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Nicole Lopanik · Niels Lindquist · Nancy Targett

Potent cytotoxins produced by a microbial symbiont protect host larvae from predation

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Abstract Larvae of the sessile marine invertebrate *Bugula neritina* (Bryozoa) are protected by an effective chemical defense. From the larvae, we isolated three bryostatin-class macrocyclic polyketides, including the novel bryostatin 20, that deterred feeding by a common planktivorous fish that co-occurs with *B. neritina*. A unique bacterial symbiont of *B. neritina*, *Endobugula sertula*, was hypothesized as the putative source of the bryostatins. We show that: (1) bryostatins are concentrated in *B. neritina* larvae and protect them against predation by fish; (2) the adults are not defended by bryostatins; and (3) *E. sertula* produces bryostatins. This study represents the first example from the marine environment of a microbial symbiont producing an anti-predator defense for its host and, in this case, specifically for the host's larval stage, which is exceptionally vulnerable to predators.

Keywords Bryostatins · *Bugula neritina* · Chemical defense · *Endobugula sertula* · Symbiosis

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N. Lopanik (✉) · N. Targett
Graduate College of Marine Studies, University of Delaware,
Lewes, DE 19958, USA
e-mail: nlopanik@umich.edu
Tel.: +1-734-6478969
Fax: +1-734-6478430

N. Lindquist
Institute of Marine Sciences, University of North Carolina at Chapel Hill,
Morehead City, NC 28557, USA

Present address:
N. Lopanik
Department of Medicinal Chemistry, University of Michigan,
Ann Arbor, MI 48109, USA

Introduction

Chemical defenses of plants and animals significantly influence the outcomes of diverse inter- and intra-specific interactions in both terrestrial and marine environments (Feeny 1991; Pawlik 1993; Hay 1996; McClintock and Baker 2001). Secondary metabolites can deter consumers and competitors, as well as inhibit the growth of pathogens, and thus affect community structure and function (Hay 1996; McClintock and Baker 2001). Some chemically mediated interactions are intricate, multi-species relationships (e.g., tritrophic interactions) that reflect a rich and complex evolutionary history (Sipura 1999; Kessler and Baldwin 2001; Shurin et al. 2002; van Poecke and Dicke 2002). Recent research has begun to highlight another type of "complex" system potentially of great importance to the field of chemical ecology: microbial symbiont production of bioactive secondary metabolites that contribute significantly to host survival. These systems have evolved, in part, through various levels of symbiont-host interactions coupled to the fitness-related costs of host exposure to symbiont chemistry and to the impact of symbiont chemistry on, for example, potential consumers of the host. The grass-fungal endophyte-herbivore systems (Cheplick and Clay 1988; Clay 1988, 2001) provide an outstanding example of symbiont chemistry contributing to the defense of its host.

Over the past decade, there has been a growing awareness that secondary metabolites isolated from some marine invertebrates, including sponges, bryozoans, and tunicates, many of which host unique communities of microbes (Vacelet and Donadey 1977; Wilkinson et al. 1981; Woollacott 1981; Kott et al. 1984), may actually be produced by a symbiont (Anthoni et al. 1990; Kobayashi and Ishibashi 1993). This idea was initially proposed based on the isolation from marine invertebrates of compounds structurally similar or identical to compounds previously reported from microorganisms (Anthoni et al. 1990; Kobayashi and Ishibashi 1993). The physical separation of symbiont cells from host tissues has provided more direct evidence for symbiont production

of some metabolites (e.g., Faulkner et al. 1999) and, in a few instances, microorganisms cultured from host material have produced compounds identical to those isolated from the host (Stierle et al. 1988; Elyakov et al. 1991).

Despite the advances in demonstrating a microbial origin for a growing number of secondary metabolites isolated from diverse marine invertebrates, we know of only two published examples for which an ecological role has been identified for a symbiont-produced marine secondary metabolite. In both cases, a symbiotic bacterium on the embryos of the shrimp *Palaemon macrodactylus* and the lobster *Homarus americanus* produced compounds that prevent infection by the pathogenic marine fungus *Lagenidium callinectes* (Gil-Turnes et al. 1989; Gil-Turnes and Fenical 1992). There are no marine examples of a symbiont producing an anti-predator chemical defense for its host. Surprisingly, few examples of this type of defensive symbiosis are known among terrestrial invertebrates. One example is pederin, a complex polyketide isolated from rove beetles (*Paederus* spp.), which deters predation on *Paederus* eggs and larvae and is produced by a symbiotic bacterium (Kellner and Dettner 1996; Kellner 2001, 2002).

The marine bryozoan *Bugula neritina* may offer the first example of an invertebrate-microbe association in the marine environment for which the symbiont chemically defends its host from potential predators. Over 20 years ago, *B. neritina* larvae were reported to host rod-shaped bacteria [later named *Endobugula sertula* (Haygood and Davidson 1997)] within a surface invagination called the pallial sinus (Woollacott 1981). *B. neritina* also possesses a unique class of macrocyclic polyketide metabolites called bryostatins. Tremendous interest developed in the bryostatins after bryostatin 1 (Fig. 1A) was reported to significantly reduce the proliferation of cancer cells (Pettit et al. 1970). To date, 19 related compounds have been found in different populations of *B. neritina* worldwide (Pettit 1996). Because the bryostatins are complex polyketides, a class of secondary metabolites found extensively in prokaryotes, several investigators have hypothesized that *E. sertula* is the true source of the bryostatin-class polyketides isolated from *B. neritina* (Haygood and Davidson 1998; Haygood et al. 1999; Davidson et al. 2001). This hypothesis, however, has not been fully tested. The presence of an active polyketide synthase gene cluster in *E. sertula* (Davidson et al. 2001) gives the symbiont the proper biosynthetic machinery to produce bryostatins, but the products of this gene complex have not been firmly established.

Although many natural marine products have been shown to act as chemical deterrents to predators (Pawlik 1993; Hay 1996; McClintock and Baker 2001), no data have been reported regarding a defensive role for the bryostatins. However, *B. neritina* larvae, as well as their extract, were shown to be unpalatable to co-occurring particle-feeding invertebrates and fishes (Lindquist 1996; Lindquist and Hay 1996). In contrast, extracts from adult *B. neritina* had no effect on fish feeding behavior (Lindquist and Hay 1996). The concentrations of bryostatins in adult colonies of *B. neritina* are typically reported to be very low (10^{-5} to 10^{-8}% wet mass; Pettit 1996), but nothing has been published about the presence of bryostatins in their larvae.

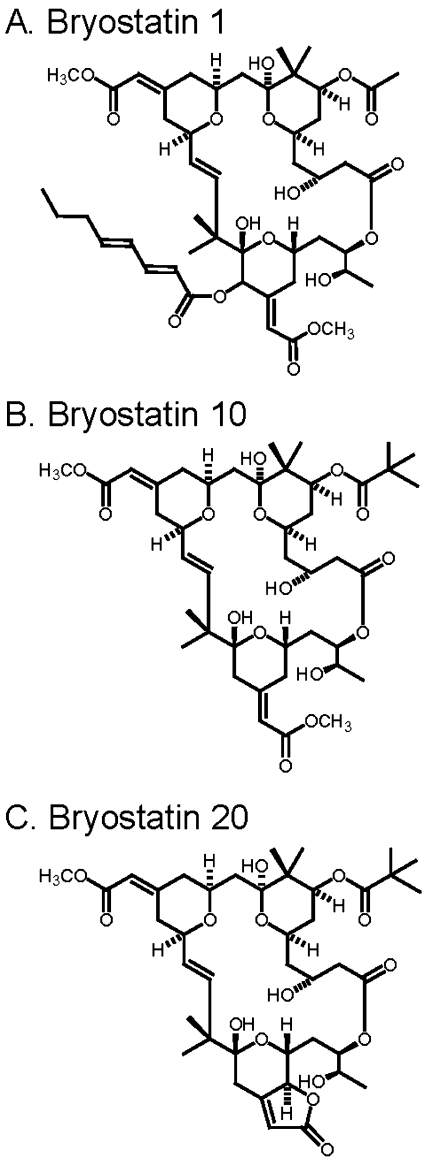

Fig. 1 Structure of **A** bryostatin 1, **B** bryostatin 10, and **C** bryostatin 20

In this paper, we report the results of our study testing the hypothesis that the bryostatins act as a chemical defense that protects the larvae of *B. neritina*, but not the adults, from potential predators. Additionally, we rigorously tested the hypothesis that the bryostatins are produced by *E. sertula*. Confirmation of this hypothesis would represent the first example from the marine environment of a symbiont producing an anti-predator defense for its host and, in this case, specifically for the host's larval stage, which is exceptionally vulnerable to predators.

Materials and methods

Study organisms

Bugula neritina (L.) is a planktivorous sessile invertebrate, which is abundant in many temperate fouling communities worldwide. Reproduction in *B. neritina* involves internal fertilization and the brooding of relatively large, non-feeding larvae that settle typically within minutes of being spawned. Despite having no morphological defenses against potential predators, the larvae are released several hours after sunrise when they are highly apparent to planktivorous fishes (Lindquist and Hay 1996). The pinfish, *Lagodon rhomboides*, is abundant in North Carolina coastal waters (Adams 1976) and has been used extensively in ecological investigations focused on how fish feeding structures benthic communities (Nelson 1979; Heck et al. 2000). Further, *B. neritina* larvae are reported to be unpalatable to pinfish, which are also deterred by the crude extract of the larvae (Lindquist and Hay 1996). Therefore, we used pinfish as the assay organism to track deterrent metabolites during the fractionation of the *B. neritina* larval extract.

Collection of *B. neritina*

B. neritina colonies were collected by SCUBA at Radio Island Jetty, Morehead City, N.C., in the spring and fall (the seasons when it is reproductive in North Carolina) of 1998–2002. Colonies were maintained in the dark in flow-through water tables at the University of North Carolina at Chapel Hill's Institute of Marine Sciences. *B. neritina* colonies from Delaware were collected by hand from floating docks near Bethany Beach, Del., in August through October 2001 and 2002, and maintained in flow-through seawater tanks at the University of Delaware's College of Marine Studies. Colonies (~1 l) placed in 4-l glass jars were stimulated to release larvae by exposing them to bright light. Larvae swam to the surface and aggregated at the rim of the jar, where they were collected with a wide-tip glass pipette and placed in ice-cold seawater. The cold seawater caused the larvae to stop swimming and sink to the bottom of the vial, after which the seawater was removed. From each jar, ~30–60 μl of larvae could be harvested each day for 5–6 days before fresh *B. neritina* needed to be collected. Larvae and adults were collected for chemical and DNA extraction.

Sample extraction and feeding assays

Larvae, as well as a collection of adults lacking larval brood chambers called ovicells, were each extracted with methanol and a 1:1 mixture of methanol and dichloromethane (DCM). The methanol and methanol/DCM extracts were combined and evaporated to dryness using a rotary evaporator to give a lipophilic extract. The larvae and adults were further extracted with distilled water, which was dried by lyophilization to give a water-soluble extract. The lipophilic and water-soluble extracts were used in feeding assays with pinfish. Fish were held individually in 1-l, perforated plastic containers placed in a flow-through water table. For each assay, the extract or fraction was dispersed in the assay food, which was prepared by pureeing together squid mantle flesh, an equal mass of distilled water, and sodium alginate at 2% of the total wet mass (=squid paste). Squid paste was used as the assay food because it has a high protein content (~35 mg/ml; Bullard and Hay 2002), like *B. neritina* larvae (~26 mg/ml; Lopanik, unpublished data). An amount of extract from a given volume of larvae or adults was stirred into that same volume of squid paste so that the assay food contained an ecologically realistic concentration of the test material. Five-microliter aliquots of the squid paste were solidified to a consistency of cooked pasta by soaking them in 0.25 M calcium chloride for 30 s. Control pellets contained an appropriate amount of carrier solvent alone. To begin an assay, a control pellet was offered to a fish. If eaten, a treatment pellet was then offered and its acceptance or rejection was recorded. Pellets that were rejected were sometimes tasted again, but they were always subsequently rejected. A second control pellet was offered to ensure that the fish remained willing to feed. If the second control pellet was not eaten, the feeding data for that replicate were discarded. Ten to 15 fish were used for each assay and feeding data were analyzed using McNemar's test for significance of changes (Sokal and Rohlf 1995). In this test, the percentage of fish that consumed the control pellets (always 100%) is compared to the percentage that subsequently consumed the extract pellets. *P* values represent the probability that the difference in the proportion of positive responses to the control food and to the extract food is significant.

Compound isolations and quantification by HPLC

To isolate deterrent compounds, the total crude larval extract was first partitioned between DCM and water, with the water further extracted with n-butanol. Deterrent compounds in the combined DCM- and butanol-soluble fractions were purified by high performance liquid chromatography (HPLC). Extracts were eluted on an analytical C18 reversed phase column (250×4.6 mm Microsorb 5 μm column, Varian, Walnut Creek, Calif.), with a gradient of water and methanol starting at 82% methanol at 0.6 ml/min and increasing to 100% at 0.75 ml/min over 27 min. The HPLC system (Waters Corp., Milford, Mass.) consisted of two pumps (model 510), an autosampler (717 Plus), a photodiode array detector (996), and a fraction collector (LC-200, Buchler). Data were acquired from 200–300 nm. All peak integrations were calculated at 229 nm, the peak of the longest UV absorption for these bryostatins. The masses of the isolated peaks were measured and used as standards for quantification of bryostatin concentrations. The structures of the isolated deterrent and non-deterrent compounds were determined by 1D and 2D ^{1}H and ^{13}C NMR, UV, and IR spectroscopy, and mass spectrometry (Lopanik 2003). To compare bryostatin and symbiont masses per larva, the symbiont dry mass was estimated by multiplying the reported number of *E. sertula* cells per larva (~2500; Haygood et al. 1999) by the dry mass of an *E. sertula* cell. The dry mass of an *E. sertula* cell was estimated by calculating its volume from its dimensions (Haygood and Davidson 1997) and assuming the bacterium has a specific density of ca. 1.0 and is 80% water by weight, as has been reported for other gram-negative bacteria (Watson 1976; Madigan et al. 1997). Bryostatin mass per larva was determined by dividing the concentration of all bryostatins contained in 1 ml larvae by the number of larvae per ml, which was calculated from the larval volume assuming a spherical larva with a diameter of 350 μm (Lindquist and Hay 1996).

Impact of larval collection method on larval chemical content

A California company, CalBioMarine Technologies, which pioneered in situ aquaculture of *B. neritina* for the production of bryostatins, collects large numbers of *B. neritina* larvae by concentrating them on sieves as they are carried on the outflow of flow-through tanks containing reproductive adults. A similar set-up was briefly used at the Institute of Marine Sciences to collect larvae, until a preliminary HPLC analysis of the extract of sieve-collected larvae showed they possessed only minute quantities of bryostatins. To more rigorously investigate the impact of the larval collection method on their bryostatin content, larvae from the same batch of adults were collected by the pipette method described above and the sieve collection method using sieves made with 100 μm NITEX. Extracts of these larvae were tested for their palatability to pinfish and for their bryostatin content. The competence of sieve-collected larvae does not appear to be compromised because they settled, metamorphosed, and grew normally for at least 1 week (Lopanik, personal observation) and larvae collected on sieves by CalBioMarine are used to "seed" *B. neritina* crops (D. Mendola, personal communication).

Creating aposymbiotic larvae

In the spring of 2002, three replicate groups of larvae from N.C. *B. neritina* and, after their settlement onto small polystyrene petri dishes, the newly metamorphosed juveniles were exposed daily for 10 h to the antibiotic gentamicin (75 μg/ml; Sigma-Aldrich, St. Louis, Mo.) over 10 consecutive days (Haygood and Davidson 1998; Davidson et al. 2001). The polystyrene dishes (n=12) were attached to a Plexiglas plate measuring approximately 10×24 cm. Each plate was considered a replicate. For the remainder of each day, the developing juveniles were suspended in an outdoor mesocosm supplied with unfiltered seawater from Bogue Sound. Recruitment of *B. neritina* into the mesocosm is rare because the pumps destroy the soft-bodied larvae. Control larvae and juveniles (n=3 replicate groups) were handled in the same manner as the treatment replicates, except they were not exposed to the antibiotic.

Following the 10th day of antibiotic treatment, treatment and control replicates were transplanted to the Indian River Inlet near Bethany Beach, Del. to grow up in situ. Delaware was chosen as the grow up site because Delaware populations are not reproductive until the late summer and early fall (Lopanik, personal observation), so there was little chance of Delaware *B. neritina* recruiting to the treatment and control groups. A recent report that Delaware *B. neritina* is a sibling species to the North Carolina and California populations (McGovern and Hellberg 2003) suggested that genetic methods could confirm that our antibiotic-treated and control groups had not become colonized by Delaware individuals. Delaware *B.* cf. *neritina* lacks *E. sertula*, suggesting that *B.* cf. *neritina* in Delaware might lack bryostatins and thus be palatable to larval predators. During the in situ grow up, the treatment and control colonies were suspended at a depth of 1 m below a floating dock. The few *B. neritina*-like recruits that settled on the sides or backs of the plates were removed.

The amount of growth in treatment and control colonies was assessed by counting the number of zooids after 10 days and the number of branch points after 5 weeks in randomly selected individuals. After 2 months in the field, larvae of the antibiotic-treated and control colonies, as well as of Delaware *B.* cf. *neritina*, were collected using the pipette method described above. The lipophilic extract of each replicate was analyzed for bryostatins by HPLC. Because of the small volume of larvae collected from each replicate, the remaining lipophilic extract from the replicates was combined for the palatability bioassays. DNA from control, treatment, and Delaware larvae was extracted (Isoquick, Orca Research, Bothell, Wash.) and quantified on a scanning spectrophotometer (Beckman, Fullerton, Wash.) at 260 nm. To ensure that the North Carolina experimental animals were not contaminated with Delaware individuals, the *B. neritina* cytochrome oxidase I (COI) gene was amplified using universal COI primers (Folmer et al. 1994). All PCR reactions were performed using a hot-start *Taq* polymerase (JumpStart Taq, Sigma-Aldrich, St. Louis, Mo.). Because of the difference in COI DNA sequences between North Carolina and Delaware individuals (Davidson and Haygood 1999; McGovern and Hellberg 2003; Lopanik, unpublished data), the restriction enzyme *SacI* (Promega, Madison, Wis.) was used to differentially cut the Delaware and North Carolina COI PCR products into two bands of ~240 and 480 bp, and ~50 and 625 bp, respectively. These fragments were visualized by agarose gel (1%) electrophoresis.

The presence or absence of 16S rDNA from *E. sertula* in both control and treatment adult and larval tissue was assessed by PCR amplification with *E. sertula*-specific primers developed by Haygood and Davidson (1997), and quantified by real-time Q-PCR (Morrison et al. 1998) using oligonucleotide primers designed specifically for North Carolina *B. neritina* COI and *E. sertula* 16S DNA. The *B. neritina* COI primers were BnCOIQf (5′-ttgatactgggggctcctgatatg-3′) and BnCOIQr (5′-aagcccgatgataagggagggta-3′); the *E. sertula* 16S primers were EBn16SQf (5′-cagcgaggaggaaaggttgacgaa-3′) and EBn16SQr (5′-tgagcccggggatttcacatctg-3′). Twenty-five ng of DNA was added to each reaction. To reduce the amount of nonspecific amplification and primer-dimer formation, a hot-start *Taq* master mix (Brilliant SYBR Green QPCR Master Mix, Stratagene, La Jolla, Calif.) was used in each reaction on an Applied Biosystems Prism 7700 Sequence Detector (Foster City, Calif.). All samples and standards were run in triplicate. To determine if the antibiotic treatment eliminated bacteria other than the symbiont, universal eubacterial primers with a GC-clamp (338f-gc and 519r-c; Muyzer et al. 1993) were used to amplify eubacterial DNA. An *E. sertula* "standard" was made by amplifying wild North Carolina larval DNA with the *E. sertula*-specific primers (Haygood and Davidson 1997), diluting and reamplifying this product with the universal bacterial primers, and running those products next to the experimental samples to determine where the *E. sertula* band occurred on the gel. The PCR products were subjected to denaturing gradient gel electrophoresis (DGGE). Certain bands, including those that appeared to be the same size as the *E. sertula* band and those that were dominant in a sample, were reamplified using the eubacterial primers and sequenced on an Applied Biosystems 310 Genetic Analyzer to verify the presence or absence of *E. sertula*.

Results

The larval lipophilic extract was highly deterrent to pinfish (P<0.001; Fig. 2), while the water-soluble extract was palatable (P=0.25). Bioassay-guided fractionation of the highly deterrent larval lipophilic extract led to the isolation of three compounds that significantly reduced pinfish feeding. Spectral analysis identified these compounds as bryostatins, and two of them specifically as bryostatin 10 and a novel bryostatin named bryostatin 20 (Fig. 1B, C) (Lopanik 2003; Lopanik, Lindquist and Gustafson, unpublished data). These bryostatins reduced pinfish feeding by 38% (unidentified bryostatin, hereafter referred to as peak D), 75% (bryostatin 10), and 33% (bryostatin 20), (P=0.032, 0.001, and 0.032, respectively). Three non-deterrent bryostatins (P>0.5), which have yet to be fully characterized, were also isolated. Each of the bryostatins comprised at least 0.1% of the larval dry mass (~0.2 mg/ml larval tissue). In total, bryostatins comprise ~1% of the larval dry mass and were readily detected in the larval extract by analytical HPLC (Fig. 3).

In contrast to the larval extract, the lipophilic extract of adult branches had no significant effect on fish feeding behavior (P=0.125; Fig. 2). When extracts obtained from identical volumes of larval and adult tissues were compared by analytical HPLC, they contained high and undetectable levels of bryostatins, respectively (Fig. 3).

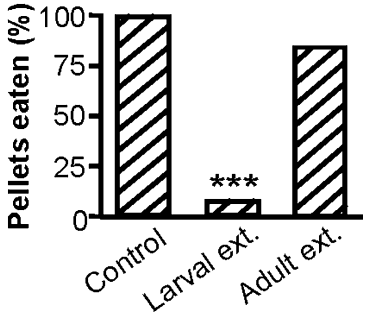

Fig. 2 Palatability of larval and adult lipophilic extracts of *Bugula neritina* to pinfish (n=13–15). *Asterisks* indicate significant differences between percentage of responses to control and extract pellets (McNemar's test; ***P<0.001)

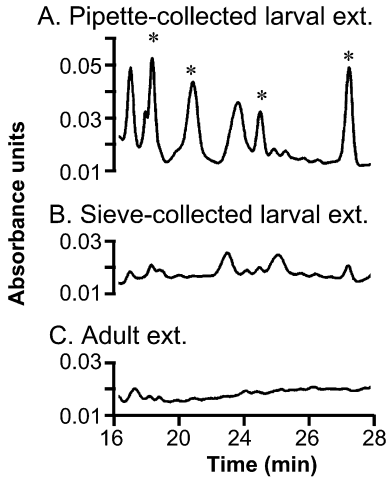

Fig. 3 Representative HPLC chromatograms (recorded at 229 nm) of **A** pipette-collected larval, **B** sieve-collected larval, and **C** adult lipophilic extracts obtained from identical volumes of tissue. Peaks in these chromatograms with *asterisks* are bryostatins

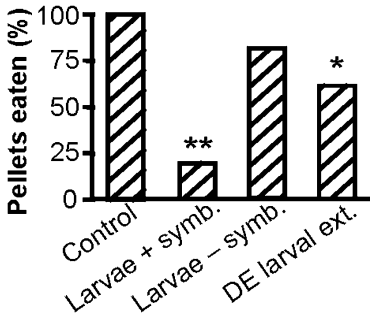

Fig. 4 Palatability of extracts of larvae obtained from North Carolina *Bugula neritina* with *Endobugula sertula* (*larvae+symb.*), North Carolina *B. neritina* without *E. sertula* (*larvae−symb.*), and Delaware *B. neritina*; n=10–13 for each assay. *Asterisks* indicate significant differences between percentage of responses to control and extract pellets (McNemar's test; *P<0.05, **P<0.01)

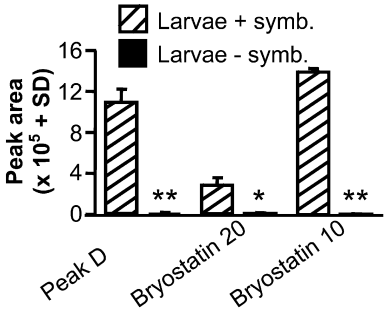

Fig. 5 Peak areas (+1 SD) of the three deterrent bryostatins in identical volumes of larvae from North Carolina *Bugula neritina* with *Endobugula sertula* (n=2) and without *E. sertula* (n=3). *Asterisks* indicate significant differences between peak areas for each compound (ANOVA, log transformed data; *P<0.05, **P<0.01)

Extracts from pipette- and sieve-collected larvae differed greatly in both palatability to pinfish and bryostatin content. The extract of pipette-collected larvae showed a trend (P=0.089; Fisher's exact test) towards being more unpalatable than the extract of sieve-collected larvae [8% (n=12) versus 40% (n=10) consumption of extract-treated food pellets]. Pipette-collected larvae contained high concentrations of bryostatins, whereas larvae collected on sieves had lost ~80% of their bryostatins (Fig. 3).

Larval/juvenile survival in both the gentamicin-exposed and control groups was high at the conclusion of the 10-day treatment period. The seawater from one control plate spilled and the juveniles did not survive the incubation. Both treatment and control groups grew well, showing no significant difference between them in the number of zooids after 1.5 weeks in the field (n=20 control and n=30 treatment colonies; P=0.502; one-way ANOVA) and branch points after 5 weeks (n=26 control and n=39 treatment colonies; P=0.207). After 10 weeks in the field, both treatment and control colonies became reproductive. A restriction enzyme analysis of COI PCR products amplified from the larval DNA confirmed that larvae of Delaware *B.* cf. *neritina* did not contaminate either the treatment or control groups. The combined extract of larvae from control colonies was highly unpalatable to pinfish, significantly reducing their consumption of food pellets by 80% (P=0.004; n=10; McNemar's test; Fig. 4). In contrast, pinfish feeding decreased by only 20% on the assay food containing the combined extract of larvae from the gentamicin-treated group (P=0.250; n=11). The extract of treatment larvae was significantly more palatable than the extract of control larvae (P=0.009; Fisher's exact test). HPLC analysis revealed a nearly complete lack of bryostatins in larvae of antibiotic-treated colonies (Fig. 5), and no bryostatins were detected in larvae of Delaware *B. neritina* (data not shown). Despite having no bryostatins, the larval extract of Delaware *B. neritina* was also significantly unpalatable (P=0.031; n=13), reducing fish feeding by 38% (Fig. 4).

Using 16S rDNA primers specific to *E. sertula* (Haygood and Davidson 1997), PCR amplification of DNA extracted from larvae of gentamicin-treated colonies did not detect the symbiont, whereas larvae from control colonies showed a strong *E. sertula* band (Fig. 6A). This *E. sertula*-specific primer set did not amplify DNA from larvae of Delaware *B.* cf. *neritina* (Fig. 6A). DGGE analysis of PCR products amplified using a general eubacterial 16S rDNA primer set showed an intense band in two independent samples of control larvae that was absent in three independent samples of treatment larvae and in three independent samples of Delaware *B.* cf. *neritina* larvae (Fig. 6B). This intense band in the control replicates was the same size as the *E. sertula* "standard" band and its nucleotide sequence was identical to the 16S rDNA sequence of *E. sertula*; six other bands sequenced from antibiotic treated and Delaware larvae (Fig. 6B) were not from *E. sertula*.

Data from real-time Q-PCR using 16S rDNA primers specific to *E. sertula* and COI primers specific to North Carolina/California *B. neritina* detected significant differences in the ratio of *E. sertula* to *B. neritina* DNA in both

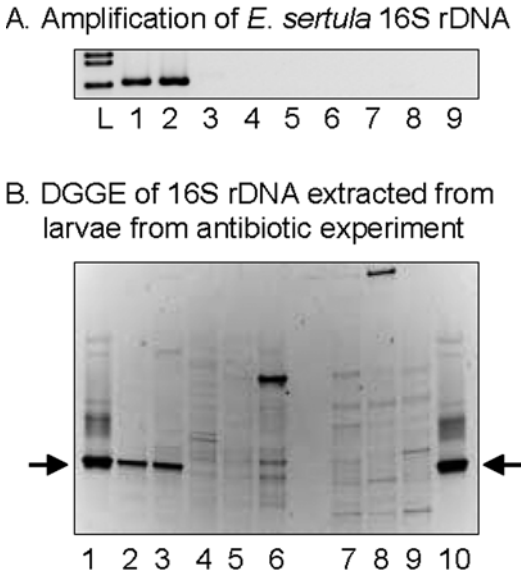

Fig. 6 **A** PCR products of DNA from larvae of North Carolina *Bugula neritina* colonies with *Endobugula sertula* (*lanes 1, 2*) and from colonies lacking *E. sertula* (*lanes 3–5*), and from larvae from colonies of Delaware *B. neritina* (*lanes 6–8*) amplified using 16S rDNA primers specific to *E. sertula* (Haygood and Davidson 1997); *lane 9* no template control, *lane L* molecular weight ladder. **B** Denaturing gradient gel electrophoresis (25–55% denaturant) of PCR-amplified DNA [universal eubacterial primers 338f-gc and 519r-c (Muyzer et al. 1993)] from larvae of North Carolina *B. neritina* colonies with *E. sertula* (*lanes 2, 3*), North Carolina colonies lacking *E. sertula* (*lanes 4–6*), and Delaware *B*. cf. *neritina* (*lanes 7–9*). *Lanes 1 and 10* are *E. sertula* "standard". *Arrows* indicate the *E. sertula* bands

larval and adult samples of gentamicin-treated versus control colonies (P=0.001 and 0.023, respectively; two-tailed t-test; see Electronic Supplementary Material). One treatment replicate was not included in this analysis due to low DNA yield from the larvae, and the DNA from the corresponding adults was not utilized. The amount of *E. sertula* DNA in treatment larvae and adults was 2–3 orders of magnitude and 1 order of magnitude lower, respectively, than in control larvae and adults.

Discussion

Recent studies have highlighted the importance of larval chemical defense among diverse taxa of benthic marine invertebrates (Lucas et al. 1979; Young and Bingham 1987; Lindquist et al. 1992; Lindquist and Hay 1995, 1996; Harvell et al. 1996; Lindquist 1996, 2002; McClintock and Baker 1997; Iyengar and Harvell 2001) and the substantial impact that larval chemical defense can have on the evolution of larval morphology (e.g., becoming large and apparent) and behavior (e.g., being day active in benthic habitats teeming with planktivorous fishes). These larval traits are thought to enhance the survival of newly settled juveniles by increasing initial juvenile size (Jackson 1985; Lindquist and Hay 1996; Lindquist 2002) and allowing larvae to use strong photic cues to choose appropriate settlement microenvironments that diminish juvenile exposure to environmental and biological stresses (Olson 1985; Young 1986; Young and Bingham 1987; Young and Chia 1987; Walters 1992; Lindquist and Hay 1996).

Results of our study demonstrate that the highly vulnerable larval stage of *B. neritina* is chemically defended from fish predation by bryostatins, including the novel bryostatin 20 (Fig. 1). The bryostatins do not, however, appear to provide anti-predator protection for the adults. With a virtual absence of bryostatins in non-reproductive branches (Fig. 3), it appears that the vast majority of these compounds in *B. neritina* are associated with the larvae. The findings that *B. neritina* larvae possess high concentrations of bryostatins, whereas the adult stage does not, is atypical because most studies of other marine invertebrates have found that both the larval and adults stages contain relatively high levels of the same secondary metabolites (Lucas et al. 1979; Lindquist and Fenical 1991; Lindquist et al. 1992; Harvell et al. 1996).

In contrast to solitary organisms, colonial animals such as *B. neritina* typically survive bouts of partial predation (Jackson 1977, 1985; Ayling 1981; Davis 1988). Lindquist (2002) has noted, however, that larvae of colonial marine invertebrates are functionally solitary organisms typically lacking morphological and behavioral defenses against predation, and thus that selection should favor chemical defense of their larvae. Furthermore, distasteful larvae are frequently rejected without apparent damage (Young and Bingham 1987; Lindquist 1996; Lindquist and Hay 1996); therefore, chemicals that function directly to deter predators must occur on the larval surface or be readily mobilized towards the surface during an attack. This appears to be the case with *B. neritina* larvae and the bryostatins because pipette-collected larvae had more bryostatins than did larvae collected by the physically rougher sieve collection method (Fig. 3). Larvae collected by either method have high rates of survival and metamorphosis. These data suggest that the bryostatins occur in an optimally defensive position on the larvae, rather than sequestered within them because disrupting the larval surface is not required to remove the bryostatins.

Although the bryostatins are complex polyketides and most likely of microbial origin, it seems hard to reconcile the high larval concentrations with the reported low number of *E. sertula* cells (~2,500) directly associated with each larva—the larval bryostatin mass is roughly three orders of magnitude greater than the mass of *E. sertula* associated with the larvae. Additionally, *E. sertula* is reported to occur throughout the bryozoan colony (Davidson et al. 2001), yet we found that non-reproductive branches do not contain detectable levels of bryostatins (Fig. 3). Two plausible explanations to account for the high larval concentrations of bryostatins are that populations of *E. sertula* in adult tissues produce bryostatins which are then transported to and concentrated on the larvae, or that larvae are the true source of the bryostatins.

After first eliminating a possible dietary origin for the bryostatins (Haygood et al. 1999), Haygood and co-workers conducted a symbiont "knock-out" experiment

using antibiotics in an attempt to eliminate *E. sertula* from *B. neritina* and thereby confirm *E. sertula* production of bryostatins (Davidson et al. 2001). Not knowing at the time about the high larval concentrations of bryostatins, they relied on indirect quantification of the bryostatins by a highly sensitive radiochemical method (Davidson et al. 2001). Genetic analysis using *E. sertula*-specific 16S rDNA primers indicated that they successfully eliminated >95% of *E. sertula* from antibiotic-treated *B. neritina* but only achieved a 50% reduction in bryostatin levels, suggesting, but not absolutely confirming, a role for *E. sertula* in bryostatin production. In our repeat of the antibiotic knock-out experiment, we evaluated the outcome by HPLC quantification of bryostatins in larvae from field-raised treatment and control colonies and determined whether or not the presence of the symbiont influenced the palatability of extracts from the larvae. The success of this experiment was demonstrated by the virtually undetectable levels of *E. sertula* DNA in the larvae of the treatment group (0.1% of that present in larvae of the control group) and by treatment adults having only 7.4% of the symbiont DNA levels found in control adults (Fig. 6A). The corresponding reduction in bryostatin levels (on average 98.6% less) for treatment versus control larvae and the palatability of the treatment larvae's extract (Figs. 4, 5) indicated that *E. sertula* is responsible for the production of the bryostatins in *B. neritina*.

Further evidence for the production of bryostatins by *E. sertula*, and thus the origins of the larval chemical defense of *B. neritina*, were the findings that the extract of larvae from Delaware *B.* cf. *neritina* contained no bryostatins, and that Delaware *B.* cf. *neritina* larvae do not possess *E. sertula* (Fig. 6). Recently, *B. neritina* from Delaware was shown to be a sibling species to *B. neritina* in California and North Carolina (McGovern and Hellberg 2003), which are closely related to each other and possess *E. sertula* (Davidson and Haygood 1999). The unpalatability of the extract of larvae from Delaware *B.* cf. *neritina* is probably due to deterrent metabolites that are not bryostatins (Fig. 4).

The weight of evidence indicates that *E. sertula* is the true source of the bryostatins, yet *E. sertula* cells directly associated with the larvae are unlikely to produce the high larval concentrations of bryostatins. However, a bacterium morphologically similar to *E. sertula* occurs in the funicular cords of adult *B. neritina* (Woollacott and Zimmer 1975). This bacterium is probably *E. sertula* because *E. sertula*-specific primers amplified DNA from adult branches lacking ovicells (Davidson et al. 2001). The funicular cords are thought to transport nutrients between zooids and from the zooid to the developing larva in the ovicell (Woollacott and Zimmer 1975). Production of bryostatins by ovicell-associated *E. sertula* would place bryostatins in the vicinity of the larvae, but the mechanism by which they would become concentrated on the larval surface is unclear.

The vast majority of reported symbioses between marine invertebrates and microorganisms involve nutritional enhancements critical to the fitness of the host. Our data suggest that *E. sertula* does not play a nutritional role for *B. neritina* because aposymbiotic colonies grew and reproduced as well as symbiotic ones. Most importantly, we demonstrated the critical roles of *E. sertula* in the production of the bryostatins and of bryostatins as a larval defense against predation. *B. neritina* is the first example from the marine environment of a symbiont producing a chemical defense for its host, and one that appears to have evolved not to protect the long-lived adults, but their short-lived, highly vulnerable larval stage. Our results, those of Fenical and co-workers (Gil-Turnes et al. 1989; Gil-Turnes and Fenical 1992), and the increasing number of secondary metabolites isolated from marine invertebrates but shown to be produced by microbial symbionts (Stierle et al. 1988; Elyakov et al. 1991; Kobayashi and Ishibashi 1993; Faulkner et al. 1999) suggest that symbiont production of ecoactive secondary compounds may be widespread among diverse taxa of marine invertebrates.

While our basic research on the chemical ecology of *B. neritina* has yielded new insights into the understudied area of defensive symbioses in the marine environment, much of the recent research on the symbiosis between *B. neritina* and *E. sertula* has focused on using state-of-the-art genetic, molecular, and microbial manipulations to optimize bryostatin production for biomedical applications (Haygood et al. 1999). Our discovery of high bryostatin levels in *B. neritina* larvae, however, suggests that current aquaculture methodologies developed for *B. neritina* (Mendola 2000) could be modified to substantially increase bryostatin yields. This increase would be accomplished not by the harvest of the adult stage, but by perpetuating the adults for their daily production of bryostatin-rich larvae which, if not captured, results in the loss of the majority of bryostatins produced over the lifetime of the colony.

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