

INVESTIGATION OF LARVAL SENSORY SYSTEMS IN THE MARINE  
BRYOZOAN, *BUGULA NERITINA*

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## ABSTRACT

Investigation of larval sensory systems in the marine bryozoan, *Bugula neritina*

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*Bugula neritina* is a sessile marine bryozoan with a pelagic larval stage. Larvae frequently settle on boat hulls, facilitating the introduction of *B. neritina* to bays and estuaries worldwide. Adrenergic agonists, such as the vertebrate hormone noradrenaline, inhibit larval settlement in a variety of marine invertebrate species, including *B. neritina*. Light also inhibits *B. neritina* larval settlement, yet the underlying mechanisms by which light and adrenergic compounds exert their effects on larvae are not well understood. Octopamine is considered the invertebrate analog of noradrenaline, and may be an endogenous hormone involved in larval settlement pathways. I observed the effects of the adrenergic agonist noradrenaline and the adrenergic antagonist phentolamine on larval settlement, and found that high concentrations of noradrenaline increased larval mortality, inhibited larval attachment, and increased larval swimming behavior. High concentrations of phentolamine also increased larval mortality, but increased larval attachment and decreased larval swimming behavior. I used fluorescent labeling and microscopy to localize sensory system components, and found that larvae possess adrenergic-like receptors, as well as tyrosine hydroxylase-like and octopamine-like immunoreactivity. I also exposed larvae to phentolamine in both dark and light conditions, and found that light significantly inhibited larval attachment, but phentolamine blocked those inhibitory effects. These results suggest that *B. neritina* larvae possess adrenergic-like receptors, which serve as the binding sites for noradrenaline and phentolamine. These are likely octopamine receptors, and octopamine may be one endogenous compound involved in controlling larval phototaxis and settlement behavior. Light may increase octopamine production, thereby stimulating ciliary activity, extending swimming behavior, and preventing larvae from attaching to a substrate. This research sheds light on previously unknown sensory mechanisms in *B. neritina* larvae, and may aid in the development of new biofouling control strategies.

Keywords: *Bugula neritina*, larval settlement, larval phototaxis, biofouling, noradrenaline, octopamine



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## Chapter I. Introduction

*Bugula neritina* (Linnaeus 1758) is a sessile marine bryozoan with a pelagic larval stage, found in warm-temperate and subtropical bays and estuaries worldwide (Ryland *et al.* 2011). *B. neritina* larvae frequently attach to boat hulls, and the species is regarded as one of the most widespread fouling bryozoans (Winston and Woollacott 2008). DNA sequencing of the mitochondrial gene cytochrome c oxidase I (COI) 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 a cosmopolitan distribution was reported for *B. neritina* as early as the 18<sup>th</sup> century (Winston and Woollacott 2008), and its range 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 larval sensory mechanisms 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.

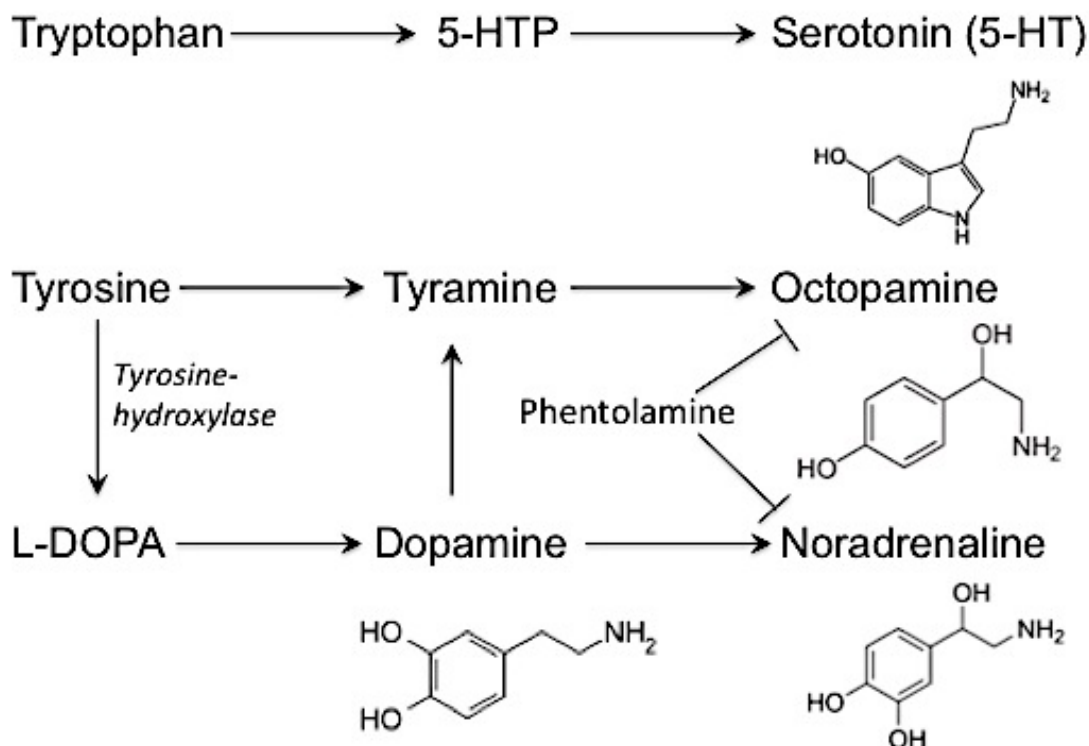
Many aspects of reproduction and development in *B. neritina* are well documented (e.g., Lynch 1947, Woollacott and Zimmer 1971). Adult colonies are comprised of branching, hermaphroditic zooids, and are typically brown to dark



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). 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 is a ciliated structure located at the narrower end of the body, surrounded by a crown of rigid cilia and the circular pallial furrow (Fig 10). The vibratile plume is another larval sensory structure, which consists of three long flagella that extend from the glandular pyriform groove (Fig. 10). 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 will then halt for a brief moment prior to eversion of the internal sac, at which point metamorphosis is initiated and the animal is permanently attached to the substratum. The newly attached morph consists of the progenitor zooid, or ancestrula, which 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 much is known about *B. neritina* larval anatomy and behavior, the underlying sensory pathways that control larval settlement remain largely

unknown. Previous research documenting the effects of fouling-deterrent compounds on larval settlement of marine invertebrates can provide insight into these pathways. Adrenergic compounds, such as the vertebrate hormone noradrenaline, inhibit larval settlement in a variety of marine invertebrates, including *B. neritina* (e.g., Gohad *et al.* 2012, Shimizu *et al.* 2000). Noradrenaline (NA) is a monoamine that binds to vertebrate adrenergic receptors and exerts a range of stimulatory effects on the sympathetic nervous system, including increased heart rate, release of glucose to the bloodstream, and increased blood flow to skeletal muscle. The underlying mechanism by which NA exerts its effects on larval settlement in marine invertebrates is not well understood, but one study on barnacle (*Balanus amphitrite*) cyprid larvae revealed the presence of adrenergic-like receptors in sensory setae on antennules. These adrenergic-like receptors may be the binding sites for NA and other adrenergic compounds (Gohad *et al.* 2012). Octopamine is considered the invertebrate analog of NA and the two compounds only differ structurally by the addition of a hydroxyl group to the benzene ring in NA (Fig. 1).



**Figure 1.** Octopamine is often considered the invertebrate equivalent of noradrenaline, and both can be synthesized from tyrosine and dopamine. Tyrosine hydroxylase is the enzyme that converts tyrosine to L-DOPA (the precursor to dopamine). Phentolamine is a pharmaceutical compound that blocks adrenergic receptors.

Octopamine regulates a variety of physiological and behavioral processes ranging from locomotion to photosensitivity in phylogenetically diverse invertebrates (Roeder 1999). Octopamine receptors have therefore been proposed as binding sites for NA and other adrenergic compounds in invertebrates (Wendt *et al.* 2013). To our knowledge, this is the first study to investigate the presence and endogenous role of octopamine in a bryozoan. In *B. neritina*, octopamine receptors may be the binding sites for NA and other 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 are photopositive upon release, but switch to become photonegative within several hours (Lynch 1943, Wendt and Woollacott 1999). As they move away from light, larvae begin the process of surface exploration that occurs 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 sensory 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, and its metabolic precursor 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).

The metabolic pathways that involve dopamine are well studied in vertebrates. 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 NA and octopamine (Fig 1). 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, noradrenaline, and the adrenergic antagonist, phentolamine, to investigate the effects of these compounds on larval attachment, behavior, and mortality. Fluorescence microscopy was also used to determine the presence and location of adrenergic-like receptors, octopamine, and tyrosine hydroxylase. Additionally, larvae were exposed to light in the presence and absence of phentolamine to observe their combined effects on larval attachment and to gain knowledge of the underlying photosensory pathway.

We hypothesized that: 1. Exposure to noradrenaline inhibits *B. neritina* larval attachment, while exposure to phentolamine induces larvae to settle more rapidly, 2. *B. neritina* larvae possess adrenergic-like receptors, which serve as the binding sites for noradrenaline, phentolamine, and other adrenergic-like compounds, 3. *B. neritina* larvae possess endogenous octopamine, as well as the tyrosine-hydroxylase enzyme, which are located in regions involved in the underlying pathway controlling larval settlement behavior, 4. Larvae exposed to phentolamine and light simultaneously will have elevated levels of settlement as compared to those only exposed to light.

## Chapter II. Materials and methods

### *Larval collection*

*Bugula neritina* colonies 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, and given no exogenous food source. In order 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.

### *Effects of noradrenaline and phentolamine on larval settlement behavior*

To observe the effects of NA (an adrenoreceptor agonist) and phentolamine (an adrenoreceptor antagonist) on larval behavior and attachment, larvae were exposed to varying concentrations of each compound in seawater and observed with a Leica EZ4D dissecting microscope. DL-Noradrenaline 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).

Live 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 filtered seawater containing 0 (control), 0.1, 1.0, 10, or 100  $\mu$ M of either NA or phentolamine.

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 12 larvae per treatment per trial. Five trials were conducted with both compounds (on the 15, 17, 22, 24, and 29 of April, 2014), for a total of 600 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. Larvae were kept at 11°C for the duration of the experiments, except during observation. Notes were also taken on larval behavior prior to attachment, and unattached larvae were classified as swimming, spinning, or dead.

#### *Effects of noradrenaline and phentolamine on larval mortality*

To further determine whether experimental exposure to exogenous NA and phentolamine solutions had an impact on larval mortality, larvae were exposed to concentrations of 0 (control), 10, or 100  $\mu$ M of NA or phentolamine in 65 mm diameter petri dishes. These concentrations were based on results from unpublished pilot experiments, and petri dishes were used to minimize larval mortality that may have occurred due to smaller wells of cell culture plates.) Two trials were conducted, with 10 larvae per treatment per trial, for a total of 60

larvae. The number of dead larvae was recorded at 24, 48, and 72 h of exposure. Mortality was assessed by lack of ciliary movement in unattached larvae, and discoloration and/or termination of metamorphosis in attached larvae.

#### *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  $\mu$ M BODIPY-FL Prazosin, a fluorescently labeled non-subtype selective  $\alpha$ -adrenergic receptor antagonist obtained from Life Technologies (Foster City, CA, USA). The solution was made up in filtered seawater (FSW) and larvae were incubated for 30 min prior to being washed three times in FSW. Larvae were then imaged on an Olympus (Center Valley, PA, USA) BX53 compound fluorescent microscope with a DP73 camera, using the 488 nm laser line and Olympus CellSens software. Unstained larvae were used to control for autofluorescence.

#### *Localization of anti-octopamine and anti-tyrosine hydroxylase-like immunoreactivity*

To determine the presence and location of tyrosine-hydroxylase-like and octopamine-like activity, *B. neritina* larvae were fixed in 4% paraformaldehyde made up in phosphate-buffered saline (PBS) for at least 2 h, then washed once in PBS. Fixed larvae were then permeabilized overnight in 0.5% Triton X-100 in PBS, and then incubated in 2% bovine serum albumin (BSA) for 4 h. BSA was pipetted off, and larvae were incubated in either anti-tyrosine hydroxylase primary antibody (1:500) or 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. DAPI was added at a 1:500 concentration and left for 15 min, prior to washing four times (5 min each) in 0.1% Triton-X in PBS. Larvae were then imaged in PBS with an Olympus FV1000 Scanning Laser Confocal Microscope using Fluoview imaging software. Unstained larvae and larvae labeled with only secondary antibodies were used as controls for autofluorescence and unspecific binding, respectively. *Drosophila* larval nervous tissue was also used as a positive control.

#### *Combined effects of light and phentolamine on larval attachment*

To investigate underlying photosensory mechanisms in *B. neritina* larvae, larval attachment rates were compared between the following groups: 1. larvae immersed in 100  $\mu$ M phentolamine and exposed to light, 2. larvae immersed in 100  $\mu$ M phentolamine and kept in the dark, 3. larvae in FSW exposed to light, and 4. larvae in FSW kept in the dark. Larvae were collected from multiple colonies, pooled, and randomly transferred to 15 ml falcon tubes containing either FSW (control) or 100  $\mu$ M phentolamine in FSW (treatment). 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 10 larvae per treatment per trial, for a total of 120 larvae.

#### *Statistical Analyses of Larval Assays*

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 at each time point. 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 statistical analyses. All analyses were conducted using JMP Pro 11 software (SAS, Cary, North Carolina, USA).

## Chapter III. Results

### *Effects of noradrenaline and phentolamine on larval attachment*

Binomial logistic regression revealed that noradrenaline (NA) significantly inhibited *B. neritina* larval attachment at 10  $\mu\text{M}$  ( $P=0.0297$ ) and 100  $\mu\text{M}$  ( $P=0.0121$ ) concentrations, but had no significant effect at 0.1 or 1.0  $\mu\text{M}$  (Fig. 2). This analysis showed that the effects of phentolamine on larval attachment were not significant at any concentration. However, a greater percentage of larvae in the 10 and 100  $\mu\text{M}$  treatments attached, while a smaller percentage of larvae in the 0.1 and 1.0  $\mu\text{M}$  concentrations attached, compared with the control group (Fig 3). *Post hoc* pairwise comparisons (Tukey's HSD) revealed significant differences between the control group and 100  $\mu\text{M}$  NA 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  $\mu\text{M}$  NA at 2 h of exposure ( $P=0.0190$ ); and between 100  $\mu\text{M}$  NA and 100  $\mu\text{M}$  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. 4).

### *Effects of noradrenaline and phentolamine on larval settlement behavior*

We examined larval behavior at 2, 4, 6, and 8 h of exposure to either NA or phentolamine, and classified each larva into one of four categories at each time point: swimming, spinning, attached, or dead. Nominal logistic regression and Cox Proportional Hazards risk ratios were used to compare each treatment to the control at every time point. Over the 8 h of sampling, significantly more larvae in 10 and 100  $\mu\text{M}$  NA treatments were swimming and spinning, while

significantly less larvae in the 10 and 100  $\mu\text{M}$  phentolamine treatments were swimming (Figs. 5, 6, 7 and 8).

#### *Effects of noradrenaline and phentolamine on larval mortality*

Noradrenaline significantly increased larval mortality over a 72 h period at concentrations of both 10  $\mu\text{M}$  ( $P<0.0001$ ) and 100  $\mu\text{M}$  ( $P<0.0001$ ). Phentolamine significantly increased larval mortality over a 72 h period at 100  $\mu\text{M}$  ( $P<0.0001$ ) (Fig. 9).

#### *Localization of adrenergic-like receptors*

Brightfield images of fixed, unstained *B. neritina* larvae provide close up views of the ciliated corona and vibratile plume and can serve as a reference for fluorescent images (Fig. 10). Strong fluorescent signals in larvae stained with BODIPY-FL Prazosin indicate that *B. neritina* larvae do possess adrenergic-like receptors, which appear to be concentrated in the apical disc, the pallial furrow, and the pyriform groove. (Fig. 11). No fluorescence was observed in control larvae that were not stained with BODIPY-FL Prazosin.

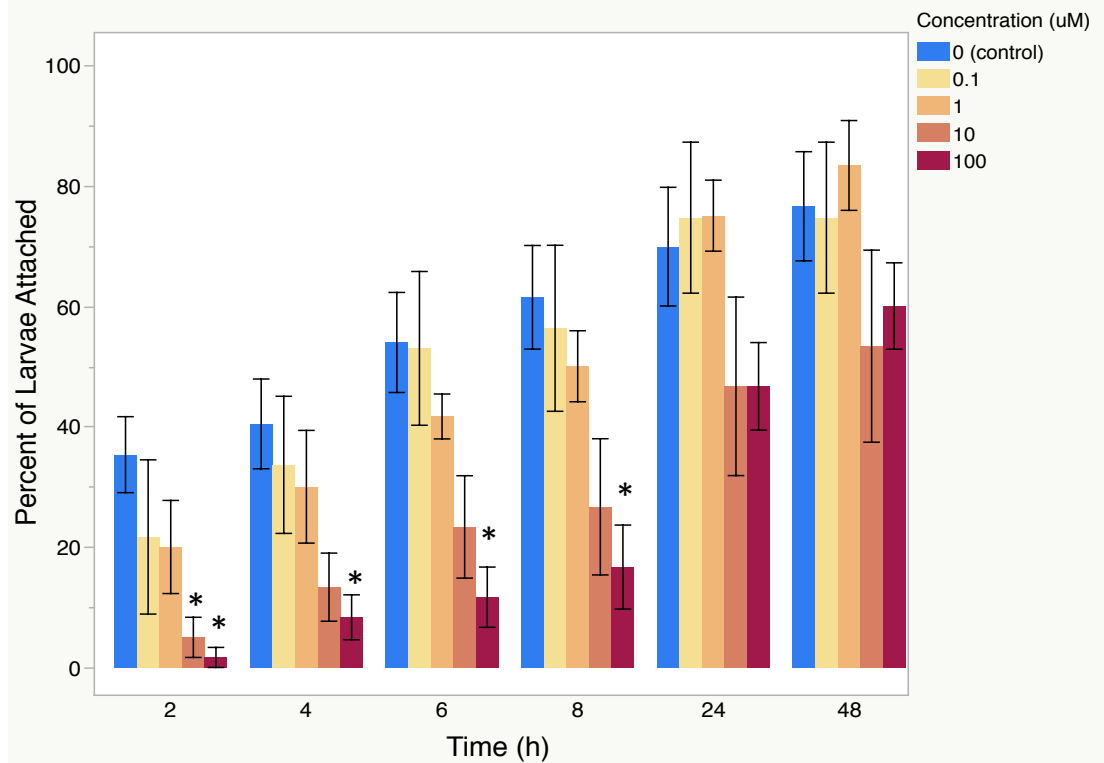
#### *Localization of anti-octopamine and anti-tyrosine hydroxylase-like immunoreactivity*

Both tyrosine hydroxylase-like and octopamine-like immunoreactivity were detected in *B. neritina* larvae, though distortion of the larvae made determination of the precise locations of these substances difficult. Tyrosine hydroxylase-like activity appeared to be concentrated in the apical disc, and in networks leading from the apical disc and the ciliated corona to the neuromuscular ring (Fig. 12). Octopamine-like activity appeared to be most prevalent in the apical disc, the

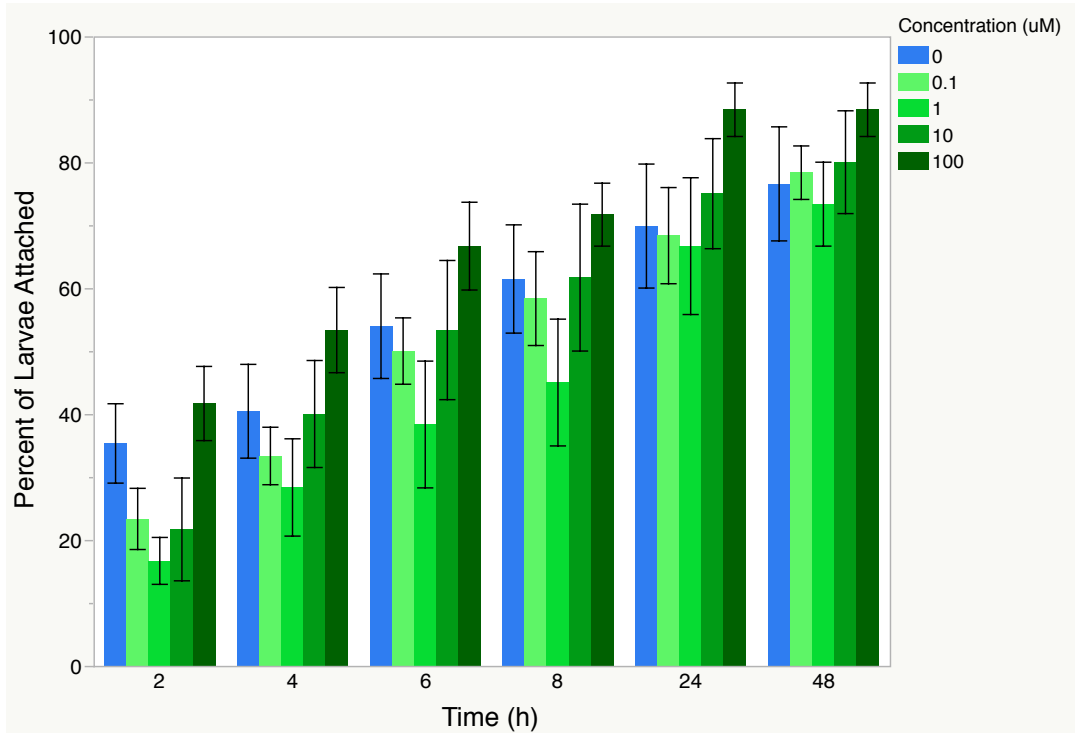
ciliated corona, and the pyriform groove (Fig. 13). No fluorescence was observed in control larvae that were not stained with primary antibodies. Our positive control, *Drosophila melanogaster* larval nervous tissue, exhibited both tyrosine hydroxylase-like and octopamine-like immunoreactivity.

*Combined effects of light and phentolamine on larval attachment*

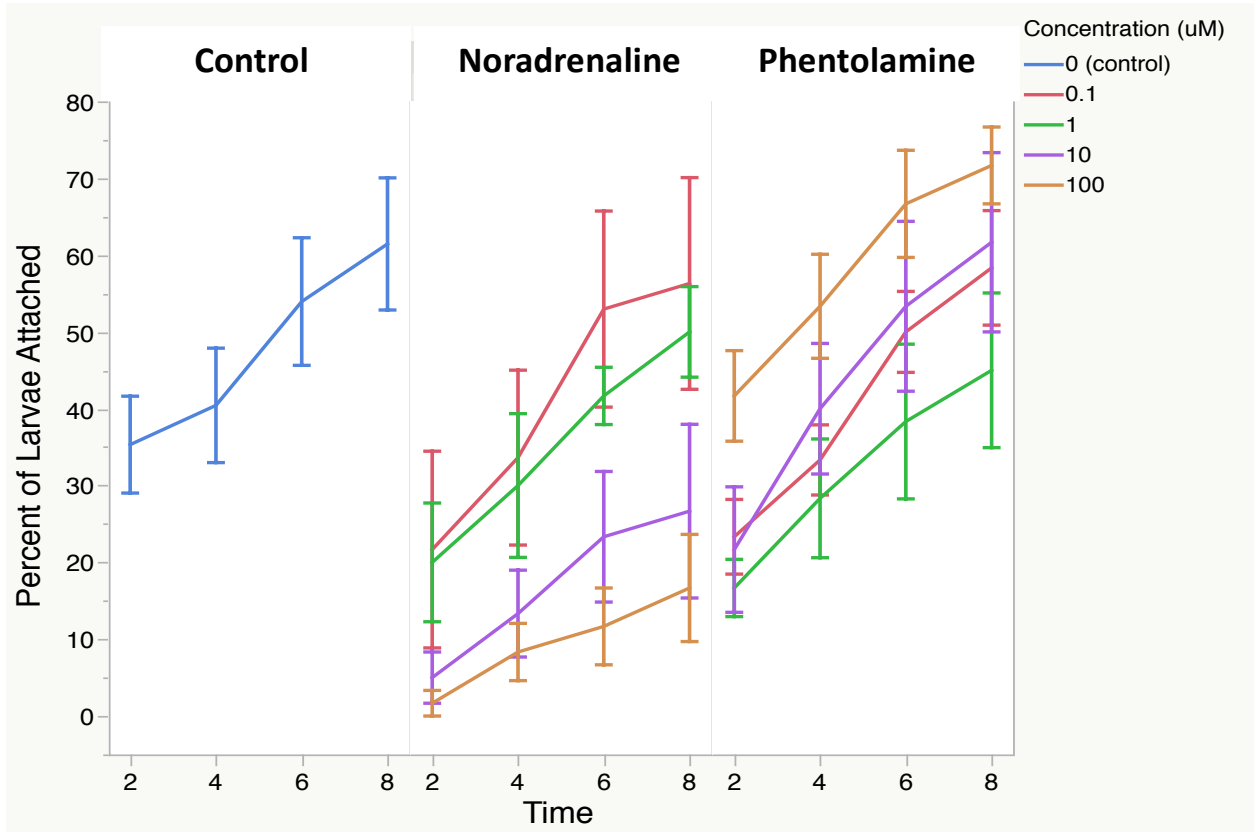
Light exposure significantly inhibited larval attachment at the 1 h, 2 h, and 3 h exposure time points ( $P < 0.0001$ ). There was a significant interaction between light and phentolamine after three hours of exposure ( $P = 0.02$ ), and the inhibitory effects of light were significantly diminished in larvae exposed to 100  $\mu\text{M}$  phentolamine. Phentolamine-exposed larvae in dark conditions also 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 (Fig. 14).



**Figure 2.** Effects of noradrenaline on *B. neritina* larval attachment over a 48 h period. Sample size was  $n=5$  replicate trials, with 12 larvae per treatment per trial, for a total of 60 larvae per treatment. Error bars represent standard errors of the means. NA significantly inhibited larval attachment at 10  $\mu\text{M}$  ( $P=0.0297$ ) and 100  $\mu\text{M}$  ( $P=0.0121$ ) concentrations. Asterisks denote significant pairwise differences from the control, determined by Tukey's HSD test.

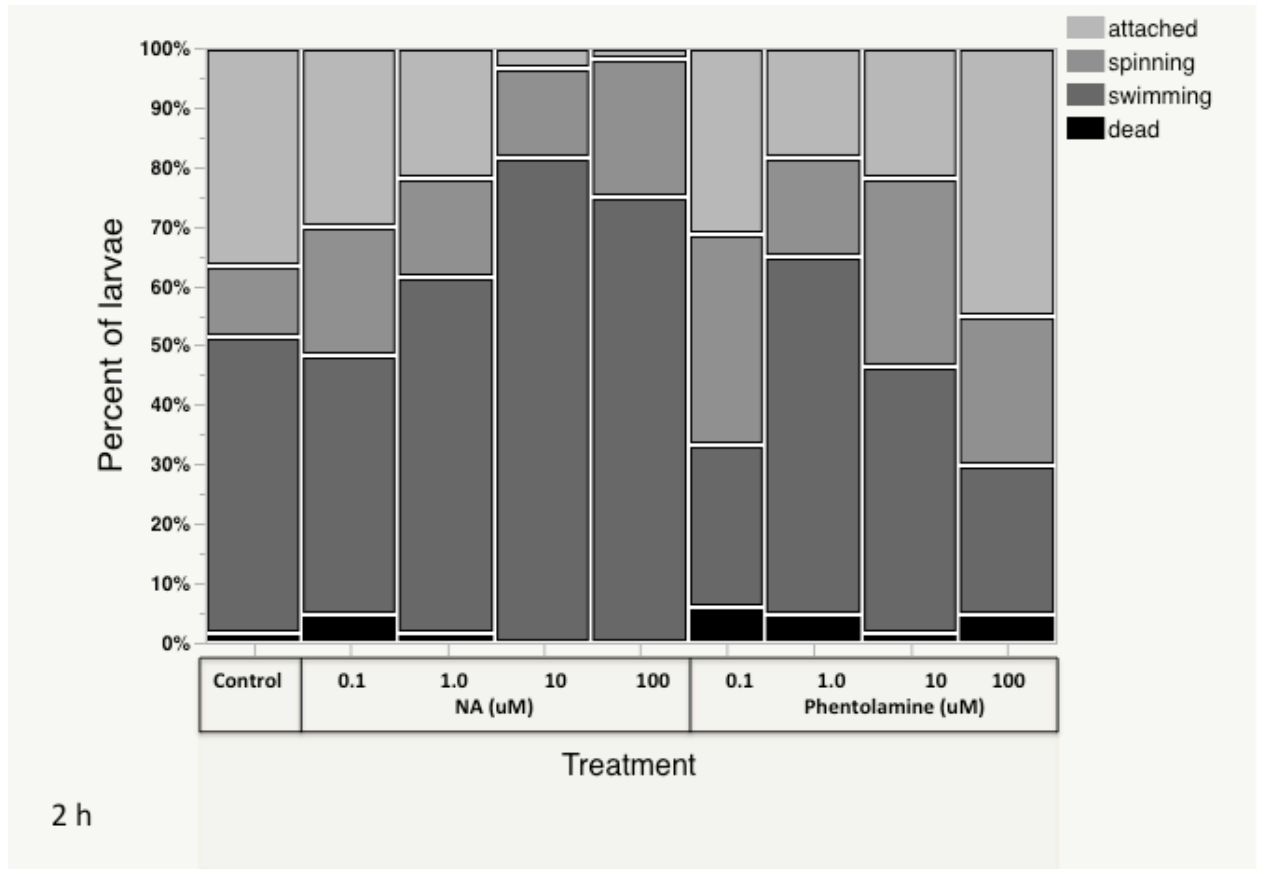


**Figure 3.** Effects of phentolamine on *B. neritina* larval attachment over 48 h period. Sample size was  $n=5$  replicate trials, with 12 larvae per treatment per trial, for a total of 60 larvae per treatment. Error bars represent standard errors of the means. There were no significant differences between treatments and control, but there was a trend of higher attachment in the 100  $\mu$ M concentration, suggesting that phentolamine may induce larval attachment.

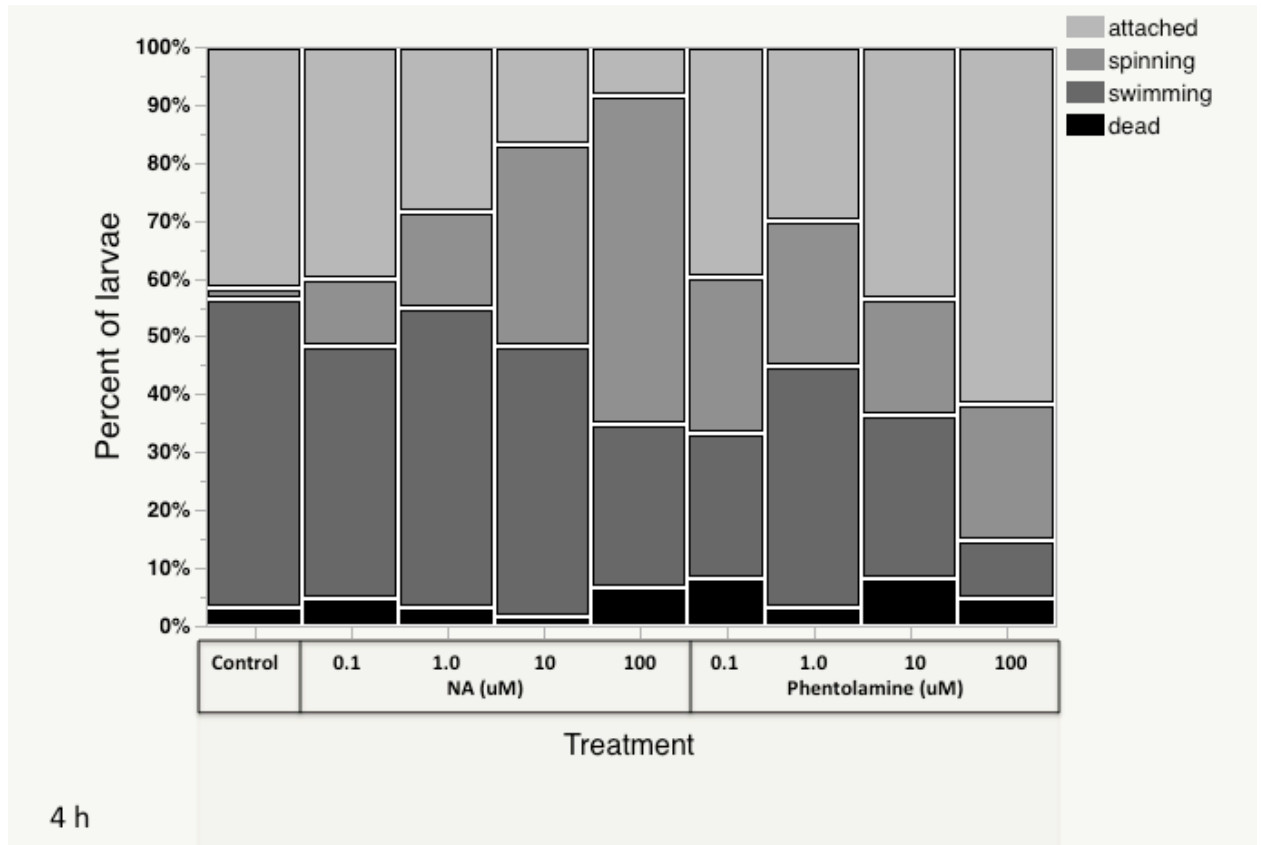


**Figure 4.** Effects of noradrenaline and phentolamine on larval attachment over first 8 h of exposure. Sample size was  $n=5$  replicate trials, with 12 larvae per treatment per trial, for a total of 60 larvae per treatment. Error bars represent standard errors of the means. NA and phentolamine had opposite effects on larval attachment at 100  $\mu\text{M}$  concentration at 2 h ( $P=0.0008$ ), 4 h ( $P=0.0006$ ), 6 h ( $P=0.0003$ ), and 8 h ( $P=0.0010$ ) of exposure.

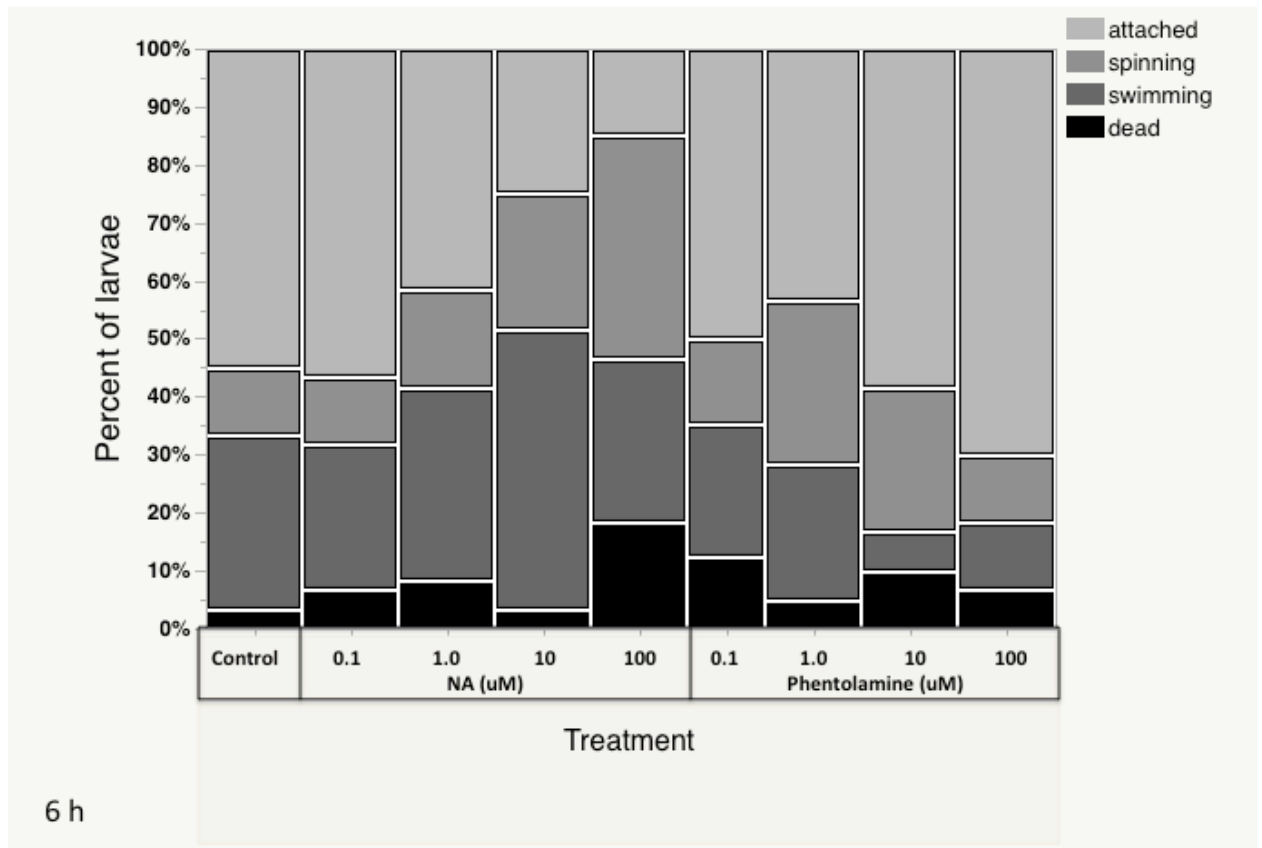




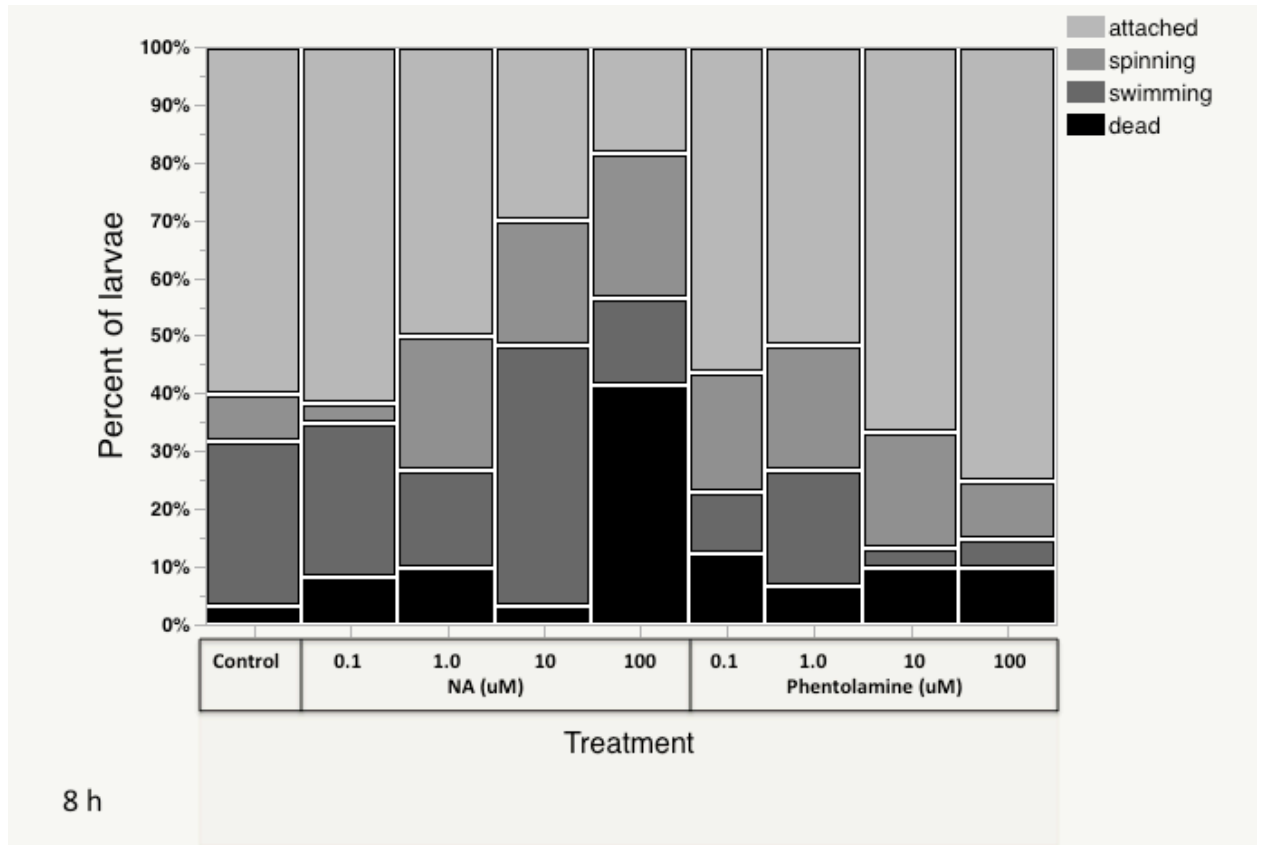
**Figure 5.** Larval behavior after 2 h of exposure to varying concentrations of noradrenaline (NA) or phentolamine. Sample size was  $n=5$  replicate trials, with 12 larvae per treatment per trial, for a total of 60 larvae per treatment. Significantly more larvae were swimming in 10  $\mu\text{M}$  ( $P<0.0001$ ) and 100  $\mu\text{M}$  ( $P=0.0027$ ) concentrations of NA compared to the control group. Significantly fewer larvae were swimming in 0.1  $\mu\text{M}$  ( $P=0.0097$ ) and 100  $\mu\text{M}$  ( $P<0.0001$ ) concentrations of phentolamine.



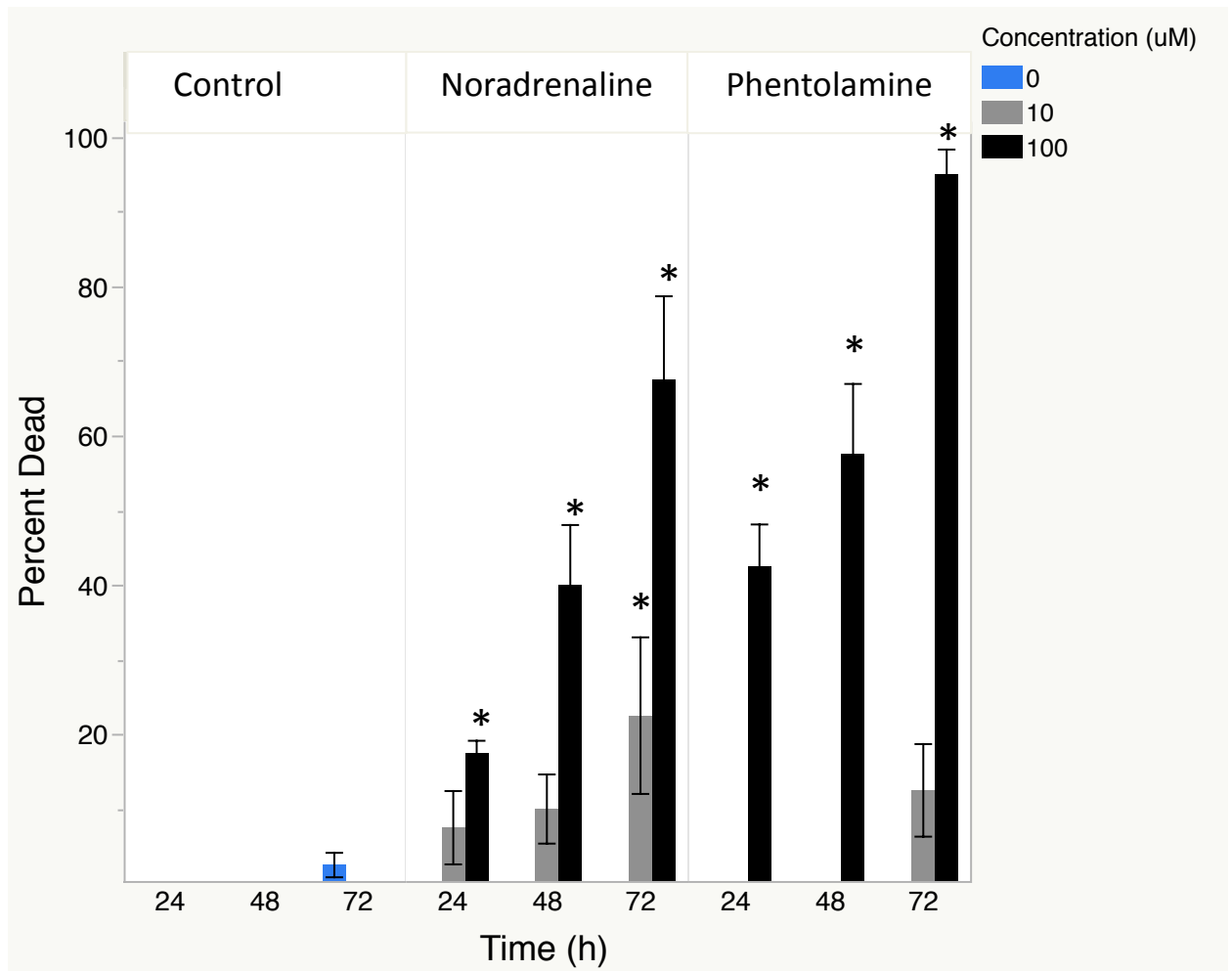
**Figure 6.** Larval behavior after 4 h of exposure to varying concentrations of noradrenaline (NA) or phentolamine. Sample size was  $n=5$  replicate trials, with 12 larvae per treatment per trial, for a total of 60 larvae per treatment. Significantly more larvae were spinning in 10 and 100  $\mu\text{M}$  NA treatments ( $P<0.0001$ ), and significantly more larvae were spinning in all phentolamine treatments ( $P<0.0132$ ).



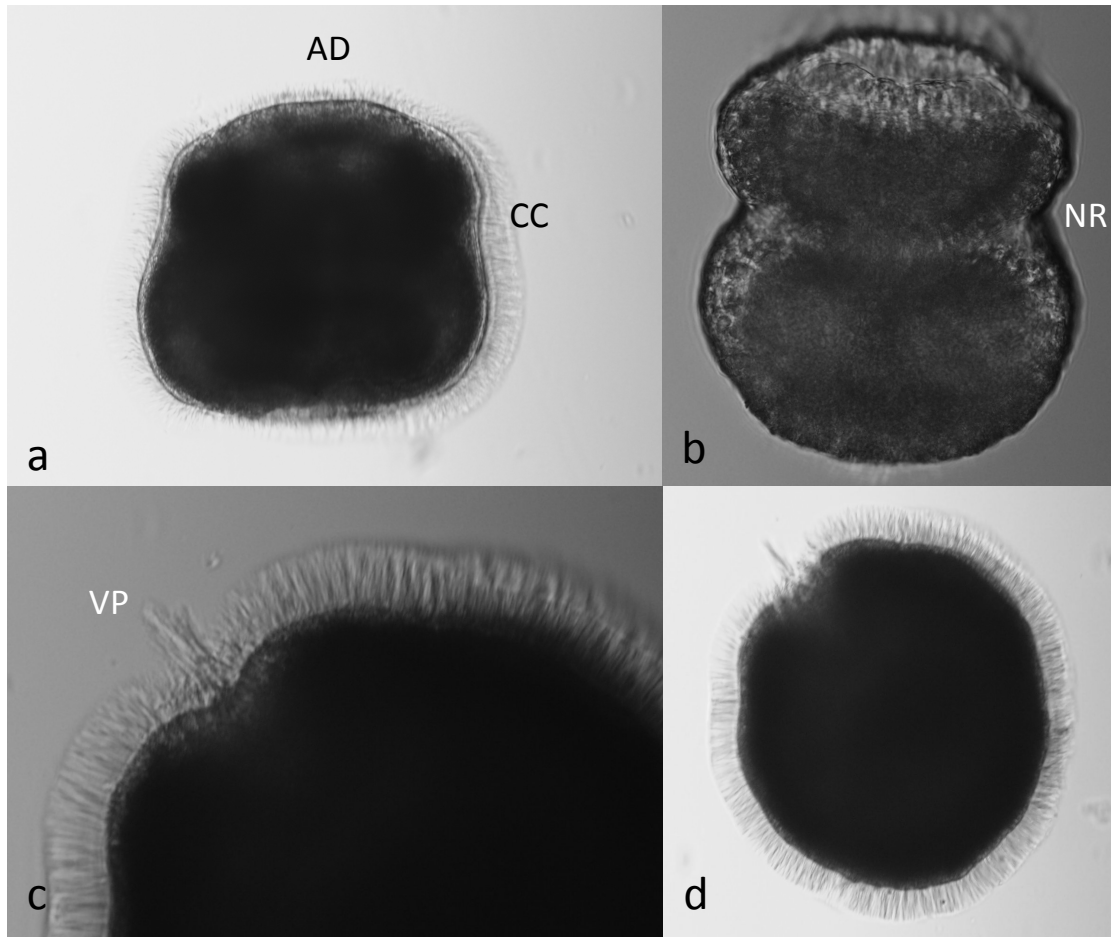
**Figure 7.** Larval behavior after 6 h of exposure to varying concentrations of noradrenaline (NA) or phentolamine. Sample size was  $n=5$  replicate trials, with 12 larvae per treatment per trial, for a total of 60 larvae per treatment. Significantly more larvae were swimming in 10  $\mu\text{M}$  NA ( $P=0.0105$ ), while significantly more larvae were spinning in 100  $\mu\text{M}$  NA ( $P=0.0002$ ). Significantly fewer larvae were swimming in 10  $\mu\text{M}$  ( $P=0.0002$ ) and 100  $\mu\text{M}$  ( $P=0.0056$ ) concentrations of phentolamine.



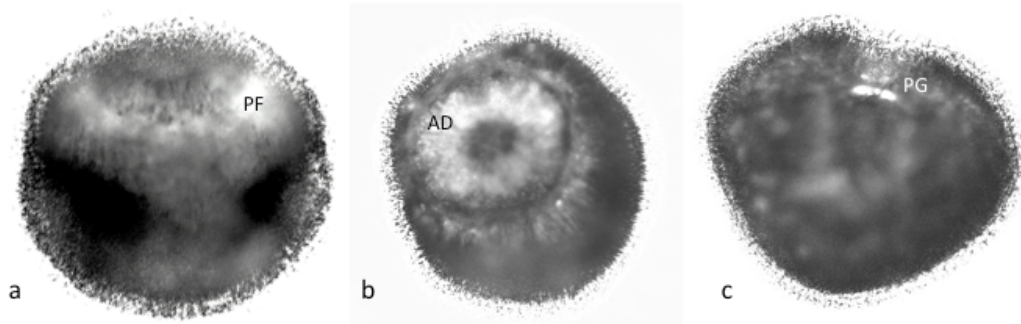
**Figure 8.** Larval behavior after 8 h of exposure to varying concentrations of noradrenaline (NA) or phentolamine. Sample size was  $n=5$  replicate trials, with 12 larvae per treatment per trial, for a total of 60 larvae per treatment. Significantly more larvae remained swimming in 10  $\mu\text{M}$  NA ( $P=0.0234$ ), while significantly more larvae remained spinning in 100  $\mu\text{M}$  NA ( $P=0.0013$ ). Significantly fewer larvae remained swimming in 10  $\mu\text{M}$  and 100  $\mu\text{M}$  phentolamine ( $P<0.0001$ ).



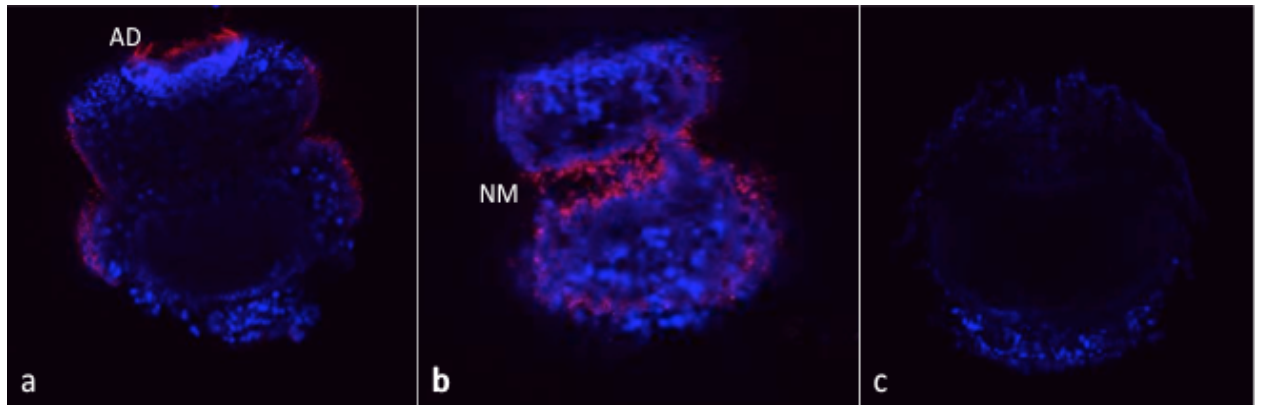
**Figure 9.** Effects of noradrenaline (NA) and phentolamine on *B. neritina* larval mortality over a 72 h period. Sample size was  $n=4$  replicate trials, with 10 larvae per treatment per trial, for a total of 40 larvae per treatment. Error bars represent standard errors of the means. Asterisks denote significant differences from the control group. NA significantly increased larval mortality over a 72 h period at concentrations of both 10  $\mu\text{M}$  ( $P<0.00001$ ) and 100  $\mu\text{M}$  ( $P<0.00001$ ). Phentolamine significantly increased larval mortality over a 72 h period at 100  $\mu\text{M}$  ( $P<0.00001$ ).



**Figure 10.** Brightfield images of *B. neritina* larvae taken with an Olympus BX53 microscope and XM10 camera using CellSens software. **a.** Barrel-shaped larvae are surrounded by a ciliated corona (CC), which acts as larval locomotory organ. The image was taken from the lateral view, with the apical disc (AD) upmost. **b.** Larvae fixed with 4% paraformaldehyde become distorted and often drop cilia. The neuromuscular ring (NR) underlies the constricted region. **c.** This image taken from the apical view offers a closer view of the ciliated corona and the vibratile plume (VP). **d.** The vibratile plume extends from the pyriform groove, and the ciliated corona is distributed across the surface of the larva.

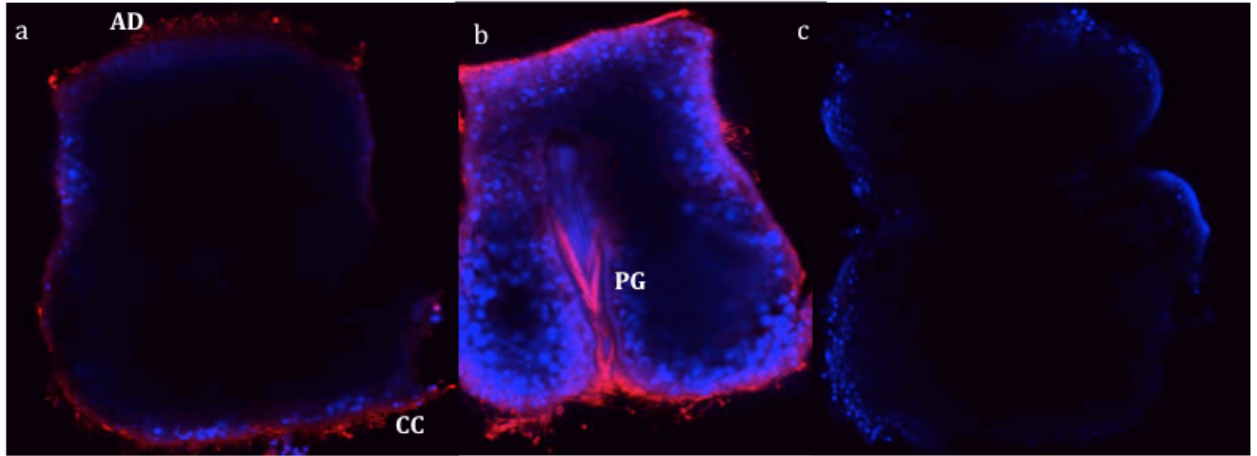


**Figure 11.** *B. neritina* larva stained with BODIPY-FL Prazosin, a fluorescently labeled adrenergic receptor antagonist. Fluorescence was observed in **a)** the region of the pallial furrow, **b)** in the region of the apical disc, and **c)** in the pyriform groove beneath the vibratile plume, suggesting that larvae possess adrenergic-like receptors in each of these regions. Images were taken with the 20X objective on an Olympus BX53 microscope and DP73 camera using CellSens software and a 488 nm laser line and converted to gray scale.

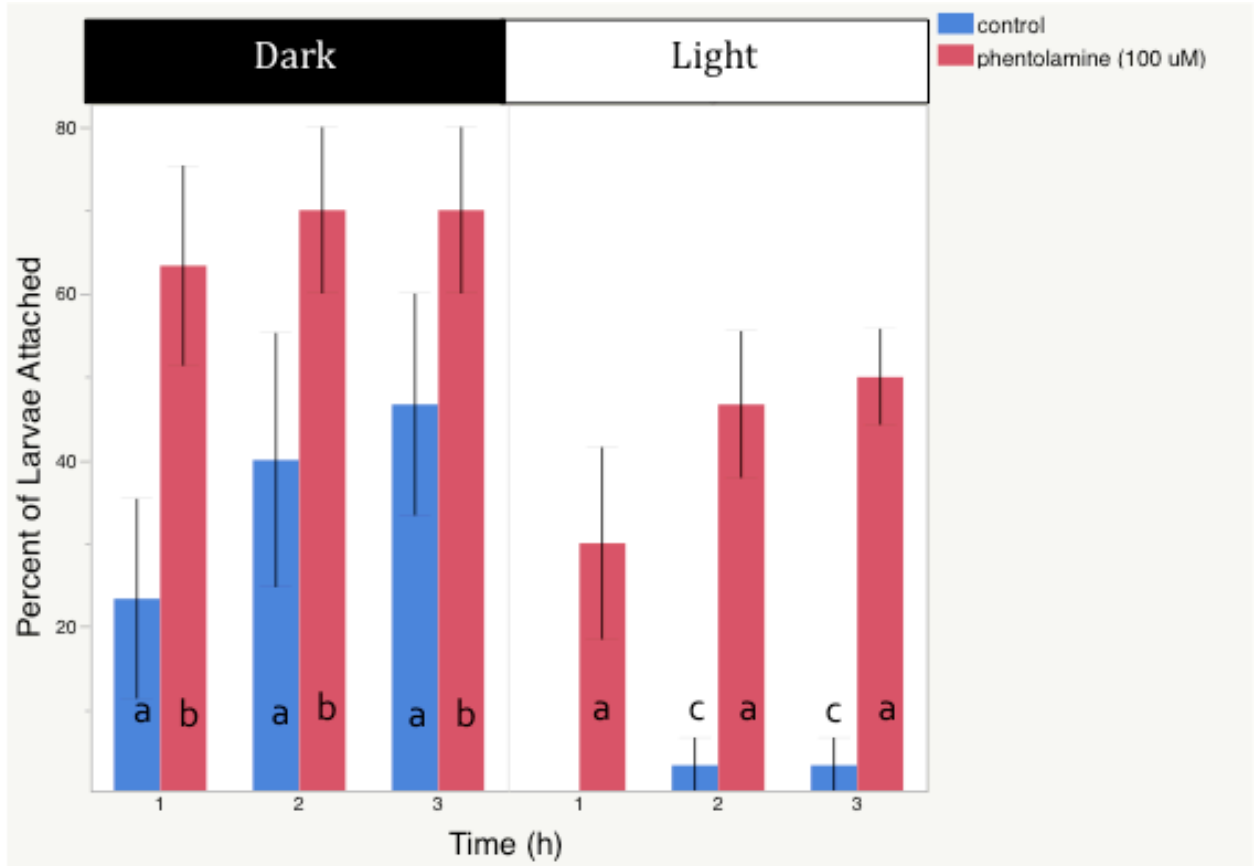


**Figure 12. a)** *B. neritina* larva stained with DAPI nuclear stain (blue), and anti-tyrosine hydroxylase (red). Tyrosine hydroxylase-like immunoreactivity localizes to the apical disc region (AD). **b)** Image taken deeper inside the larva shows tyrosine hydroxylase-like immunoreactivity (red) in the region of the neuromuscular ring (NM). **c)** Control larva stained with only DAPI nuclear stain and secondary antibody. All images were taken on an Olympus FV1000 Scanning Laser Confocal Microscope using Fluoview imaging software and the 20X objective. A 405 nm laser line at 8% transmissivity was used for DAPI excitation (blue), and a 559 nm laser line at 12% transmissivity was used for Alexafluor-568 excitation (red).





**Figure 13. a)** *B. neritina* larva stained with DAPI nuclear stain (blue), and anti-octopamine (red). Octopamine-like immunoreactivity localizes to the regions of the apical disc (AD) and the ciliated corona (CC). **b)** A frame from deeper inside the larva reveals octopamine-like activity in the region of the pyriform groove (PG), which houses the vibratile plume. **c)** Control larvae stained with only DAPI and secondary antibody. All images were taken on an Olympus FV1000 Scanning Laser Confocal Microscope using Fluoview imaging software and the 20X objective. A 405 nm laser line at 8% transmissivity was used for DAPI excitation (blue), and a 559 nm laser line at 30% transmissivity was used for Alexafluor-594 excitation (red).



**Figure 14.** Effects of light and phentolamine on larval attachment. Sample size of  $n=3$  replicate trials, with 10 larvae per treatment per trial, for a total of 30 larvae per treatment. Error bars represent standard errors of the mean. Different letters represent groups that are significantly different from each other. Light significantly inhibited larval attachment in control larvae at each timepoint ( $P<0.0001$ ). The effect of light was significantly diminished by phentolamine (an adrenergic receptor blocker) after 3 h of exposure ( $P=0.02$ ), suggesting that adrenergic-like receptors play a role in the photosensory pathway.

## Chapter IV. Discussion

### *Effects of noradrenaline and phentolamine on larval attachment, behavior, and mortality*

Our results confirm previous reports of the inhibitory effects of NA on the larval settlement of marine invertebrates (e.g. Shimizu *et al.* 2000, Gohad *et al.* 2012). *B. neritina* larval attachment was significantly hindered by 10 and 100  $\mu\text{M}$  concentrations of the adrenergic agonist, an effect that was detected at the first time sampling point of 2 h, and lasted up to 8 h (Fig 2). Both 10 and 100  $\mu\text{M}$  concentrations of NA increased larval mortality over a 72 h period, and even larvae that successfully attached often died shortly thereafter (Fig. 9).

Although not statistically significant, phentolamine exhibited a seemingly biphasic effect on larval attachment based on dose concentration, in that the 1  $\mu\text{M}$  concentration inhibited attachment, while the 100  $\mu\text{M}$  concentration increased attachment compared to the control (Fig. 4). The 100  $\mu\text{M}$  concentration of phentolamine also increased larval mortality over a 72 h period, and most larvae died shortly after attachment (Fig. 9).

In a separate experiment, larvae exposed to 100  $\mu\text{M}$  phentolamine had significantly higher rates of attachment than control larvae (Fig. 14). These results contradict those from an experiment conducted by Dahms *et al.* (2004), a discrepancy that may be explained by the method of counting attached larvae. In our experiment, 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. Considering these larvae as unattached would have significantly altered the results of the experiment.

NA, an adrenergic receptor agonist, inhibited larval attachment at 100  $\mu\text{M}$ , while phentolamine, an adrenergic receptor antagonist, augmented larval attachment at 100  $\mu\text{M}$ , suggesting that larvae possess adrenergic-like receptors that are involved in the sensory pathways underlying settlement. Analyses of *B. neritina* larval behavior in response to NA and phentolamine offer further insight into the mechanism by which these chemicals exert their effects. NA (at 10 and 100  $\mu\text{M}$  concentrations) increased larval swimming and spinning behavior, while phentolamine (at 100  $\mu\text{M}$  concentration) decreased swimming behavior over an 8 h period (Figs. 5-8). These findings suggest that NA has a stimulatory effect on *B. neritina* larval cilia, thus prohibiting larvae from entering into a period of quiescence often observed prior to attachment. In vertebrate vascular smooth muscle, NA causes an increase in intracellular  $\text{Ca}^{2+}$  levels (Godfraind 1976), which can thereby increase airway ciliary activity (Lansley and Sanderson 1999). In invertebrates, such as the mollusc *H. trivolvis*,  $\text{Ca}^{2+}$  also causes an increase in ciliary beat frequency (Christopher *et al.* 1996). In barnacle cyprid larvae, exposure to octopamine, the invertebrate analog of NA, results in concentration-dependent increases in intracellular  $\text{Ca}^{2+}$  levels and significantly increases the speed of leg kicking (Lind *et al.* 2010). Octopamine exposure also inhibits *B. neritina* larval settlement in a similar fashion to NA (Shimizu *et al.* 2000). In *B. neritina* larvae, we therefore propose that endogenous octopamine regulates

ciliary activity, and that the adrenergic-like receptors which bind NA and phentolamine are octopamine receptors.

#### *Localization of adrenergic-like receptors*

Results from fluorescent labeling and microscopy provide more evidence for the presence of octopamine receptors and endogenous octopamine in *B. neritina* larvae. Localization patterns were expected in sensory structures, such as the apical disc and the vibratile plume. Images of larvae stained with the fluorescently-labeled adrenergic receptor antagonist BODIPY-FL Prazosin, indicate that larvae possess adrenergic-like receptors in the apical disc, the pallial furrow (a groove which surrounds the apical disc), and the pyriform groove, which houses the vibratile plume (Fig. 11). The apical disc and the vibratile plume are both held in advance of larvae as they move through the water, and are the first structures to come into contact with a substratum (Lynch 1947). These have long been considered the primary sensory structures in *B. neritina* larvae, and the presence of adrenergic-like receptors beneath the apical disc and in the glandular region underlying the vibratile plume provides evidence for their sensory role.

#### *Localization of anti-octopamine and anti-tyrosine hydroxylase-like immunoreactivity*

Images of larvae stained with anti-tyrosine hydroxylase provide further evidence for the sensory role of these structures. Anti-tyrosine hydroxylase-like immunoreactivity localized to the apical disc, to the ciliated corona, and to cells that appear to form a network between these regions and the neuromuscular ring

(Fig. 12). Tyrosine hydroxylase is responsible for converting tyrosine to L-DOPA, the precursor to many hormones, including NA and octopamine (Fig. 1).

Tyrosine-hydroxylase-like immunoreactivity is therefore considered indicative of neuroactive tissue. 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 our results support his claim.

Images of larvae stained with anti-octopamine further support this hypothesis, and suggest that *B. neritina* larvae do possess endogenous octopamine. Octopamine-like immunoreactivity was localized to the apical disc, to the ciliated corona, to the pyriform groove, and to networks of cells that appear to connect all of these structures (Fig. 13). These results provide the first evidence for endogenous octopamine in a bryozoan, and may provide an indication of how adrenergic compounds exert their effects on marine invertebrate larvae, even though NA might not be synthesized by these species themselves.

#### *Combined effects of light and phentolamine on larval attachment*

Results confirm that light significantly inhibits larval attachment. The inhibitory effect of light was significantly diminished when adrenergic-like receptors were blocked by phentolamine (Fig. 14), suggesting that these receptors play a role in the photosensory pathway. It is likely that these receptors are octopamine receptors, and that octopamine is involved in controlling phototaxis in *B. neritina* larvae. In locusts (*Schistocerca gregaria*), the

greatest density of octopamine receptors is found in optic lobes (Roeder and Nathanson 1993), and in honey bees (*Apis spp.*) exposure to octopamine increases positive phototaxis (Scheiner *et al.* 2014). Our results, combined with earlier work investigating the effects of dopamine and serotonin on *B. neritina* larvae (Pires and Woollacott 1997) suggest that dopamine and octopamine may both be involved in the photosensory pathway. It is possible that light stimulates production of dopamine, which is then converted to octopamine.

Based on previous evidence and the results from the current study, we have constructed a conceptual model of some of the underlying sensory pathways that may control *B. neritina* larval behavior (Fig. 15). We propose that endogenously produced octopamine triggers an influx of calcium into ciliary cells, stimulating their activity and resulting in swimming behavior. Exogenous exposure to adrenergic agonists like NA results in overstimulation of octopamine receptors, preventing larvae from becoming still and thereby inhibiting their attachment to a substratum. Conversely, exogenous exposure to adrenergic antagonists like phentolamine can 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 the same pattern of increased ciliary activity and settlement inhibition.

#### *Future work*

To further test this conceptual model (Fig. 15), video microscopy software could be used to calculate ciliary beat frequencies and larval swimming speeds in

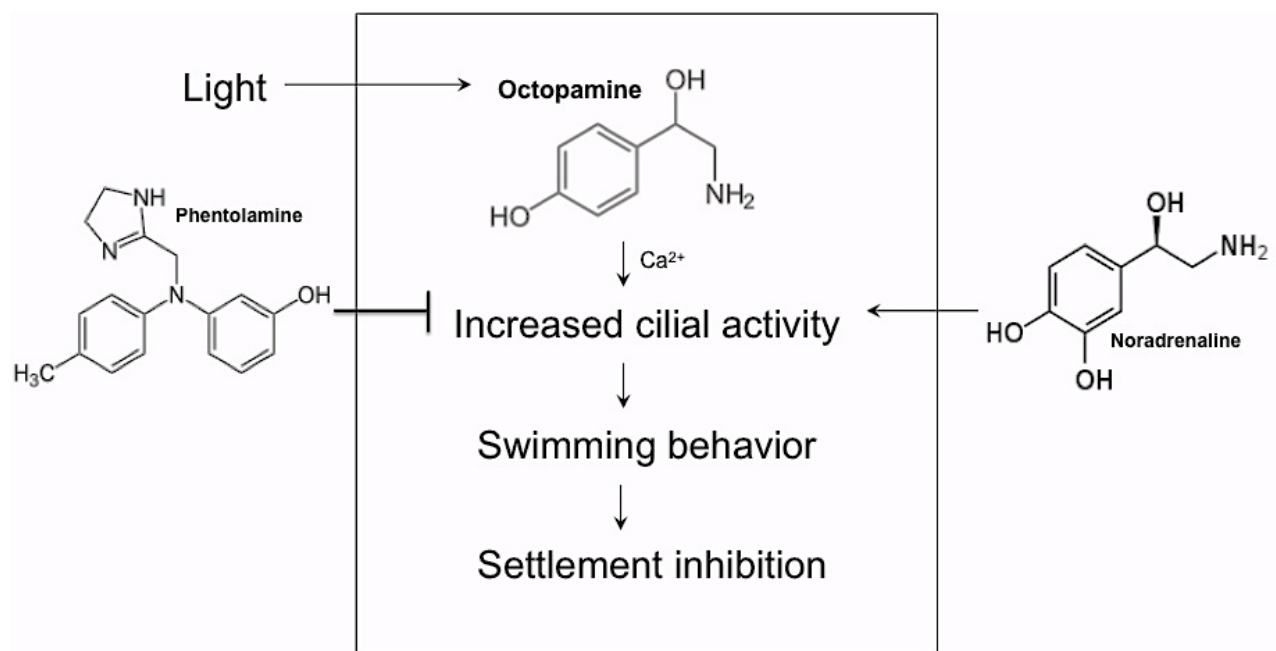
*B. neritina* larvae exposed to light, octopamine, noradrenaline, and phentolamine. We predict that light and octopamine-exposed larvae would have significantly greater ciliary beat frequencies and swimming speeds compared to control larvae, and that phentolamine-exposed larvae would have significantly lower ciliary beat frequencies and swimming speeds. To gain more knowledge of the biosynthesis of endogenous octopamine, immunohistochemistry could be performed with an antibody targeting tyramine-beta hydroxylase, the enzyme that converts tyramine to octopamine (Fig 1). The presence of this enzyme is indicative of octopamine production, and would provide further information on the precise location of the hormone within *B. neritina* larvae. A comparison of octopamine levels between larvae exposed to light and kept in the dark using High Pressure Liquid Chromatography (HPLC) would shed further light on *B. neritina* larval photosensory pathways. We predict that larvae exposed to light would have significantly higher levels of endogenous octopamine compared with larvae kept in the dark. This experimental result would provide further evidence for our proposed hypothesis that light stimulates octopamine production.

### *Conclusion*

Our investigations of *B. neritina* sensory systems offer insight into the underlying mechanisms controlling larval settlement behaviors that have been reported for over a century, 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 sensory system components 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.



**Figure 15.** Putative *B. neritina* larval sensory mechanism. Light stimulates octopamine production, which thereby increases intracellular levels of  $\text{Ca}^{2+}$  and causes larval cilia to beat. Beating cilia result in swimming behavior, which prevents larvae from entering into a period of quiescence necessary for attachment. Noradrenaline binds octopamine receptors, stimulating cilia activity, while phentolamine blocks octopamine receptors, preventing larval swimming.

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