels can detach from collectors after initial settlement, in a process known as secondary settlement (Buchanan and Babcock 1997; Alfaro and Jeffs 2003). Moreover, mussels can move from the substrate of settlement and metamorphosis through sequential production and release of byssal threads. A secondary dispersal mechanism through byssus drifting has also been observed in mussels (Lane et al. 1985) as well as in other bivalves such as cockles, clams, tellinids, and scallops (e.g., Baker and Mann 1997). Thus it is important to ensure that the surface of spat collectors is attractive for mussel attachment, so that mussels stay at the substrate of initial settlement.

In this work, we used juveniles of the mussel *Perna perna* for the discovery of attachment-promoting natural substances. Although the extrapolation of these data to larval settlement in the field may seem unrealistic, other studies have shown that laboratory-reared mussel larvae seldom settle and metamorphose even in the presence of the preferred field substrate (S. Dobretsov, *pers. obs*., quoted in Dobretsov and Qian 2003). In addition, da Gama et al. (2003) showed a good agreement between laboratory assays using young *Perna perna* and field antifouling assays, suggesting that the laboratory results may be reproduced in field assays. However, the activity of epitaondiol as a settlement inducer of mussel larvae still needs further confirmation through field experiments.

For most marine invertebrates such as echinoids (Pearce and Scheibling 1990), ascidians (Young and Braithwaite 1980), and prosobranch molluscs (McGee and Targett 1989), a single cue is known, while for others, separate cues are indicated for settlement and metamorphosis. In response to the aquaculture challenge of producing constant numbers of juveniles of cultured species, natural chemical inducers have been screened in the laboratory for their ability to induce larval settlement and metamorphosis (Doroudi and Southgate 2002; Gallardo and Buen 2003; Zhao et al. 2003). Most of these investigations explored the effects of some compounds to promote larval settlement and/or metamorphosis. For example, the secondary metabolite jacaranone isolated from the ethanolic extracts of the red seaweed *Delesseria sanguinea* stimulated settlement of the scallop *Pecten maximus* (Yvin et al. 1985). The mollusc *Alderia modesta* is induced to settle and metamorphose by carbohydrates found in the alga *Vaucheria longiccaulis* (Krug and Manzi 1999). Chemical cues from some red and green seaweeds accelerated metamorphosis of larvae of the blue crab *Callinectes sapidus* (Brumbaugh and McConaugha 1995; Forward et al. 1996). Low molecular weight compounds (δ-tocotrienol and epoxi-δ-tocotrienol) found in *Sargassum tortile* effectively induced not only settlement, but also attachment and metamorphosis of larvae of the hydroid *Coryne uchidai* (Kato et al. 1975). New promoters may be very useful to aquaculture technology, increasing settling rates of cultured organisms on desired surfaces. Future studies can further evaluate the potential of epitaondiol from *S. zonale* for use in aquaculture technology through more ecologically realistic assays in the field in order to improve mussel spat collection.

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