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14. ABSTRACT <p><i>Bugula neritina</i> is a sessile marine bryozoan with a pelagic larval stage. Larvae frequently settle on boat hulls, facilitating the introduction of <i>B. neritina</i> to bays and estuaries worldwide. Adrenergic agonists, such as noradrenaline, inhibit larval settlement in a variety of marine invertebrate species, including <i>B. neritina</i>. Light also inhibits <i>B. neritina</i> larval settlement, yet the underlying mechanisms by which light and adrenergic compounds exert their effects on larvae are largely unknown. Octopamine is considered the invertebrate analog of noradrenaline, and may be involved in larval settlement pathways. In this study, we observed the effects of noradrenaline and the adrenergic antagonist phentolamine on larval settlement, and found that high concentrations of noradrenaline inhibited larval attachment and increased larval swimming behavior. High concentrations of phentolamine increased larval attachment and decreased larval swimming behavior. We used fluorescent labeling and microscopy to localize sensory system components, and found that larvae possess adrenergic-like receptors and octopamine-like immunoreactivity. We also exposed larvae to phentolamine in both dark and light conditions, and found that light inhibited larval attachment, but phentolamine blocked those inhibitory effects. Based on these results, we put forth a putative sensory pathway that explains the effects of both light and adrenergic compounds on <i>B. neritina</i> larval settlement behavior. This study sheds light on previously unknown larval sensory mechanisms and may aid in the development of effective, non-toxic biofouling control strategies.</p>					
15. SUBJECT TERMS marine invertebrate larval settlement, <i>Bugula neritina</i> , octopamine, noradrenaline, phentolamine, adrenergic receptors, larval phototaxis, biofouling, investigation of larval sensory mechanisms					
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December 18, 2015

Defense Technical Information Center
8725 John J Kingman Road, Ste. 0944
Fort Belvoir, VA 22060-6218

Re. Award No. N00014-12-1-0432

Greetings,

Please find enclosed the Final Technical Report, with the SF298, for the above referenced grant.

If you have any questions or require further information, please do not hesitate to contact me by telephone at (805) 756-5348 or by email at lrebik@calpoly.edu.

Sincerely,



Leslie Rebik
Contract & Grant Analyst

Enclosures

1 **Final Report (Award number: N000141210432)**

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

6 **Keywords and phrases:** marine invertebrate larval settlement, *Bugula neritina*,
7 octopamine, noradrenaline, phentolamine, adrenergic receptors, larval phototaxis,
8 biofouling, investigation of larval sensory mechanisms

9
10 **Abstract**

11
12 *Bugula neritina* is a sessile marine bryozoan with a pelagic larval stage. Larvae
13 frequently settle on boat hulls, facilitating the introduction of *B. neritina* to bays and
14 estuaries worldwide. Adrenergic agonists, such as noradrenaline, inhibit larval settlement
15 in a variety of marine invertebrate species, including *B. neritina*. Light also inhibits *B.*
16 *neritina* larval settlement, yet the underlying mechanisms by which light and adrenergic
17 compounds exert their effects on larvae are largely unknown. Octopamine is considered
18 the invertebrate analog of noradrenaline, and may be involved in larval settlement
19 pathways. In this study, we observed the effects of noradrenaline and the adrenergic
20 antagonist phentolamine on larval settlement, and found that high concentrations of
21 noradrenaline inhibited larval attachment and increased larval swimming behavior. High
22 concentrations of phentolamine increased larval attachment and decreased larval
23 swimming behavior. We used fluorescent labeling and microscopy to localize sensory
24 system components, and found that larvae possess adrenergic-like receptors and
25 octopamine-like immunoreactivity. We also exposed larvae to phentolamine in both dark
26 and light conditions, and found that light inhibited larval attachment, but phentolamine
27 blocked those inhibitory effects. Based on these results, we put forth a putative sensory

28 pathway that explains the effects of both light and adrenergic compounds on *B. neritina*
29 larval settlement behavior. This study sheds light on previously unknown larval sensory
30 mechanisms and may aid in the development of effective, non-toxic biofouling control
31 strategies.

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34 Introduction

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

52 Many aspects of reproduction and development in *B. neritina* are well documented
53 (e.g., Lynch 1947, Woollacott and Zimmer 1971). Adult colonies are comprised of
54 branching, hermaphroditic zooids, and are typically brown to dark purple in color. Sexually
55 reproduced embryos are brooded in modified zooids called ovicells, which release larvae
56 that are non-feeding (aplankotrophic, Wendt 1996) and typically spend less than 24 h as
57 plankton prior to settling (e.g., Wendt and Woollacott 1999). Larvae swim through the
58 water column using cilia that cover most of the surface of their barrel-shaped bodies,
59 collectively referred to as the ciliated corona (Woollacott and Zimmer 1971). Larvae often
60 swim in a spiraling motion, and hold sensory structures in advance as they move through
61 the water and begin exploration of a substratum. The sensory apical disc is located at the
62 narrower end of the body, surrounded by a crown of rigid cilia and a circular cleft called
63 the pallial furrow (Fig 1). The vibratile plume is another larval sensory structure, which
64 consists of three long cilia that extend from the glandular pyriform groove (Fig. 1). Prior to
65 attachment, larvae alight on a surface and spin counter-clockwise for 5-10 min, actively
66 feeling the substratum with the vibratile plume (Lynch 1947). All visible ciliary activity then
67 halts for a brief moment prior to eversion of the internal sac, at which point
68 metamorphosis is initiated and the animal is permanently attached to the substratum. The
69 newly attached morph consists of the progenitor zooid, or ancestrula, which gives rise to all
70 other zooids in the colony *via* asexual budding (Lynch 1947). Colonies can become
71 reproductive and release larvae within just twelve days of metamorphosis (Wendt 1998).

72 While many aspects of *B. neritina* larval anatomy and behavior are well documented,
73 the underlying sensory pathways that control larval settlement remain largely unknown.
74 Literature on the effects of fouling-deterrent compounds on larval settlement in marine

75 invertebrates can provide insight into these pathways. Adrenergic compounds, such as the
76 hormone noradrenaline, inhibit larval settlement in a variety of marine invertebrates,
77 including *B. neritina* (e.g., Gohad *et al.* 2012, Shimizu *et al.* 2000). Noradrenaline (NA) is a
78 monoamine that binds to vertebrate adrenergic receptors and exerts a range of stimulatory
79 effects on the sympathetic nervous system, including increased heart rate, release of
80 glucose to the bloodstream, and increased blood flow to skeletal muscle. The underlying
81 mechanism by which NA exerts its effects on larval settlement in marine invertebrates is not
82 well understood, but one study on barnacle (*Balanus amphitrite*) cyprid larvae revealed the
83 presence of adrenergic-like receptors in sensory setae on antennules. These adrenergic-
84 like receptors may be the binding sites for NA and other adrenergic compounds (Gohad *et*
85 *al.* 2012). Octopamine is considered the invertebrate analog of NA and the two compounds
86 only differ structurally by the addition of one hydroxyl group to the benzene ring in NA
87 (Fig. 2).

88 Octopamine regulates a variety of physiological and behavioral processes ranging
89 from locomotion to photosensitivity in phylogenetically diverse invertebrates (Roeder
90 1999). Octopamine receptors have therefore been proposed as binding sites for adrenergic
91 compounds in invertebrates (Wendt *et al.* 2013). In *B. neritina*, octopamine receptors may
92 be the binding sites for NA and other adrenergic compounds, and endogenous octopamine
93 may be a neuroactive compound that modulates many aspects of larval behavior; including
94 locomotion, settlement, and phototaxis.

95 Photosensory systems play an important role in *B. neritina* larval behavior. Larval
96 release is induced in the laboratory by exposing dark-acclimated adult colonies to light (e.g.
97 Woollacott and Zimmer 1971, Wendt 1996), and continued light exposure inhibits larval

98 settlement (Wendt 1996). Larvae are photopositive upon release, but switch to become
99 photonegative within several hours (Lynch 1943, Wendt and Woollacott 1999). As they
100 move away from light, larvae begin the process of surface exploration that occurs prior to
101 attachment and metamorphosis. Thus, there is an inverse relationship between positive
102 phototaxis and initiation of metamorphosis (Wendt and Woollacott 1999). While the
103 effects of light on *B. neritina* larvae are well documented, the underlying sensory pathways
104 controlling these phenomena are still not well understood. One study investigated the
105 mechanisms underlying phototaxis by exposing *B. neritina* larvae to the monoamines
106 dopamine and serotonin. Dopamine exposure extended the period of positive phototaxis,
107 while serotonin, or 5-hydroxytryptophan (5HT), made larvae immediately photonegative.
108 5HT-like activity was also found in tracts connecting eyespots to the larval locomotory
109 organ (Pires and Woollacott 1997).

110 The metabolic pathways that involve dopamine are well studied in vertebrates.
111 Tyrosine is first converted by the enzyme tyrosine hydroxylase (TH) into L-DOPA, which is
112 then converted into dopamine. Dopamine is the precursor to several other monoamines,
113 including NA and octopamine (Fig 2). Therefore, it is possible that light exerts its effects on
114 *B. neritina* larvae *via* an underlying chemical pathway that involves both dopamine and
115 octopamine.

116 In the present study, *B. neritina* larvae were exposed to various concentrations of
117 the adrenergic agonist, NA, and the adrenergic antagonist, phentolamine, to investigate the
118 effects of these compounds on larval attachment, behavior, and mortality. Fluorescent
119 labeling and microscopy were used to determine the presence and location of adrenergic-
120 like receptors, octopamine, and tyrosine hydroxylase. Larvae were also exposed to light in

121 the presence and absence of phentolamine to observe their combined effects on larval
122 attachment and to gain knowledge of the underlying photosensory pathway.

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

131 **Materials and methods**

132

133 *Larval collection*

134 *Bugula neritina* colonies were collected by hand from floating docks in two separate
135 locations in Morro Bay, CA USA (35.3708, -120.8580; 35.3461, -120.8432) from March 19,
136 2014 through May 1, 2015. Colonies were maintained in captivity in a dark, aerated
137 container of raw seawater at 11°C for 2 to 10 days, and given no exogenous food source. In
138 order to induce larval release, dark-acclimated colonies were exposed to light (both natural
139 and incandescent). Larvae were then collected and transferred by pipette within 1 h of
140 release. Larvae were pooled from multiple colonies to foster genetic heterogeneity for all
141 experiments.

142 *Effects of noradrenaline and phentolamine on larval attachment and behavior*

143 To observe the effects of NA (an adrenoreceptor agonist) and phentolamine (an
144 adrenoreceptor antagonist) on larval behavior and attachment, larvae were exposed to

145 varying concentrations of each compound in seawater and observed with a Leica EZ4D
146 dissecting microscope. DL-Noradrenaline Hydrochloride ($\geq 97\%$) was obtained from Sigma-
147 Aldrich (St. Louis, MO, USA). Phentolamine-Hydrochloride ($\geq 98\%$) was obtained from
148 Santa Cruz Biotechnology (Dallas, TX, USA). Larvae were released from multiple adult
149 colonies (collected on three different days from two Morro Bay sites), pooled in a beaker,
150 and transferred to 15 ml Falcon tubes with filtered seawater containing 0 (control), 0.1, 1.0,
151 10, or 100 μM of either NA or phentolamine.

152 Larvae were immediately transferred from Falcon tubes to 24 well polystyrene cell
153 culture plates, one larva per well in 1 ml of solution, with a total of twelve larvae per
154 treatment per trial. Five trials were conducted with both compounds (on the 15, 17, 22, 24,
155 and 29 of April, 2014), for a total of 600 larvae used in the experiment. Fresh treatment
156 solutions were made up for each trial. The number of larvae attached was recorded at 2, 4,
157 6, 8, 24, and 48 h following the start of treatment solution exposure. Larvae were
158 designated as attached if they had settled on the polystyrene and could not be moved by a
159 pipetted stream of water, or if they had settled on the air-water interface and begun to
160 metamorphose. Larvae were kept at 11°C for the duration of the experiments, except
161 during observation. Notes were also taken on larval behavior prior to attachment, and
162 unattached larvae were classified as swimming, spinning, or dead.

163 *Effects of noradrenaline and phentolamine on larval mortality*

164 To specifically determine whether experimental exposure to exogenous NA and
165 phentolamine solutions had an impact on larval mortality, larvae were exposed to
166 concentrations of 0 (control), 10, or 100 μM of NA or phentolamine in 65 mm diameter
167 petri dishes. These concentrations were based on results from unpublished pilot

168 experiments, and petri dishes were used to minimize larval mortality that may have
169 occurred due to smaller wells of cell culture plates. Two trials were conducted, with ten
170 larvae per treatment per trial, for a total of sixty larvae. The number of dead larvae was
171 recorded at 24, 48, and 72 h of exposure. Mortality was assessed by lack of ciliary movement
172 in unattached larvae, and discoloration and/or termination of metamorphosis in attached
173 larvae.

174 *Localization of adrenergic-like receptors*

175
176 Fluorescent labeling and microscopy were used to determine the presence and
177 location of adrenergic-like receptors within whole mount *B. neritina* larvae. Live larvae
178 were incubated in 10 μ M BODIPY-FL Prazosin, a fluorescently labeled non-subtype
179 selective α -adrenergic receptor antagonist obtained from Life Technologies (Foster City,
180 CA, USA). The solution was made up in filtered seawater (FSW) and larvae were incubated
181 for 30 min prior to being washed three times in FSW. Larvae were then imaged on an
182 Olympus (Center Valley, PA, USA) BX53 compound fluorescent microscope with a DP73
183 camera, using the 488 nm laser line and Olympus CellSens software. Unstained larvae were
184 used as controls for autofluorescence.

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

186
187 To determine the presence and location of tyrosine-hydroxylase-like and
188 octopamine-like immunoreactivity, *B. neritina* larvae were fixed in 4% paraformaldehyde
189 made up in phosphate-buffered saline (PBS) for at least 2 h, then washed once in PBS. Fixed
190 larvae were then permeabilized overnight in 0.5% Triton X-100 in PBS, and incubated in
191 2% bovine serum albumin (BSA) for 4 h. BSA was pipetted off, and larvae were incubated
192 in either anti-tyrosine hydroxylase primary antibody (1:500) or anti-octopamine primary

193 antibody (1:500) in PBS overnight at 4°C. Anti-tyrosine hydroxylase was obtained from
194 Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA) and anti-octopamine was
195 obtained from Millipore (Billerica, MA, USA). Larvae were washed in 0.1% Triton X-100 in
196 PBS four times (5 min each) and those incubated with anti-tyrosine hydroxylase were
197 transferred to Alexafluor 568 anti-mouse secondary antibody (1:500), while those
198 incubated in anti-octopamine were transferred to Alexafluor 594 anti-rabbit secondary
199 antibody (1:500) and incubated overnight at 4°C. Both secondary antibodies were
200 obtained from Life Technologies. Larvae were incubated with the nuclear stain DAPI for 15
201 min (1:500), prior to washing four times (5 min each) in 0.1% Triton-X in PBS. Larvae were
202 then imaged in PBS in chambered coverglass with an Olympus FV1000 Scanning Laser
203 Confocal Microscope using Fluoview imaging software. Unstained larvae and larvae labeled
204 with only secondary antibodies were used as controls for autofluorescence and unspecific
205 binding, respectively.

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

207 To investigate underlying photosensory mechanisms in *B. neritina* larvae, larval
208 attachment rates were compared between the following groups: 1. larvae immersed in 100
209 μM phentolamine and exposed to light, 2. larvae immersed in 100 μM phentolamine and
210 kept in the dark, 3. larvae in FSW exposed to light, and 4. larvae in FSW kept in the dark.
211 Larvae were collected from multiple colonies, pooled, and randomly transferred to 15 ml
212 falcon tubes containing either FSW (control) or 100 μM phentolamine (treatment) in FSW.
213 Each tube was transferred to a separate 65 mm diameter petri dish, and placed in either
214 dark or light conditions for 4 hours at room temperature. The number of larvae attached

215 was recorded at 1, 2, and 3 h of exposure. Three trials were conducted, with ten larvae per
216 treatment per trial, for a total of 120 larvae.

217 *Statistical Analyses*

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

228 **Results**

229 *Effects of noradrenaline and phentolamine on larval attachment*

230 Binomial logistic regression revealed that noradrenaline (NA) significantly inhibited
231 *B. neritina* larval attachment at 10 μM ($P=0.0297$) and 100 μM ($P=0.0121$), but had no
232 significant effect at 0.1 or 1.0 μM (Fig. 3). In this experiment, the effects of phentolamine
233 on larval attachment were not statistically significant at any concentration. However, a
234 greater percentage of larvae in the 10 and 100 μM treatments attached, while a smaller
235 percentage of larvae in the 0.1 and 1.0 μM concentrations attached compared with the
236 control group (Fig 3). *Post hoc* pairwise comparisons (Tukey's HSD) revealed significant
237 differences between the control group and 100 μM NA at 2 h ($P=0.0066$), 4 h ($P=0.0284$), 6

238 h ($P=0.0090$), and 8 h ($P=0.0111$) of exposure; between the control group and 10 μM NA
239 at 2 h of exposure ($P=0.0190$); and between 100 μM NA and 100 μM phentolamine at 2 h (P
240 $=0.0008$), 4 h ($P=0.0006$), 6 h ($P=0.0003$), and 8 h ($P=0.0010$) of exposure (Fig. 3).

241

242

243 *Effects of noradrenaline and phentolamine on larval behavior*

244 We examined larval behavior at 2, 4, 6, and 8 h of exposure to either NA or
245 phentolamine, and classified each live, unattached larva as either swimming or spinning.
246 Nominal logistic regression and Cox Proportional Hazards risk ratios were used to compare
247 each treatment to the control at every time point. After 2 h of exposure, significantly more
248 larvae were swimming in 10 μM ($P<0.0001$) and 100 μM ($P=0.0027$) NA compared to the
249 control group, while significantly fewer larvae were swimming in 0.1 μM ($P=0.0097$) and
250 100 μM phentolamine ($P<0.0001$) (Fig. 4). After 4 h of exposure, significantly more larvae
251 were spinning in 10 μM ($P<0.0001$) and 100 μM ($P<0.0001$) NA, significantly more larvaew
252 were swimming in 100 μM NA ($P=0.0058$), and significantly fewer larvae were swimming
253 in 10 μM ($P=0.0058$) and 100 μM ($P<0.0001$) phentolamine. After 6 h of exposure,
254 significantly more larvae were swimming in 10 μM NA ($P=0.0105$), while significantly more
255 larvae were spinning in 100 μM NA ($P=0.0002$). Significantly fewer larvae were swimming
256 in 10 μM ($P=0.0002$) and 100 μM ($P=0.0056$) phentolamine. After 8 h, significantly more
257 larvae remained swimming in 10 μM NA ($P=0.0234$) and significantly more larvae
258 remained spinning in 100 μM NA ($P=0.0013$). Significantly fewer larvae remained
259 swimming in 10 μM ($P<0.0001$) and 100 μM ($P<0.0001$) phentolamine.

260 *Effects of noradrenaline and phentolamine on larval mortality*

261 Noradrenaline significantly increased larval mortality over a 72 h period at both 10
262 μM ($P < 0.0001$) and 100 μM ($P < 0.0001$). Phentolamine significantly increased larval
263 mortality over a 72 h period at 100 μM ($P < 0.0001$).

264

265 *Localization of adrenergic-like receptors*

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

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

272
273 Both tyrosine hydroxylase-like and octopamine-like immunoreactivity were
274 detected in *B. neritina* larvae, though some distortion of the larvae made determination of
275 the precise locations of these substances difficult. Tyrosine hydroxylase-like
276 immunoreactivity appeared to be concentrated in the apical disc, and the neuromuscular
277 ring (Fig. 6). Octopamine-like immunoreactivity appeared to be most prevalent in the
278 apical disc, the ciliated corona, and the pyriform groove (Fig. 7). No fluorescence was
279 observed in control larvae that were not stained with primary antibodies.

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

281 Light exposure significantly inhibited larval attachment at the 1 h, 2 h, and 3 h
282 exposure time points ($P < 0.0001$). Phentolamine-exposed larvae in dark conditions also
283 had significantly higher rates of attachment than control larvae in dark conditions after 1 h

284 ($P=0.0004$) and 2 h ($P=0.004$) of exposure. There was a significant interaction between
285 light and phentolamine after three hours of exposure, and the inhibitory effects of light
286 were significantly diminished in larvae exposed to 100 μM phentolamine ($P=0.02$) (Fig. 8).

287

288 **Discussion**

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

290

291 Our results confirm previous reports of the inhibitory effects of the adrenergic
292 agonist NA on the larval attachment of marine invertebrates (e.g. Shimizu *et al.* 2000,
293 Gohad *et al.* 2012). We expected an adrenergic antagonist to have the opposite effect, and
294 though not statistically significant, higher concentrations of phentolamine increased larval
295 attachment in our initial experiment (Fig. 3). Later experiments examining the effects of
296 both light and phentolamine allowed us to confirm this trend of increased attachment in
297 the highest concentration of phentolamine. Larvae exposed to 100 μM phentolamine had
298 significantly higher rates of attachment than control larvae in both light and dark
299 conditions (Fig. 8). These results on the effects of phentolamine on *B. neritina* larval
300 attachment contradict those from an experiment conducted by Dahms *et al.* (2004); a
301 discrepancy that may be explained by the method of counting attached larvae. More than
302 25% of attached larvae in the phentolamine treatments settled and began metamorphosis
303 on the air-water interface, never attaching to the polystyrene container. We counted these
304 larvae as attached, and considering them as unattached would have significantly altered
305 the results of the experiment.

306 That NA, an adrenergic receptor agonist, inhibited larval attachment at 100 μM ,
307 while phentolamine, an adrenergic receptor antagonist, augmented larval attachment at

308 100 μ M, supports our initial hypotheses and suggests that larvae do possess adrenergic-
309 like receptors that are involved in the chemosensory pathways underlying settlement.

310 Analyses of *B. neritina* larval behavior in response to NA and phentolamine offer
311 further insight into the mechanism by which these chemicals exert their effects. Our
312 analysis of larval behavior was complicated by the fact that larvae can alternate between
313 swimming and spinning during the period of surface exploration prior to attachment, and
314 future analyses comparing ciliary activity (in both swimming and spinning larvae) between
315 treatment and control groups would provide further insight into the mechanisms
316 underlying the effects of adrenergic compounds. However, our analysis revealed that NA
317 (at 10 and 100 μ M) increased swimming behavior, while phentolamine (at 100 μ M)
318 decreased swimming behavior after just 2 h of exposure (Fig. 4), and this pattern continued
319 through the 8 h sampling period. These findings suggest that NA has a stimulatory effect on
320 *B. neritina* larval cilia, thus prohibiting larvae from entering into a period of quiescence
321 often observed prior to attachment. In vertebrate vascular smooth muscle, NA causes an
322 increase in intracellular Ca^{2+} levels (Godfraind 1976), which can thereby increase airway
323 ciliary activity (Lansley and Sanderson 1999). In invertebrates, such as the mollusc *H.*
324 *trivolvis*, Ca^{2+} also causes an increase in ciliary beat frequency (Christopher *et al.* 1996). In
325 barnacle cyprid larvae, exposure to octopamine, the invertebrate analog of NA, results in
326 concentration-dependent increases in intracellular Ca^{2+} levels and significantly increases
327 the speed of leg kicking (Lind *et al.* 2010). Octopamine exposure also inhibits *B. neritina*
328 larval settlement in a similar fashion to NA (Shimizu *et al.* 2000). In *B. neritina* larvae, we
329 therefore propose that endogenous octopamine regulates ciliary activity, and that the
330 adrenergic-like receptors which bind NA and phentolamine are octopamine receptors.

331 While multiple studies have looked at the effects of adrenergic compounds on
332 marine invertebrate larval settlement (e.g. Gohad *et al.* 2012, Shimizu *et al.* 2000), few have
333 examined their effects on metamorphic success or larval survival over a longer duration of
334 time. In our study, both NA (at 10 and 100 μM) and phentolamine (at 100 μM) significantly
335 increased larval mortality over a 72 h period, and many larvae that successfully attached
336 died shortly thereafter. These results suggest that adrenergic agonists and antagonists do
337 more than simply extend or abbreviate the duration of larval swimming, and highlight the
338 need for research into the long-term effects of these compounds on a range of organisms
339 and ecosystems prior to their widespread use as biofouling controls.

340 *Localization of adrenergic-like receptors*

341 Results from fluorescent labeling and microscopy provide more evidence for the
342 presence of octopamine receptors and endogenous octopamine in *B. neritina* larvae.
343 Localization patterns were expected in sensory structures, such as the apical disc and the
344 vibratile plume. Images of larvae stained with the fluorescently-labeled adrenergic
345 receptor antagonist BODIPY-FL Prazosin, indicate that larvae possess adrenergic-like
346 receptors in and around the apical disc, and in the pyriform groove, which houses the
347 vibratile plume (Fig. 5). The apical disc and the vibratile plume are both held in advance of
348 larvae as they move through the water, and are the first structures to come into contact
349 with a substratum (Lynch 1947). These have long been considered the primary sensory
350 structures in *B. neritina* larvae, and the presence of adrenergic-like receptors beneath the
351 apical disc and in the glandular region underlying the vibratile plume provides evidence for
352 their sensory role.

353

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

355
356 Images of larvae stained with anti-tyrosine hydroxylase provide further evidence
357 for the sensory role of these structures. Anti-tyrosine hydroxylase-like immunoreactivity
358 localized to the apical disc, to the ciliated corona, and to cells that appear to form a network
359 between these regions and the neuromuscular ring (Fig. 6). Tyrosine hydroxylase is
360 responsible for converting tyrosine to L-DOPA, the precursor to many hormones, including
361 NA and octopamine (Fig. 2), and tyrosine-hydroxylase-like immunoreactivity is therefore
362 considered indicative of neuroactive tissue. As early as 1890, Prouho asserted that the
363 apical disc, the ciliated corona, and the pyriform organ in bryozoan larvae were all
364 connected by nervous tissue, and our results support his claim.

365 Images of larvae stained with anti-octopamine further support this hypothesis, and
366 suggest that *B. neritina* larvae do possess endogenous octopamine. Octopamine-like
367 immunoreactivity localized to the apical disc, the ciliated corona, the pyriform groove and
368 to underlying networks that appear to connect these structures (Fig. 7). Control larvae
369 stained with only secondary fluorescent antibodies did not exhibit any red fluorescence,
370 indicating that results seen in larvae stained with both primary and secondary antibodies
371 were not due to autofluorescence or unspecific binding. While the specific location of
372 octopamine was difficult to determine from our images, the pattern of immunoreactivity
373 we observed was consistent with our expectations. These results provide the first evidence
374 for endogenous octopamine in a bryozoan. The presence of octopamine within *B. neritina*
375 may provide an explanation for how adrenergic compounds like NA exert their effects on
376 marine invertebrate larvae, even though NA might not be synthesized by these species
377 themselves.

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

379 Our results confirm that light significantly inhibits larval attachment. The inhibitory
380 effect of light was significantly diminished when adrenergic-like receptors were blocked by
381 phentolamine (Fig. 8), suggesting that these receptors are involved in the photosensory
382 pathway. It is likely that these receptors are octopamine receptors, and that octopamine
383 plays a role in controlling phototaxis in *B. neritina* larvae. In locusts (*Schistocerca*
384 *gregaria*), the greatest density of octopamine receptors is found in optic lobes (Roeder and
385 Nathanson 1993), and in honey bees (*Apis spp.*) exposure to octopamine increases positive
386 phototaxis (Scheiner *et al.* 2014). Our results, combined with earlier work investigating the
387 effects of dopamine and serotonin on *B. neritina* larvae (Pires and Woollacott 1997),
388 suggest that dopamine and octopamine may both be involved in the photosensory
389 pathway. Light may stimulate the production of dopamine, which is then converted to
390 octopamine. While we did not investigate the role of serotonin in this study, it is possible
391 that the hormone also plays a role in controlling *B. neritina* larval phototactic behavior.

392 *Conceptual model of B. neritina larval sensory systems*

393 Based on previous evidence and the results from the current study, we have
394 constructed a conceptual model of some of the underlying sensory pathways that may
395 control *B. neritina* larval behavior (Fig. 9). We propose that endogenously produced
396 octopamine triggers an influx of calcium into ciliary cells, stimulating their activity and
397 resulting in swimming behavior. Exogenous exposure to adrenoceptor agonists like NA
398 results in stimulation of octopamine receptors, preventing larvae from becoming still and
399 thereby inhibiting their attachment to a substratum. Conversely, exogenous exposure to
400 adrenoceptor antagonists like phentolamine can block octopamine receptors, preventing

401 larval swimming and causing larvae to attach more rapidly. Light exposure may naturally
402 increase octopamine production within *B. neritina* larvae, stimulating the same pathway of
403 increased ciliary activity and settlement inhibition. This may also explain how light
404 stimulates the release of larvae from the ovicells in which they are brooded, offering a
405 mechanistic explanation for a phenomenon that has been observed and exploited in the
406 laboratory for decades.

407 *Future work*

408 To further test this conceptual model (Fig. 9), video microscopy software could be
409 used to calculate ciliary beat frequencies and larval swimming speeds in *B. neritina* larvae
410 exposed to light, octopamine, noradrenaline, and phentolamine. We predict that light and
411 octopamine-exposed larvae would have significantly greater ciliary beat frequencies and
412 swimming speeds compared to control larvae, and that phentolamine-exposed larvae
413 would have significantly lower ciliary beat frequencies and swimming speeds. To gain more
414 knowledge of the biosynthesis of endogenous octopamine, immunohistochemistry could be
415 performed with an antibody targeting tyramine-beta hydroxylase, the enzyme that
416 converts tyramine to octopamine (Fig 2). The presence of this enzyme is indicative of
417 octopamine production, and would provide further information on the precise location of
418 the hormone within *B. neritina* larvae. A comparison of octopamine levels between larvae
419 exposed to light and kept in the dark using High Pressure Liquid Chromatography (HPLC)
420 would shed further light on *B. neritina* larval photosensory pathways. We predict that
421 larvae exposed to light would have significantly higher levels of endogenous octopamine
422 compared with larvae kept in the dark. This experimental result would provide further
423 evidence for our proposed hypothesis that light stimulates octopamine production.

424 *Conclusion*

425 Our investigations of *B. neritina* sensory systems offer insight into the underlying
426 mechanisms controlling larval settlement behaviors that have been reported for decades,
427 but not fully understood. An enhanced understanding of the larval biology of marine
428 invertebrates like *B. neritina* not only expands our knowledge of the evolution of sensory
429 system components across taxa, but can also aid in the development of new approaches to
430 control biofouling and prevent the further spread of invasive species, which will benefit
431 coastal ecosystems worldwide.

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437 commercial distributors, as stated in the Materials and Methods.

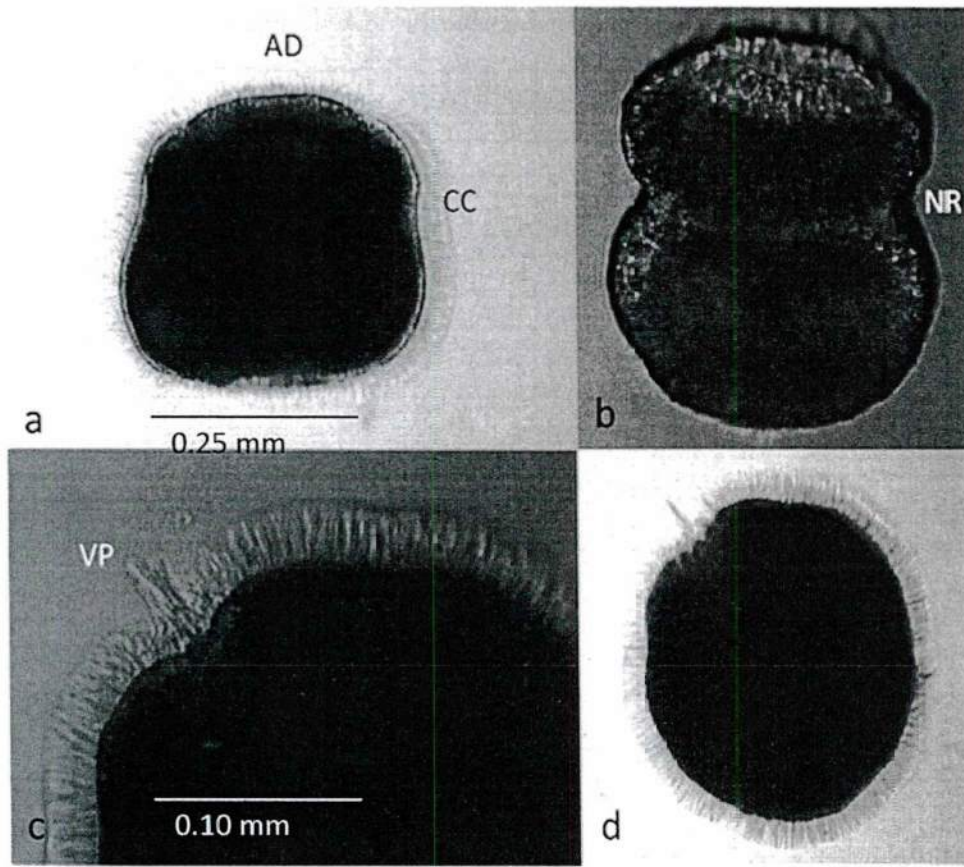
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443 **Literature Cited**

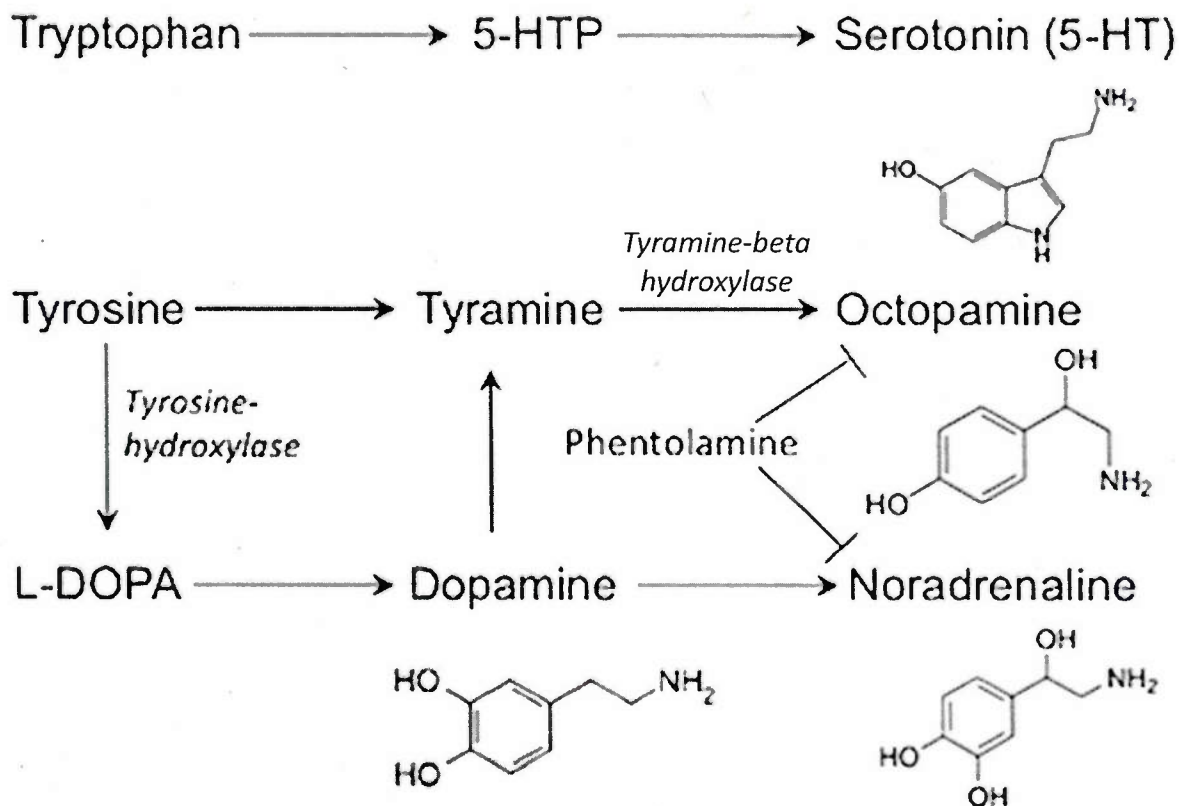
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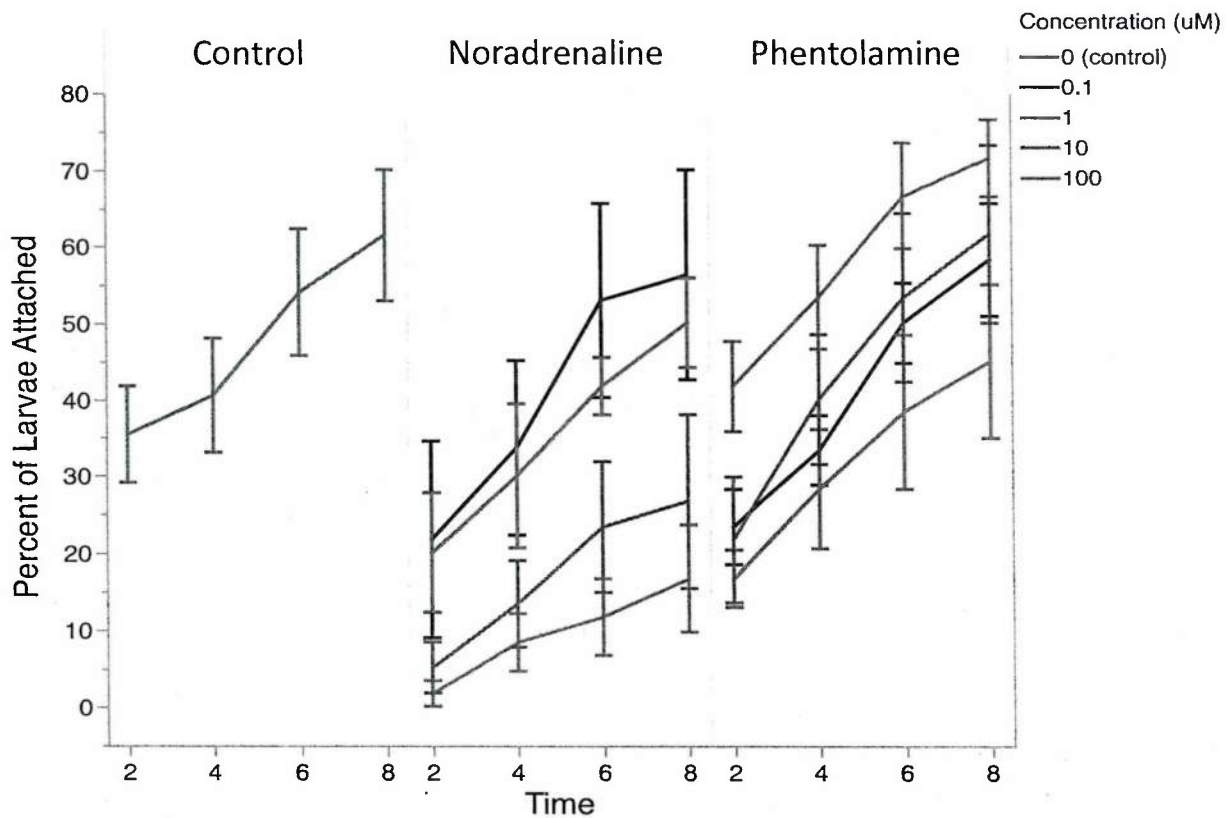


536
 537
 538 **Figure 1.** Brightfield images of *B. neritina* larvae taken with an Olympus BX53 microscope
 539 and DP73 camera using Cel.Sens software. **a.** Barrel-shaped larvae are surrounded by a
 540 ciliated corona (CC), which acts as larval locomotory organ. The image was taken from the
 541 lateral view, with the apical disc (AD) upmost. **b.** Larvae fixed with 4% paraformaldehyde
 542 can become distorted and drop cilia. The neuromuscular ring (NR) underlies the
 543 constricted region. **c.** This image taken from the apical view offers a closer view of the
 544 ciliated corona and the vibratile plume (VP). **d.** The vibratile plume extends from the
 545 pyriform groove, and the ciliated corona is distributed across the surface of the larva.
 546

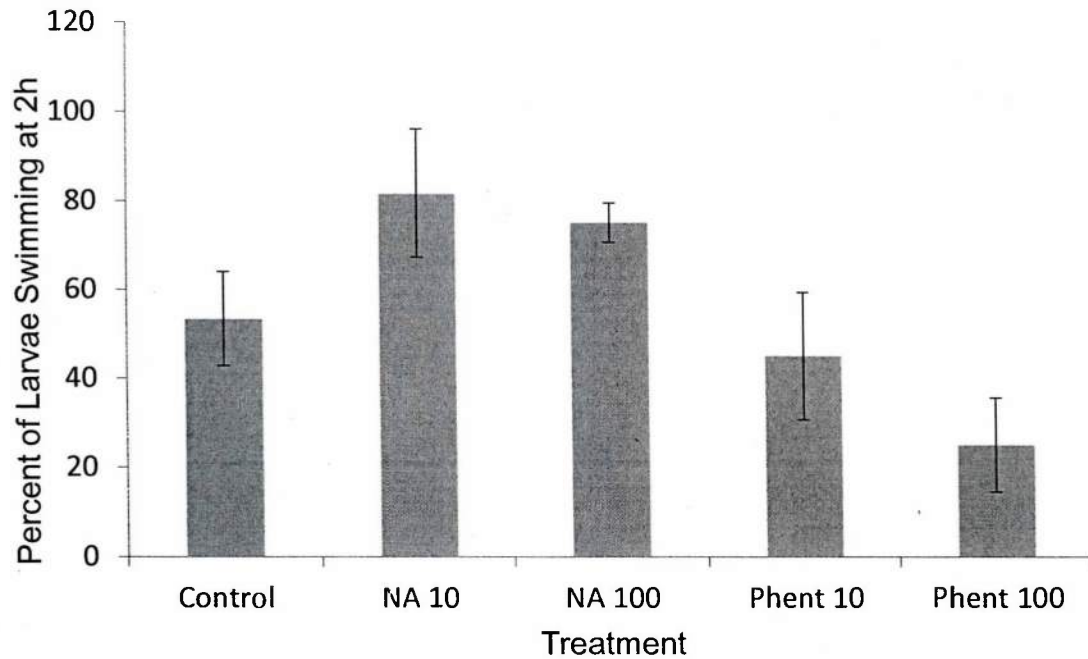


547
 548 **Figure 2.** Biosynthesis of some of the neuroactive compounds likely involved in controlling
 549 *B. neritina* larval behavior. Noradrenaline, an adrenergic receptor agonist, inhibits larval
 550 settlement in many marine invertebrates, including *B. neritina*. Phentolamine is a
 551 pharmaceutical compound that blocks adrenergic receptors. Octopamine is often
 552 considered the invertebrate equivalent of noradrenaline, and both monoamines can be
 553 synthesized from tyrosine and dopamine. Tyrosine hydroxylase is the enzyme that converts
 554 tyrosine to L-DOPA (the precursor to dopamine). Dopamine and serotonin influence *B.*
 555 *neritina* larval phototaxis.

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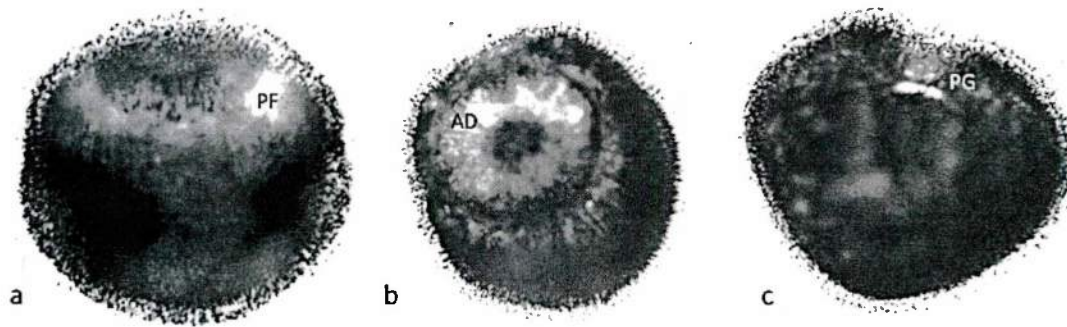


557
 558 **Figure 3.** Effects of noradrenaline and phentolamine on larval attachment over 8 h of
 559 treatment exposure. Sample size was $n=5$ replicate trials, with 12 larvae per treatment per
 560 trial, for a total of 60 larvae per treatment. Error bars represent standard errors of the
 561 means. NA significantly inhibited larval attachment at 10 μM ($P=0.0297$) and 100 μM
 562 ($P=0.0121$). The effects of phentolamine were not significant, but there was a trend of
 563 lower attachment at 0.1 and 1.0 μM , and higher attachment at 10 and 100 μM .
 564



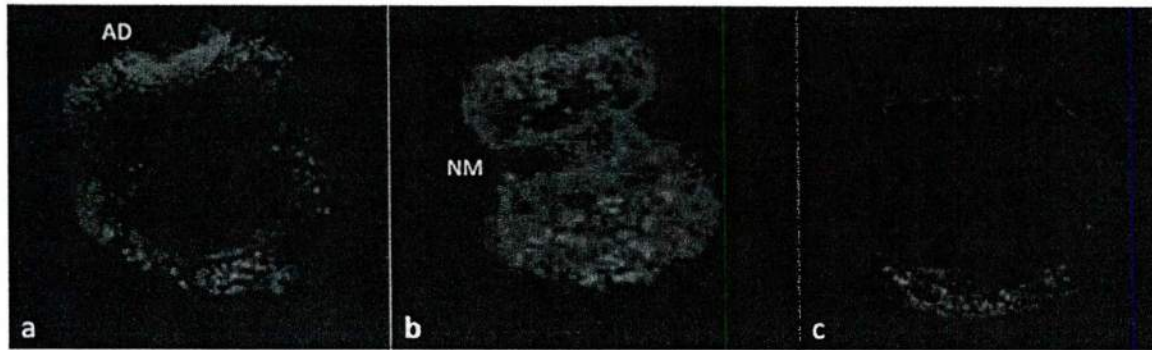
Figure

565
566 **4.** Effects of noradrenaline and phentolamine on larval swimming behavior at 2 h of
567 exposure. Sample size was $n=5$ replicate trials, with 12 larvae per treatment per trial, for a
568 total of 60 larvae per treatment. Error bars represent standard errors of the means. NA
569 significantly increased swimming behavior at 10 and 100 μM , while phentolamine
570 significantly decreased swimming behavior at 100 μM .
571



572 **Figure**
573 **5.** *B. neritina* larva stained with BODIPY-FL Prazosin, a fluorescently labeled adrenergic
574 receptor antagonist. Fluorescence was observed in: **a.** the region surrounding the apical
575 disc, **b.** the apical disc, and **c.** the pyriform groove, suggesting that larvae possess
576 adrenergic-like receptors in each of these regions. Images were taken with the 20X
577 objective on an Olympus BX53 microscope, using a DP73 camera and CellSens software.
578 The 488 nm laser line was used, and images were then converted to gray scale.
579

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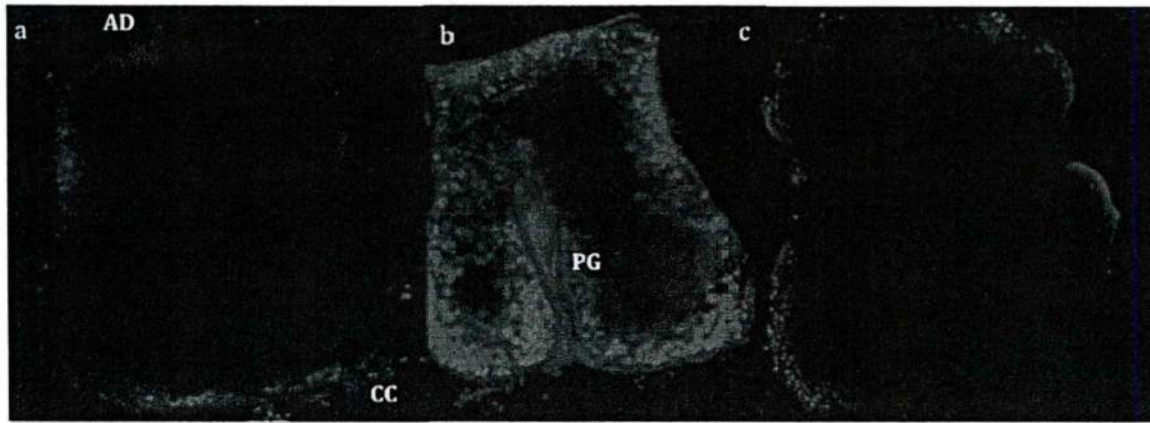


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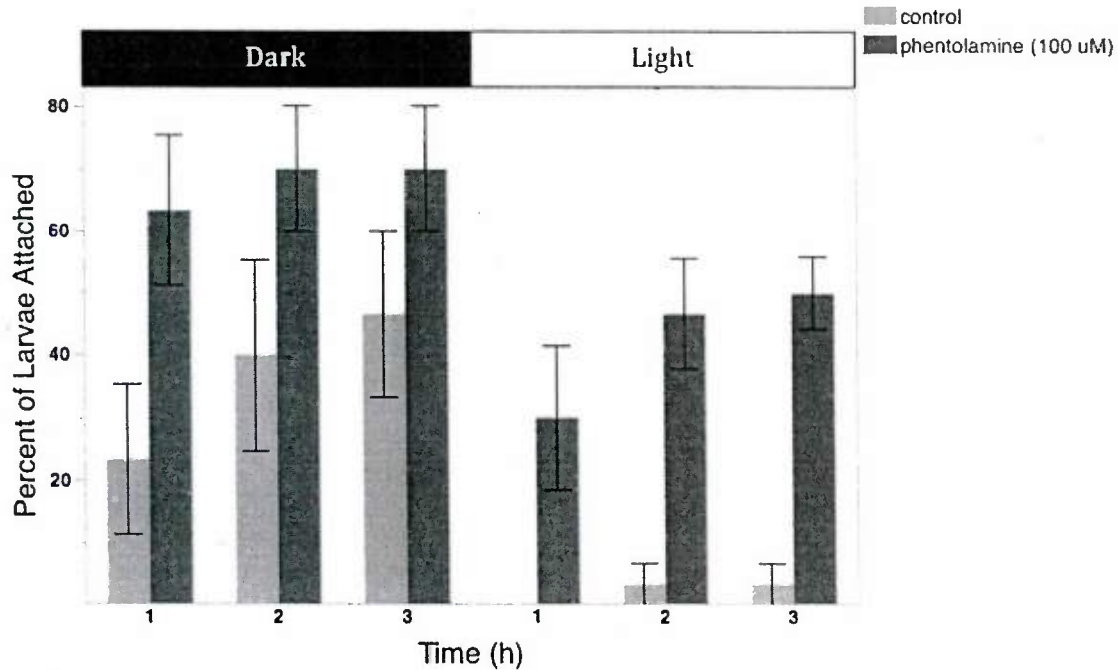
582 **Figure 6. a)** *B. neritina* larva stained with DAPI nuclear stain (blue), and anti-tyrosine
583 hydroxylase (red). At the surface, tyrosine hydroxylase-like immunoreactivity localized to
584 the apical disc region (AD). **b)** An image taken deeper inside the larva shows tyrosine
585 hydroxylase-like immunoreactivity (red) in the region of the neuromuscular ring (NM).
586 **c)** Control larva stained with only DAPI nuclear stain and secondary antibody. All images
587 were taken on an Olympus FV1000 Scanning Laser Confocal Microscope using Fluoview
588 imaging software and the 20X objective. A 405 nm laser line at 8% transmissivity was used
589 for DAPI excitation (blue), and a 559 nm laser line at 12% transmissivity was used for
590 Alexafluor-568 excitation (red). A 3D z-stack video file of this larva is provided in the
591 supplemental materials.

592

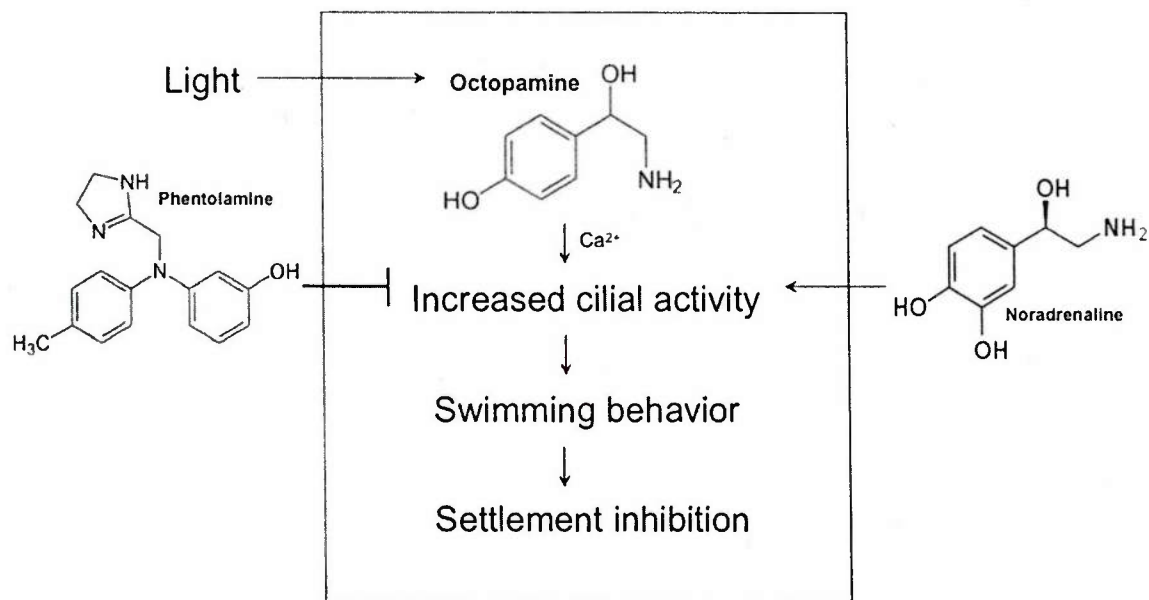
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594
595 **Figure 7. a)** *B. neritina* larva stained with DAPI nuclear stain (blue), and anti-octopamine
596 (red). At the surface, octopamine-like immunoreactivity localized to the apical disc (AD)
597 and the ciliated corona (CC). **b)** An image from deeper inside the larva reveals octopamine-
598 like activity in the region of the pyriform groove (PG), which houses the vibratile plume.
599 **c)** Control larvae stained with only DAPI and secondary antibody. All images were taken on
600 an Olympus FV1000 Scanning Laser Confocal Microscope using Fluoview imaging software
601 and the 20X objective. A 405 nm laser line at 8% transmissivity was used for DAPI
602 excitation (blue), and a 559 nm laser line at 30% transmissivity was used for Alexafluor-
603 594 excitation (red). A 3D z-stack video file of this larva is provided in the supplemental
604 materials.
605



606
 607 **Figure 8.** Effects of light and phentolamine on larval attachment. Sample size of $n=3$
 608 replicate trials, with 10 larvae per treatment per trial, for a total of 30 larvae per treatment.
 609 Error bars represent standard errors of the mean. Light significantly inhibited larval
 610 attachment in control larvae at each timepoint. The effect of light was significantly
 611 diminished by phentolamine (an adrenergic receptor blocker) after 3 h of exposure,
 612 suggesting that adrenergic-like receptors play a role in the photosensory pathway.
 613



614 **Figure**

615 **9.** Putative *B. neritina* larval sensory mechanism. Light stimulates octopamine production,

616 which thereby increases intracellular levels of Ca²⁺ and causes larval cilia to beat. Beating

617 cilia result in swimming or spinning behavior, which prevents larvae from entering into a

618 necessary period of quiescence prior to attachment. Noradrenaline binds octopamine

619 receptors, stimulating cilia activity, while phentolamine blocks octopamine receptors,

620 preventing larval swimming.

621