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Investigation of larval settlement pathways in the marine bryozoan, *Bugula neritina*

Heather L. Price^{a*}, Neeraj V. Gohad^b, Andrew S. Mount^b, and Dean E. Wendt^a

^a Center for Coastal Marine Sciences, California Polytechnic State University, 1 Grand Ave, San Luis Obispo, CA 93407, USA; ^b The Department of Biological Sciences, 132 Long Hall, Clemson University, Clemson, SC 29634, USA

*To whom correspondence should be addressed. Email: heatherleighprice@gmail.com

1. Introduction

Found in warm-temperate and subtropical waters worldwide, *Bugula neritina* (Linnaeus 1758) is a sessile marine bryozoan with a pelagic larval stage (Ryland *et al.* 2011). Larvae frequently attach to boat hulls, and the species is regarded as one of the most widespread fouling bryozoans. A cosmopolitan distribution was reported for *B. neritina* as early as the 18th century, and shipping likely played a role in its introduction to bays and estuaries around the globe (Winston and Woollacott 2008). Sequencing of the mitochondrial gene cytochrome c oxidase I suggests that *B. neritina* is actually a complex of three cryptic species (Mackie *et al.* 2006, Davidson & Haygood 1999), which may have distinct native ranges (Fehlauer-Ale *et al.* 2014). Native and non-native boundaries for *B. neritina* therefore remain unclear, but the range of the bryozoan is expanding (Winston and Woollacott 2008). (From this point on, *B. neritina* will be used to refer to the species complex, or *sensu lato* definition of the organism.) Increased knowledge of the mechanisms controlling larval settlement behavior in fouling organisms like *B. neritina* will allow us to better understand factors that are responsible for their success as invasive species, and will enable us to develop improved strategies for preventing biofouling and further anthropogenic transport of non-native species to coastal ecosystems worldwide.

Many aspects of larval anatomy and behavior of *B. neritina* are well documented (e.g., Lynch 1947, Woollacott and Zimmer 1971). Adult colonies are comprised of branching, hermaphroditic zooids, and are typically brown to dark purple in color. Sexually reproduced embryos are brooded in modified zooids called ovicells, which release larvae that are non-feeding (aplankotrophic, Wendt 1996) and typically spend less than 24 h as plankton prior to settling (e.g., Wendt and Woollacott 1999). There is an inverse relationship between duration of larval swimming and metamorphic success (Wendt

1996). Larvae swim through the water column using cilia that cover most of the surface of their barrel-shaped bodies, collectively referred to as the ciliated corona (Woollacott and Zimmer 1971). Larvae often swim in a spiraling motion, and hold sensory structures in advance as they move through the water and begin exploration of a substratum.

The sensory apical disc consists of a central sensory cap made up of neural plate cells surrounded by a ring of ciliated ray cells and a crown of rigid cilia (Lynch 1947, Santagata 2008). The neural plate cells of the sensory cap extend basally into paraxial nerve cords, which connect with the neuromuscular ring (Santagata 2008). Nerve fibers also extend from the neuromuscular ring to the pyriform organ, which consists of three long cilia that extend from the glandular pyriform groove (Fig. 1). These cilia, collectively referred to as the vibratile plume, are held in advance as larvae swim and spin through the water, and, along with the apical disc, are thought to play a chemosensory role during both the pelagic phase and the early stages of *B. neritina* settlement (Lynch 1947).

Prior to attachment, larvae alight on a surface and spin counter-clockwise for 5-10 min, actively feeling the substratum with the vibratile plume (Lynch 1947). All visible ciliary activity then halts for a brief moment prior to eversion of the internal sac. This invaginated epithelium is held within the body of planktonic larvae, and functions as an adhesive organ during settlement. Once the internal sac is everted, metamorphosis is initiated and the animal is permanently attached to the substratum. The newly metamorphosed progenitor zooid, or ancestrula, gives rise to all other zooids in the colony *via* asexual budding (Lynch 1947). Colonies can become reproductive and release larvae within just twelve days of metamorphosis (Wendt 1998).

While many aspects of larval anatomy and behavior of *B. neritina* are well documented, the underlying pathways that control larval settlement remain largely

unknown. Antifouling research into natural substances that deter marine invertebrate settlement can provide insight into these pathways. Adrenergic compounds, such as norepinephrine (NE), reduce larval settlement in *B. neritina* (Shimizu *et al.* 2000) and a variety of other marine invertebrates; including the bivalve *Crassostrea virginica*, and the crustacean *Balanus Amphitrite* (Gohad *et al.* 2010). That these compounds exert similar effects on species from an array of phyla suggests that there may be a conserved mechanism of reception across invertebrate taxa. While many studies have looked at the effects of adrenergic compounds on marine invertebrate settlement, Gohad *et al.* offer some of the first insight into the underlying mechanisms of this inhibition. The effects of adrenergic compounds on larval settlement are typically tested in solution, but NE also deters barnacle cyprid (*Balanus Amphitrite*) settlement when covalently conjugated to a polymer surface (Gohad *et al.* 2010). A further study revealed the presence of adrenergic-like receptors in sensory setae on cyprid antennules (Gohad *et al.* 2012). These findings suggest that NE may be detected in cyprid larvae by adrenergic-like receptors located in sensory structures.

It is possible that *B. neritina* larvae, like *B. amphitrite* cyprid larvae, possess adrenergic-like receptors within sensory structures that bind exogenous NE, thereby triggering a pathway of settlement inhibition. It is also possible that NE acts by permeating larval tissue and binding endogenous receptors involved in controlling settlement pathways. These two explanations need not be mutually exclusive, as NE could conceivably act as a sensory stimulus, and also mimic the action of an endogenous neuroactive compound.

While it is unknown whether marine invertebrates possess receptors specific for NE, a multitude of invertebrate species possess receptors for a similar compound,

octopamine (Roeder 1999). Octopamine is considered the invertebrate analog of NE and the two compounds only differ structurally by the addition of one hydroxyl group to the benzene ring in NE (Fig. 2). Octopamine regulates a variety of physiological and behavioral processes ranging from locomotion to photosensitivity in phylogenetically diverse invertebrates (Roeder 1999). Octopamine receptors are known targets for adrenergic antagonists in insects (Evans 1981) and have been proposed as binding sites for adrenergic compounds in marine invertebrates (e.g. Wendt *et al.* 2013). It is therefore possible that NE exerts its inhibitory effects on marine invertebrate larvae by permeating cells and acting on octopamine receptors, which are likely part of an endogenous pathway controlling settlement. In *B. neritina*, octopamine receptors may be one of the binding sites for adrenergic compounds, and endogenous octopamine may be a neuroactive compound that modulates many aspects of larval behavior; including locomotion, settlement, and phototaxis.

Photosensory systems play an important role in *B. neritina* larval behavior. Larval release is induced in the laboratory by exposing dark-acclimated adult colonies to light (e.g. Woollacott and Zimmer 1971, Wendt 1996), and continued light exposure inhibits larval settlement (Wendt 1996). Larvae detect light with two posterolateral ocelli, which are connected to the neuromuscular ring by axonal nerve fibers (Santagata 2008), and are photopositive upon release, but switch to photonegativity within several hours (Lynch 1947, Wendt and Woollacott 1999). As they move away from light, larvae begin the process of surface exploration prior to attachment and metamorphosis. Thus, there is an inverse relationship between positive phototaxis and initiation of metamorphosis (Wendt and Woollacott 1999). While the effects of light on *B. neritina* larvae are well documented, the underlying pathways controlling these phenomena are still not well understood. One

study investigated the mechanisms underlying phototaxis by exposing *B. neritina* larvae to the monoamines dopamine and serotonin. Dopamine exposure extended the period of positive phototaxis, while serotonin, or 5-hydroxytryptophan (5HT), made larvae immediately photonegative. 5HT-like activity was also found in tracts connecting eyespots to the larval locomotory organ (Pires and Woollacott 1997). In both vertebrates and invertebrates, the metabolic pathways involving dopamine are well studied. Tyrosine is first converted by the enzyme tyrosine hydroxylase (TH) into L-DOPA, which is then converted into dopamine. Dopamine is the precursor to several other monoamines, including NE and octopamine (Fig 2). Therefore, it is possible that light exerts its effects on *B. neritina* larvae *via* an underlying chemical pathway that involves both dopamine and octopamine.

In the present study, *B. neritina* larvae were exposed to various concentrations of the adrenergic agonist, NE, and the adrenergic antagonist, phentolamine, to investigate the effects of these compounds on larval settlement behavior and offer insight into the potential role of adrenergic-like receptors in settlement pathways. Fluorescent labeling and microscopy were used to determine the presence and location of adrenergic-like receptors, octopamine, and tyrosine hydroxylase. Larvae were also exposed to light in the presence and absence of phentolamine to observe the combined effects of these stimuli on larval attachment and to gain knowledge about the underlying photosensory pathway.

2. Materials and methods

2.1 Larval collection

Colonies of *Bugula neritina* were collected by hand from floating docks in two separate locations in Morro Bay, CA USA (35.3708, -120.8580; 35.3461, -120.8432) from March 19, 2014 through May 1, 2015. Colonies were maintained in captivity in a dark, aerated container of raw seawater at 11°C for 2 to 10 days. To induce larval release, dark-acclimated colonies were exposed to light (both natural and incandescent). Larvae were then collected and transferred by pipette within 1 h of release. Larvae were pooled from multiple colonies to foster genetic heterogeneity for all experiments.

2.2 Effects of norepinephrine and phentolamine on larval attachment and behavior

To observe the effects of NE (an adrenoreceptor agonist) and phentolamine (an adrenoreceptor antagonist) on larval behavior and attachment, larvae were bath-applied with varying concentrations of each compound in seawater and observed with a Leica EZ4D dissecting microscope. DL- Norepinephrine Hydrochloride ($\geq 97\%$) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Phentolamine-Hydrochloride ($\geq 98\%$) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Larvae were released from multiple adult colonies (collected on three different days from two Morro Bay sites), pooled in a beaker, and transferred to 15 ml Falcon tubes with 0.1 μM filtered seawater containing 0 (control), 0.1, 1.0, 10, or 100 μM of either NE or phentolamine. These concentrations were chosen based on those used in previous studies (e.g. Shimizu *et al.* 2000, Gohad *et al.* 2012) and also those that would be biologically relevant.¹

¹ While endogenous concentrations of monoamines within bryozoan larvae are unknown, one study revealed a wide range of octopamine concentrations between tissues of different invertebrate taxa (Roeder 1999), and because NE and phentolamine were not thought to be found endogenously, we used them at higher concentrations than those of monomines that would likely be found in nature, in order for them to successfully compete with the endogenous ligands at the receptor binding sites.

Larvae were immediately transferred from Falcon tubes to 24 well polystyrene cell culture plates, one larva per well in 1 ml of solution, with a total of twelve larvae per treatment per trial. Individual wells were used to eliminate the effects of gregariousness on settlement behavior. Five trials were conducted with both compounds (on the 15, 17, 22, 24, and 29 of April, 2014), for a total of 300 larvae used in the experiment. Fresh treatment solutions were made up for each trial. The number of larvae attached was recorded at 2, 4, 6, 8, 24, and 48 h following the start of treatment solution exposure. Larvae were designated as attached if they had settled on the polystyrene and could not be moved by a pipetted stream of water, or if they had settled on the air-water interface and begun to metamorphose. We examined larval behavior at 2, 4, 6, and 8 h of exposure and observed how many live, unattached larvae were swimming in each treatment. Larvae were kept at 11°C for the duration of the experiments, except during observation.

2.3 Localization of adrenergic-like receptors

Fluorescent labeling and microscopy were used to determine the presence and location of adrenergic-like receptors within whole mount *B. neritina* larvae. Live larvae were incubated in 10 μ M BODIPY-FL Prazosin, a fluorescently labeled non-subtype selective α -adrenergic receptor antagonist obtained from Life Technologies (Foster City, CA, USA). The solution was made up in 0.1 μ M filtered seawater (FSW) and larvae were incubated for 30 min prior to being washed three times in FSW. Larvae were imaged with the 20X objective on an Olympus (Center Valley, PA, USA) BX53 microscope, using a DP73 camera and CellSens software. The 488 nm laser line was used, and images were converted to gray scale. Unstained larvae were used as controls for autofluorescence. Brightfield images of *B. neritina* larvae were taken with an Olympus BX53 microscope and DP73 camera using CellSens software to provide a reference of basic larval morphology.

2.4 Localization of anti-octopamine and anti-tyrosine hydroxylase-like immunoreactivity

To determine the presence and location of tyrosine-hydroxylase-like and octopamine-like immunoreactivity, *B. neritina* larvae were fixed in 4% paraformaldehyde made up in phosphate-buffered saline (PBS) for at least 2 h, then washed once in 1X PBS. Fixed larvae were then permeabilized overnight in 0.5% Triton X-100 in PBS, and incubated in 2% bovine serum albumin (BSA) for 4 h. This serum was pipetted off, and larvae were incubated in either an anti-tyrosine hydroxylase primary antibody (1:500) or an anti-octopamine primary antibody (1:500) in PBS overnight at 4°C. Anti-tyrosine hydroxylase was obtained from Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA) and anti-octopamine was obtained from Millipore (Billerica, MA, USA). Larvae were washed in 0.1% Triton X-100 in PBS four times (5 min each) and those incubated with anti-tyrosine hydroxylase were transferred to Alexafluor 568 anti-mouse secondary antibody (1:500), while those incubated in anti-octopamine were transferred to Alexafluor 594 anti-rabbit secondary antibody (1:500) and incubated overnight at 4°C. Both secondary antibodies were obtained from Life Technologies. All larvae were incubated with the nuclear stain DAPI for 15 min (1:500), prior to washing four times (5 min each) in 0.1% Triton-X in PBS. Larvae were imaged with the 20X objective in PBS using Nunc Lab-Tek 4-chambered coverglass (obtained from Sigma-Aldrich) on an Olympus FV1000 Scanning Laser Confocal Microscope using Fluoview imaging software. A 405 nm laser line at 8% transmissivity was used for DAPI excitation, and a 559 nm laser line was used at 12% transmissivity for Alexafluor-568 excitation and at 30% transmissivity for Alexafluor-594 excitation. Untreated larvae and larvae labeled with only secondary antibodies were used as controls for autofluorescence and unspecific binding, respectively.

2.5 Combined effects of light and phentolamine on larval attachment

To investigate underlying photosensory mechanisms in *B. neritina* larvae, larval attachment was compared between larvae immersed in phentolamine and larvae in FSW, in both dark and light conditions. Larvae were collected from multiple colonies, pooled, and transferred to 15 ml falcon tubes containing either FSW (control) or 100 μ M phentolamine in FSW. Each tube was transferred to a separate 65 mm diameter petri dish, and placed in either dark or light conditions for 4 hours at room temperature. The number of larvae attached was recorded at 1, 2, and 3 h of exposure. Three trials were conducted, with ten larvae per treatment per trial, for a total of 120 larvae.

2.6 Statistical Analyses

Binomial logistic regression and Tukey's HSD *post hoc* test were used to determine whether rates of larval attachment differed significantly between treatment and control groups. Nominal logistic regression and Cox Proportional Hazards risk ratios were used to compare larval behavior between treatment and control groups. Analysis of Variance (ANOVA) and Dunnett's test were used to determine whether rates of larval attachment differed significantly between treatment and control groups in dark and light conditions. For experiments that sampled individual larvae at multiple time points, separate analyses were performed at each time point to avoid pseudo-replication. Residuals were normally distributed, so data were not transformed prior to analyses. All statistical analyses were conducted using JMP Pro 11 software (SAS, Cary, North Carolina, USA).

3. Results

3.1 Effects of norepinephrine and phentolamine on larval attachment

Binomial logistic regression revealed that norepinephrine (NE) significantly reduced larval attachment of *B. neritina* at 10 μM ($P=0.0297$) and 100 μM ($P=0.0121$) compared to the control group, but had no significant effect at 0.1 or 1.0 μM (Fig. 3). Pairwise *post hoc* comparisons (Tukey's HSD) revealed significant differences between the control group and 100 μM NE at 2 h ($P=0.0066$), 4 h ($P=0.0284$), 6 h ($P=0.0090$), and 8 h ($P=0.0111$) of exposure; between the control group and 10 μM NE at 2 h of exposure ($P=0.0190$); and between 100 μM NE and 100 μM phentolamine at 2 h ($P=0.0008$), 4 h ($P=0.0006$), 6 h ($P=0.0003$), and 8 h ($P=0.0010$) of exposure (Fig. 3).

3.2 Effects of norepinephrine and phentolamine on larval behavior

We examined larval behavior at 2, 4, 6, and 8 h of exposure to either NE or phentolamine, and observed how many live, unattached larvae were swimming. Nominal logistic regression and Cox Proportional Hazards risk ratios were used to compare each treatment to the control at every time. After 2 h of exposure, significantly more larvae were swimming in 10 μM ($P<0.0001$) and 100 μM ($P=0.0027$) NE, while significantly fewer larvae were swimming in 0.1 μM ($P=0.0097$) and 100 μM phentolamine ($P<0.0001$) compared to the control group (Fig. 4). After 4 h of exposure, significantly more larvae were swimming in 100 μM NE ($P=0.0058$), and significantly fewer larvae were swimming in 10 μM ($P=0.0058$) and 100 μM ($P<0.0001$) phentolamine compared to the control group. After 6 h of exposure, significantly more larvae were swimming in 10 μM NE ($P=0.0105$), while significantly fewer larvae were swimming in 10 μM ($P=0.0002$) and 100 μM ($P=0.0056$) phentolamine compared to the control group. After 8 h, significantly more

larvae remained swimming in 10 μ M NE ($P=0.0234$) and significantly fewer larvae remained swimming in 10 μ M ($P<0.0001$) and 100 μ M ($P<0.0001$) phentolamine compared to the control group (Fig. 4).

3.3 Localization of adrenergic-like receptors

Fluorescent signals detected in larvae labeled with BODIPY-FL Prazosin indicate that *B. neritina* larvae do possess adrenergic-like receptors, which appear to be concentrated in and around the apical disc and the pyriform groove (Fig. 5). No fluorescence was observed in control larvae that were not treated with BODIPY-FL Prazosin.

3.4 Localization of anti-octopamine and anti-tyrosine hydroxylase-like immunoreactivity

Both tyrosine hydroxylase-like and octopamine-like immunoreactivity were detected in *B. neritina* larvae, though some distortion of the larvae made determination of the precise locations of these substances difficult. Tyrosine hydroxylase-like immunoreactivity appeared to be concentrated in the apical disc and the neuromuscular ring (Fig. 6). Octopamine-like immunoreactivity appeared to be most prevalent in the apical disc, the ciliated corona, and the pyriform groove (Fig. 7). No immunoreactivity was observed in control larvae that were not treated with primary antibodies.

3.5 Combined effects of light and phentolamine on larval attachment

Light exposure significantly reduced larval attachment at 1 h, 2 h, and 3 h of exposure ($P<0.0001$). Phentolamine-exposed larvae in dark conditions had significantly higher rates of attachment than control larvae in dark conditions after 1 h ($P=0.0004$) and 2 h ($P=0.004$) of exposure. There was a significant interaction between light and

phentolamine after three hours of exposure, as the inhibitory effects of light were significantly diminished in larvae exposed to 100 μ M phentolamine ($P=0.02$) (Fig. 8).

4. Discussion

4.1 Effects of norepinephrine and phentolamine on larval attachment and behavior

Results from this study confirm previous reports of the inhibitory effects of the adrenergic agonist NE on larval attachment in marine invertebrates (e.g. Shimizu *et al.* 2000, Gohad *et al.* 2012). While initial settlement experiments did not reveal significant differences between control and phentolamine-treated groups, a further experiment examining the effects of both light and phentolamine revealed that larvae exposed to 100 μ M phentolamine had significantly higher rates of attachment than control larvae in both light and dark conditions (Fig. 8). That NE, an adrenergic receptor agonist, reduced larval attachment at 100 μ M, and phentolamine, an adrenergic receptor antagonist, augmented larval attachment at 100 μ M, supports the hypothesis that *B. neritina* larvae possess adrenergic-like receptors that are involved in settlement pathways.

The effects of phentolamine on *B. neritina* larval attachment observed in this study differ from those in an experiment conducted by Dahms *et al.* (2004); a discrepancy that may be explained by the method of counting attached larvae. In this study, if larvae were attached at the time of observation, they were classified as such, even if they subsequently died and failed to complete metamorphosis. Also, more than 25% of attached larvae in the phentolamine treatments settled and began metamorphosis on the air-water interface, never attaching to the polystyrene container. These larvae were counted as attached, and considering them as unattached would have significantly altered the results of the experiment. While it is not unusual for *B. neritina* larvae to attach to the air-water

interface, exposure to high concentrations of phentolamine did increase this behavior. A more in-depth investigation of this observation may yield further interesting insights into the mechanism of adrenergic antagonists on larval settlement.

Analyses of *B. neritina* larval behavior in response to NE and phentolamine offer further insight into the mechanism by which these chemicals exert their effects. Classification of larval behavior was complicated by the fact that larvae can alternate between swimming and spinning during the period of surface exploration prior to attachment, but it was clear that NE (at 10 μ M) increased swimming behavior, while phentolamine (at 10 μ M) decreased swimming behavior over the 8 h sampling period (Fig. 4). One possible explanation for these observations is that NE binds receptors located within sensory structures, thereby triggering a signaling cascade that would ultimately stimulate larval ciliary action and prevent larvae from entering a period of quiescence necessary for attachment. It is possible that adrenergic compounds are structurally similar to natural environmental cues encountered by bryozoans, and that NE may be acting as a stimulus that indicates to *B. neritina* larvae that a location is not suitable for settlement. Phentolamine might bind and block these sensory receptors, preventing a natural cue from being received, thereby slowing ciliary beating, and causing larvae to settle more rapidly.

Bryozoan larvae receive sensory input from numerous positions on the surface of the larval body, and typically process the information in a central nerve nodule located beneath the pyriform organ before sending signals to effector organs such as the musculature and locomotory ciliated corona (Santagata 2008). Some of the sensory neurons found in *B. neritina* larvae include the border cells of the pyriform groove, the ciliated ray cells and neural plate cells of the apical disc, and the ciliated whorls adjacent to

cells of the ciliated corona known as intercoronal cells (Santagata 2008). Therefore, if adrenergic compounds are binding sensory receptors, these receptors are likely located within the pyriform organ, the apical disc, and/or intercoronal cells. Interestingly, intercoronal cells can receive various types of sensory stimuli and exert direct control over the ciliary beat of adjacent coronal cells through shared chemical synapses without any central processing through the nerve nodule (Reed, 1988). Therefore, an external chemical stimulus received by intercoronal cells could have a very rapid effect on larval swimming behavior.

While this hypothesis offers a mechanistic explanation for the action of NE on *B. neritina* larval behavior, it is still unclear why larvae would possess sensory receptors for adrenergic-like compounds. A wide array of chemical compounds may act to either deter or encourage marine invertebrate larvae to settle, including those exuded from conspecifics and microbial films (e.g. Hadfield and Paul 2001). In barnacle cyprid larvae, NE appears to act as a sensory stimulus (Gohad *et al.* 2010), yet an analogous compound that occurs naturally in the marine environment has yet to be found. Another possible explanation for the effects of NE on *B. neritina* larvae is that the monoamine permeates larval tissue and mimics the action of an endogenous neuroactive compound. Bath-applied NE may be entering cells and directly binding receptors within neural tissue. The proximity of intercoronal cells to the surface of the larval body, along with their direct synaptic connection to locomotory cilia, make them especially likely targets for this mechanism of action by NE. Phentolamine may likewise be permeating larval tissue and exerting its effects by blocking endogenous receptors. This seems a more plausible explanation for the action of the adrenergic antagonist, especially given the limited number of natural cues that

would be received by larvae within the filtered water used in laboratory experiments.

While it is possible that phentolamine blocks *B. neritina* larvae from binding a sensory 'do not settle' cue received by control larvae, it seems more likely the compound is interfering with endogenous control of ciliary movement within neural tissue.

Whether these compounds are acting by way of sensory reception or permeation of neural tissue, they likely exert their effects by triggering or blocking influxes of calcium that are ultimately responsible for controlling larval ciliary action. In vertebrate vascular smooth muscle, NE causes an increase in intracellular Ca^{2+} levels (Godfraind 1976), which can thereby increase airway ciliary activity (Lansley and Sanderson 1999). In invertebrates, such as the mollusk *H. trivolvis*, Ca^{2+} also causes an increase in ciliary beat frequency (Christopher *et al.* 1996). In barnacle cyprid larvae, exposure to octopamine, the invertebrate analog of NE, results in concentration-dependent increases in intracellular Ca^{2+} levels and significantly increases the speed of leg kicking (Lind *et al.* 2010).

Octopamine exposure also reduces *B. neritina* larval settlement in a similar fashion to NE (Shimizu *et al.* 2000), though the mechanism for this effect has not been reported. In *B. neritina* larvae, endogenous octopamine may be involved in regulating ciliary activity, and endogenous octopamine receptors may be the binding sites for NE and other adrenergic compounds. While these bath-applied experiments do not clarify whether NE is binding sensory receptors or mimicking endogenous octopamine, it is clear that adrenergic-like receptors are involved in larval settlement behavior, and could therefore be useful targets for bryozoan biofouling controls.

4.2 Localization of adrenergic-like receptors

Images of larvae labeled with fluorescently-labeled BODIPY-FL Prazosin, a non

subtype-selective α -adrenergic antagonist, indicate that larvae possess adrenergic-like receptors in and around the ciliated corona, the apical disc, and the pyriform groove (Fig. 5). The resolution of our images does not allow for the distinction between cell types, and it remains unclear whether these adrenergic-like receptors are located on the surface of sensory neurons or deeper within the larval tissue of these structures. This is the first time adrenergic-like receptors have been located within bryozoan tissue, however, and their presence within regions of known neural activity provides further evidence for the role of these receptors in larval settlement behavior, and as binding sites for NE and other adrenergic compounds.

4.3 Localization of anti-octopamine and anti-tyrosine hydroxylase-like immunoreactivity

Images of larvae incubated with anti-tyrosine hydroxylase provide further evidence for the mechanisms underlying larval settlement behavior. Anti-tyrosine hydroxylase-like immunoreactivity localized to the apical disc, to the ciliated corona, and to what appear to be axonal fibers that connect these regions to the neuromuscular ring (Fig. 6). Tyrosine hydroxylase is responsible for converting tyrosine to L-DOPA, the precursor to many monoamines, including NE and octopamine (Fig. 2). As early as 1890, Prouho asserted that the apical disc, the ciliated corona, and the pyriform organ in bryozoan larvae were all connected by nervous tissue, and many researchers have since confirmed his assertion (e.g. Santagata 2008, Pires and Woollacott 1997, Reed *et al.* 1988). The presence of tyrosine hydroxylase-like activity within these regions suggests that *B. neritina* larvae possess the enzyme, and that it is involved in the production of endogenous neuroactive compounds.

Images of larvae incubated with anti-octopamine suggest that octopamine may be one of those endogenously produced neuroactive compounds. Octopamine-like

immunoreactivity localized to the apical disc, the ciliated corona, the pyriform groove and what appear to be axonal fibers that underlie these structures (Fig. 7). While the images do not allow for visualization of octopamine within specific cell types, the observed pattern of immunoreactivity within regions of known neural activity was consistent with expectations. The presence of octopamine within these regions offers more evidence for the hypothesis that NE exerts its effects on *B. neritina* larvae by mimicking the action of endogenous octopamine. In the barnacle *Balanus improvisus*, cloned β -octopamine receptors are activated by both octopamine and the alpha-adrenergic receptor agonist medetomidine, resulting in intracellular increases of calcium (Lind *et al.* 2010). Therefore, it is possible that NE works by a similar mechanism in *B. neritina* larvae; binding octopamine receptors located within neural tissue and stimulating cilia by triggering intracellular increases in calcium. These results provide the first evidence for endogenous octopamine in a bryozoan, and suggest that it is one neuroactive compound involved in controlling *B. neritina* larval settlement behavior. Octopamine receptors may therefore prove useful as specific targets for the control of bryozoan biofouling.

4.4 Combined effects of light and phentolamine on larval attachment

Results from this study confirm that light significantly inhibits *B. neritina* larval attachment. The inhibitory effect of light was significantly diminished when adrenergic-like receptors were blocked by phentolamine (Fig. 8), suggesting that these receptors are involved in the photosensory pathway. It is possible that these receptors are octopamine receptors, and that octopamine plays a role in the photosensory pathway of *B. neritina* larvae. The role of octopamine in photosensory behavior has been documented in many other invertebrate species. In honeybees (*Apis spp.*), exposure to octopamine increases

positive phototaxis (Scheiner *et al.* 2014). In locusts (*Schistocerca gregaria*), the greatest density of octopamine receptors is found in optic lobes (Roeder and Nathanson 1993), and in horseshoe crabs (*Limulus spp.*) octopamine enhances dark-adaptation of ventral photoreceptors (O'Day and Lisman 1985). In *B. neritina* larvae, it is therefore possible that light entering the two photosensory ocelli stimulates a rise in octopamine that thereby triggers locomotory cilia to beat.

Results from this study, combined with earlier work investigating the effects of dopamine and serotonin on *B. neritina* larvae (Pires and Woollacott 1997), suggest that dopamine, serotonin, and octopamine may all be involved in the photosensory pathways of *B. neritina* larvae. Octopamine is synthesized from β -hydroxylation of tyramine, which is typically produced by decarboxylation of the amino acid tyrosine (Fig. 2). While this simple two-step process appears to be most common in invertebrates, tyramine can also be formed by dehydroxylation of dopamine in insects (Roeder 1999). If this alternative pathway is employed in *B. neritina* larvae, then dopamine exposure may result in elevated levels of octopamine, thereby leading to increased ciliary activity and an extended photopositive period.

4.5 Conceptual model of *B. neritina* larval settlement pathway

Previous evidence and the results from the current study were used to construct a conceptual model of some of the underlying pathways that may control *B. neritina* larval settlement behavior (Fig. 9). In this model, octopamine (likely released at the chemical synapses shared by intercoronal cells and cells of the ciliated corona) triggers an influx of calcium into ciliary cells, stimulating their activity and resulting in swimming behavior. Exogenous exposure to adrenoreceptor agonists like NE results in stimulation of

octopamine receptors, either *via* binding to receptors within sensory structures and stimulating a signaling cascade, or by permeating larval tissue and binding directly to octopamine receptors within neural tissue. Larvae therefore remain swimming and are thus inhibited from beginning the process of attachment to a substratum. Exogenous exposure to adrenoreceptor antagonists like phentolamine may block octopamine receptors, preventing larval swimming and causing larvae to attach more rapidly. Light exposure may naturally increase octopamine production within *B. neritina* larvae, stimulating increased ciliary activity and resulting in settlement inhibition. This may also help explain how light stimulates the release of larvae from the ciliated ovicells in which they are brooded, offering a mechanistic explanation for a phenomenon that has been observed and exploited in the laboratory for decades.

4.7 Conclusion

These investigations of *B. neritina* larvae offer insight into the underlying mechanisms controlling larval phototactic and settlement behaviors that have been reported for decades, but not fully understood. An enhanced understanding of the larval biology of marine invertebrates like *B. neritina* not only expands our knowledge of the evolution of larval settlement pathways across taxa, but can also aid in the development of new approaches to control biofouling and prevent the further spread of invasive species, which will benefit coastal ecosystems worldwide.

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Figure Captions

Figure 1. a) A lateral view of a *Bugula neritina* larva shows the ciliated corona (CC), which functions as the larval locomotory organ, and the sensory apical disc (AD), which is kept foremost during larval swimming and surface exploration. **b)** Larvae fixed with 4% paraformaldehyde can become distorted and drop cilia. The neuromuscular ring (NR) underlies the constricted region of the larval body. Two pigmented eyespots, or ocelli, (though not visible in this black and white image), lie just above the neuromuscular ring on the posterolateral side of the larval body (opposite the apical disc and pyriform groove). **c)** An apical view of a larva shows the ciliated corona and the sensory vibratile plume (VP). **d)** The vibratile plume extends from the pyriform groove, while the cells of the ciliated corona occur across most of the entire surface of the larval body.

Figure 2. Biosynthesis of some of the neuroactive compounds likely to be involved in controlling *B. neritina* larval behavior. Norepinephrine, an adrenergic receptor agonist, inhibits larval settlement in many marine invertebrates, including *B. neritina*. Phentolamine is a pharmaceutical compound that blocks adrenergic receptors. Octopamine is often considered the invertebrate equivalent of norepinephrine, and both monoamines can be synthesized from tyrosine and dopamine. Tyrosine hydroxylase is the enzyme that converts tyrosine to L-DOPA (the precursor to dopamine). Dopamine and serotonin influence *B. neritina* larval phototaxis.

Figure 3. Effects of norepinephrine and phentolamine on larval attachment over 8 h of treatment exposure. N=5. Error bars are standard errors of the means. Norepinephrine (NE) significantly reduced larval attachment at 10 μ M ($P=0.0297$) and 100 μ M ($P=0.0121$).

Figure 4. Effects of 10 μ M norepinephrine (NE) and 10 μ M phentolamine on larval swimming behavior after 2, 4, 6 and 8 h of exposure. N=5. Error bars are standard errors of the means. Asterisks denote significant differences from the control group. NE significantly increased swimming behavior at 2, 6, and 8 h ($P<0.001$, $P= 0.0105$, $P= 0.0234$, respectively), while phentolamine significantly decreased swimming behavior at 4, 6, and 8 h ($P=0.0058$, $P=0.0002$, $P<0.0001$, respectively).

Figure 5. *B. neritina* larva stained with BODIPY-FL Prazosin, a fluorescently labeled adrenergic receptor antagonist. Fluorescence was observed in: **a)** the apical disc, and **b)** the pyriform groove, suggesting that larvae possess adrenergic-like receptors in these regions.

Figure 6. a) *B. neritina* larva incubated with DAPI nuclear stain (blue), and anti-tyrosine hydroxylase (red). At the surface, tyrosine hydroxylase-like immunoreactivity localized to the apical disc (AD). **b)** An image taken deeper inside the larva shows tyrosine hydroxylase-like immunoreactivity (red) in the region of the neuromuscular ring (NR). **c)** Control larva incubated with only DAPI nuclear stain and secondary antibody. A 3D z-stack video of this specimen is provided in the supplemental materials. (*Color for both web and print.*)

Figure 7. a) *B. neritina* larva incubated with DAPI nuclear stain (blue), and anti-octopamine (red). At the surface, octopamine-like immunoreactivity localized to the apical disc (AD) and the ciliated corona (CC). **b)** An image from deeper inside the larva reveals octopamine-like immunoreactivity in the region of the pyriform groove (PG), which houses the vibratile plume. **c)** Control larvae incubated with only DAPI and secondary antibody. A 3D z-stack video of this specimen is provided in the supplemental materials. (*Color for both web and print.*)

Figure 8. Effects of light and phentolamine on larval attachment. N=3. Error bars are standard errors of the mean. Light significantly reduced larval attachment in control larvae at each timepoint. The effect of light was significantly diminished by phentolamine (an adrenergic receptor blocker) after 3 h of exposure, suggesting that adrenergic-like receptors play a role in the photosensory pathway.

Figure 9. Putative settlement pathway in *B. neritina* larvae. Light stimulates octopamine production, which thereby increases intracellular levels of Ca^{2+} and causes larval cilia to beat. Beating cilia result in swimming or spinning behavior, which prevents larvae from entering into a necessary period of quiescence prior to attachment. Norepinephrine may bind octopamine receptors, stimulating ciliary activity, while phentolamine may block octopamine receptors, preventing larval swimming.

Supplementary Video Captions

Supplementary Video 1. Animated 3D z-stack of a *B. neritina* larva incubated with DAPI nuclear stain (blue), and anti-tyrosine hydroxylase (red). At the surface, tyrosine hydroxylase-like immunoreactivity localized to the apical disc (AD). Images taken deeper inside the larva reveal tyrosine hydroxylase-like immunoreactivity (red) in the region of the neuromuscular ring (NR). (Should be viewed in color.)

Supplementary Video 2. Animated 3D z-stack of a *B. neritina* larva incubated with DAPI nuclear stain (blue), and anti-octopamine (red). At the surface, octopamine-like immunoreactivity localized to the apical disc (AD) and the ciliated corona (CC). **b)** Images deeper inside the larva reveal octopamine-like activity in the region of the pyriform groove (PG), which houses the vibratile plume. (Should be viewed in color.)

Figure

Figure

Tryptophan $\longrightarrow$ 5-HTP $\longrightarrow$ Serotonin (5-HT)

Tyrosine $\longrightarrow$ Tyramine $\longrightarrow$ Octopamine

*Tyrosine-
hydroxylase*

L-DOPA $\longrightarrow$ Dopamine $\longrightarrow$ Noradrenaline

Phentolamine

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