

Locomotory behavior of competent cyphonautes larvae in response to positive and negative cues in flow

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Abstract

Previous research has shown evidence of chemosensory behavioral responses in invertebrate larvae across species and using varying stimuli. Diverse exploratory behaviors in larval *Membranipora* spp. Cyphonautes on suitable settlement substrates (macroalgae) have been defined (Matson et al. 2010), but little is known about the behavioral mechanisms used to find settling habitats in the first place. We investigated the behavioral swimming response to hypothesized positive and negative chemical cues in late-stage cyphonautes larvae (*Membranipora* spp.) to determine whether cyphonautes have noticeably different behavioral responses to chemical cue plumes. We compared the response of larvae to a control (filtered seawater) cue, a positive cue (the red alga, *Mazzaella splendens*), and a negative cue (predator, *Corambe* spp.). Larvae were collected from plankton tows and exposed to plumes of cue water for five minutes under a unidirectional, laminar flow regime. Locomotory behaviors (swimming direction relative to flow, circling behavior, crawling behavior, tank wall interactions) were recorded. The results suggest that cyphonautes larvae swim more frequently toward the source of the cue when it is a positive cue, but do not respond negatively to predator cues (from a predator of their adult form). These results are consistent with the hypothesis that competent cyphonautes larvae actively use chemosensation to locate algal blades for settlement. Additionally, the results suggest that circular swimming and swimming perpendicular to flow, in addition to swimming towards a stimulus source, might aid cyphonautes larvae in finding substrate for settlement.

Introduction

Settlement behavior in larval forms has been an area of scientific interest for over 70 years, with studies conducted on substrate preference and response to potential cues in various species (Wilson 1952). These various findings suggest that there is some advantage to picky behavior in choosing a substrate for settling as a competent larva. Planktotrophic larvae often disperse far from their site of fertilization and therefore must drift and/or swim until they find a suitable adult habitat (Salinas-de-León 2012). Proposed mechanisms for larval detection of a suitable settling habitat include the recognition of changes in turbulence regimes in oysters (Fuchs et al. 2015), chemosensing in cue plumes of adult prey type in the nudibranch *Phestilla sibogae* (Hadfield & Koehl 2004), passive drifting until a suitable substratum is found (Keough & Downes 1982), and others. Diverse and interesting exploratory behaviors in response to settling substrata have been described in the larval bryozoan *Membranipora* (Matson et al. 2010), but little is known about potential behavioral mechanisms to find suitable substrate in the first place in this species. We hypothesize that cyphonautes larvae use chemosensation in the water column in settlement selection and predator avoidance during their competency period.

We were interested in testing whether or not cyphonautes larvae altered their locomotory behavior in response to plumes of suspected positive and negative cue in a flow environment. The process of navigation through fluids to find a substrate has been studied in other animals and forms of larvae. Parasitic barnacle nauplii have been shown to follow plumes of host scent through the water in order to locate a host for settlement (Pasternak et al. 2004). Additionally, larvae of the genus *Mytilus* will selectively follow or avoid scent plumes depending on whether the scent is associated with a positive stimulus (like settlement substrate) or a negative stimulus (like the scent

of a predator) (Morello & Yund 2016). Chemosensation in other animals is well-documented. Members of *Virbia lamae*, the bog moth, use chemosensation to find mates (Cardé et al. 2011). Larvae have demonstrated similar capabilities during settlement selection (Pasternak et al. 2004, Morello & Yund 2016). Chemosensation has been shown to play a role in predator avoidance in many animals (Woodin et al 1995, Yohe et al. 2018, Morello & Yund 2016).

We are curious as to whether settlement cues extend beyond those encountered once the larvae reach the algae, particularly how the larvae manage to navigate to the algae in the first place. We are also curious to see how the presence of a predator cue, from the nudibranch predator *Doridella steinbergae*, which eats adult *Membranipora*, impacts behavior of cyphonautes larvae (Yoshioka 1986). The overall goal of this study is to assess the role that chemosensation plays in the locomotory behavior of competent cyphonautes larvae.

Methods

Later stage cyphonautes larvae were obtained with a plankton net (mesh size 150 μm) via row boat off the main dock at Friday Harbor Labs (FHL) in San Juan County, Washington. Plankton samples were immediately sorted under dissecting microscopes. Cyphonautes that could be identified using the lowest magnification setting were plated on microscope slides for photographs. Selected larvae were photographed under a compound microscope to assess competence (considered to be a function of size, per Nielsen and Worsaae 2010) and placed into well plates filled with 0.45 μm filtered seawater (SW) (Figure 1). Larvae were then allowed to acclimate for at least 5 hours in the sea table before swimming trials commenced. Fifty-two larvae were isolated and photographed for use in the experiment, but 16 were either lost during transfer,

or settled in the well plate before use. 12 individuals were allocated for each treatment: 1) Control
2) Positive Algal Cue, and 3) Negative Predator Cue.



Figure 1: photograph of cyphonautes C25, at 4X magnification under a compound microscope.
Scale bar is 300um.

To create our negative settlement cue, the nudibranch *Doridella steinbergae*, a predator of adult *Membranipora* zooids, was collected from colonies of *Membranipora* on red algae attached to the dock at FHL. Six adult nudibranchs were kept in 0.5L of 0.45 μ m filter SW for 36 hours in order to condition the water with their scent. A similar procedure for conditioning SW was a predator cue showed that 3 live *D. steinbergae* in 1L of water was a strong enough cue to induce maximum defensive spine growth in adult *Membranipora* (Harvell 1998, Iyengar & Harvell 2002). This conditioned SW was then filtered through a 15 μ m sieve and 125mL of cue was added to 5mL of SW containing Fluorescein. To create our positive settlement cue, we collected pieces of colony-free red alga *Mazzaella splendens* off the FHL docks and let it sit in a container of 0.45 μ m filtered SW for 36 hours. The conditioned algal SW was also filtered through a 15 μ m sieve and 125mL of conditioned water was added to 5mL of SW containing Fluorescein. Control cues consisted of 125mL of 0.45 μ m filtered SW added to 5mL of SW containing Fluorescein. Fluorescein came from a single source of previously diluted stock, so the exact concentration was not known but the same amount was used for all trials. Control trials with filtered SW not containing Fluorescein were not run due to time constraints, but a study using cue plumes stained with Fluorescein showed no behavioral effects in nudibranch veligers between control water with and without Fluorescein, so we expect any response to Fluorescein to be minimal (Hadfield & Koehl 2004).

Our swimming arena consisted of a 2.25 cm x 10 cm PlexiGlass tank connected via plastic tubing to a reservoir of 0.45 μ m filtered seawater kept at approximately ambient local sea temperature in a sea table. An aquarium pump set at 3.2V was used to ensure a steady yet gentle flow for the larvae and incoming cue. To produce laminar flow through our arena, a diffuser made from a Scrubbie sponge was put in front of the incoming seawater port which directed water relatively evenly across the area of interest in the arena (Figure 2, structure A). A wall on the end

opposite of flow with two bottom openings directed the water downward at the base of the arena to slow water flow and keep the larvae from getting washed completely out of the arena (Figure 2, structure B). Above the arena, a USB camera with a close-up lens was affixed to a movable track so the larva could be viewed across the length of the arena (Figure 2). A 23 gauge needle attached to PE 50 tubing was used to deliver a small steady volume of our cue. Volumetric flow rate of cue was controlled using a Gilmont syringe. Water was terminally released through a v-shaped port on the opposite wall from flow (Figure 2).

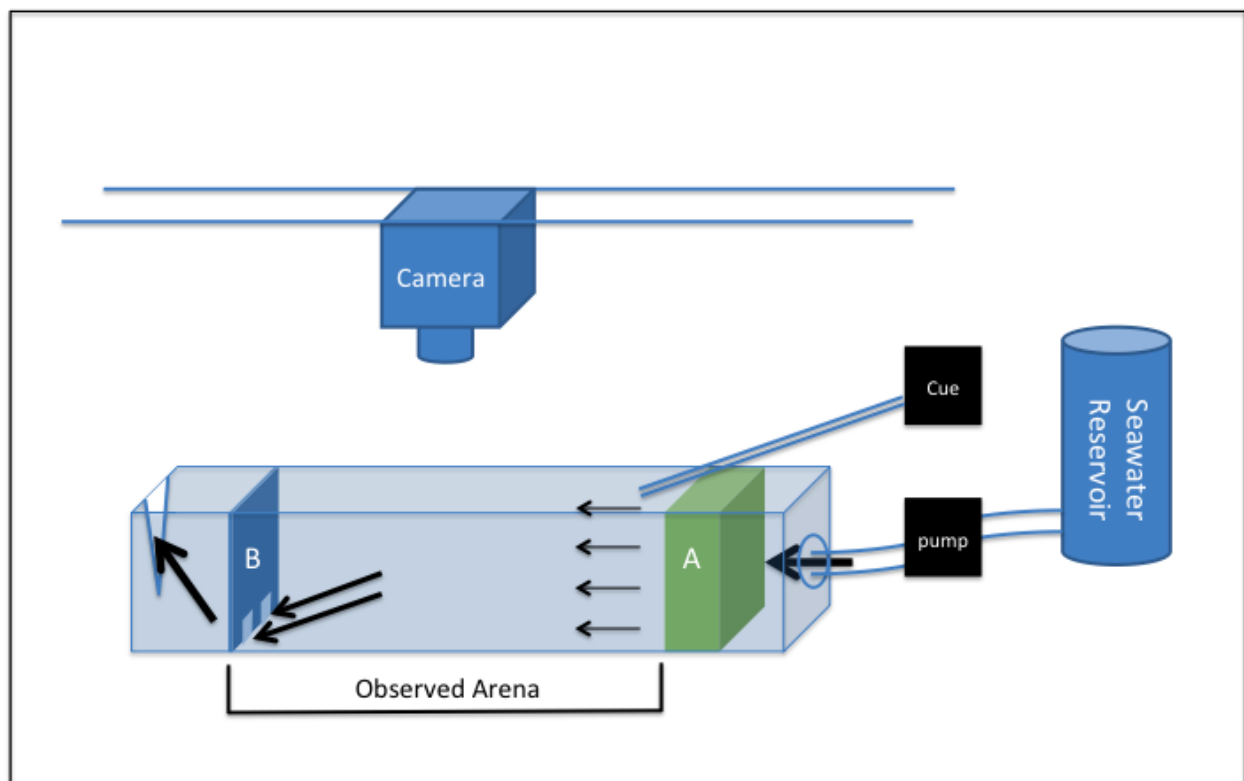


Figure 2. Diagram of experimental set-up depicting swimming arena with A) Scrubie sponge flow diffuser, B) wall flow modulator that directs flow toward the base of the tank. Black arrows depict water flow, with thicker arrows representing faster, more restricted flow. Observed arena stretched between Structures A and B.

For phase one of experimentation, flow was established prior to putting the cyphonautes into the tank, and stimulus was introduced as a steady plume (via a Gilmont syringe) upstream at the start of the trial. Larvae were placed individually in the middle of the tank and recorded until 5 minutes had passed or until the cyphonautes left the tank via a small series of openings at the bottom of the solid flow barrier (Figure 1, Structure B). Video footage was recorded using the microcamera connected to an Ubuntu 16.04 linux laptop running Cheese (version 3.32.1) software. All 12 trials for each treatment were run consecutively in a single session lasting approximately 3hrs. Between each individual trial, the SW reservoir, arena water, and cue were switched with water/cue maintained at ambient SW temperature in a flow-through sea table to stabilize temperature across trials. Between treatments, all instruments were cleaned and the cue line was flushed with the next cue.

For phase two of experimentation, individuals from each group were randomly selected to undergo cross-treatment with a second stimulus. Two individuals initially exposed to the control plume during phase one trials were run through positive cue trials and two through negative cue trials. Two larvae from phase one negative cue trials were run through positive cue trials, and two larvae from phase one positive cue individuals were run through negative cue trials. We conducted this second portion of the experiment to evaluate individual-specific behavioral responses to being subjected to trials. More cross trials were not possible due to loss of larvae during phase one trials, and due to time constraints.

Videos were visually assessed and time spent moving along the tank bottom towards or away from incoming flow of cue (upstream vs. downstream swimming), against the tank walls, moving perpendicular to the direction of flow, and moving in circles were calculated. Whether or not the individuals exhibited crawling along the bottom of the tank was also noted. We determined

whether a cyphonautes was crawling by assessing whether it was on the bottom of the tank (and had no shadow), in an upright position, and moving consistently in that position for more than 3 seconds. Circular swimming was defined as movement in a distinctly circular path that maintained the larva in the same area in the flow-through arena. When circling, individuals were either oriented horizontally when viewed in relation to the camera with the non-ciliated point directed toward the center of the circle, or oriented upright with the apical end closest to the camera. Statistical analyses (one-way ANOVA and Tukey mean comparison) and data visualization were completed in RStudio (RStudio version 1.1.463). An α level of 0.05 used to ascertain statistical significance.

Results

Phase One: Individual Trials

The data showed significant differences in time spent travelling in a direction perpendicular to flow, time spent moving toward the stimulus, and time spent circling (Figure 3, Table 1). Additionally, differences in the presence or absence of crawling behavior was significant, with crawling behavior observed only in the positive (algae) cue group. This “exploratory” crawling behavior previously described by Matson et al. 2010 occurred in four out of twelve algae-exposed larvae and was not observed in the two other trial groups. Time spent swimming away from the stimulus and time spent at the arena walls (when the larvae were largely at the surface and difficult to detect during video analysis) were not significantly different between treatments. Due to small sample size and large spread of the data, there is a large standard deviation, which is worthy of note when discussing our results (Table 1).

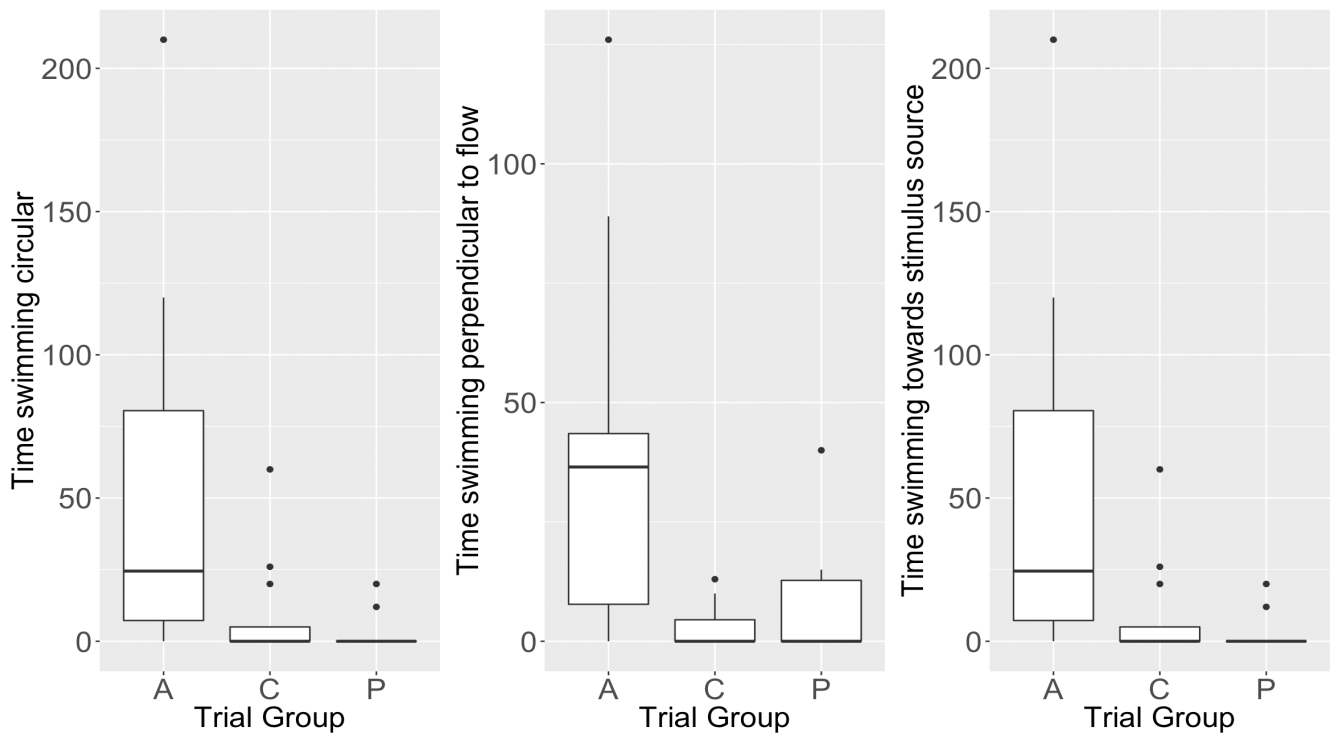


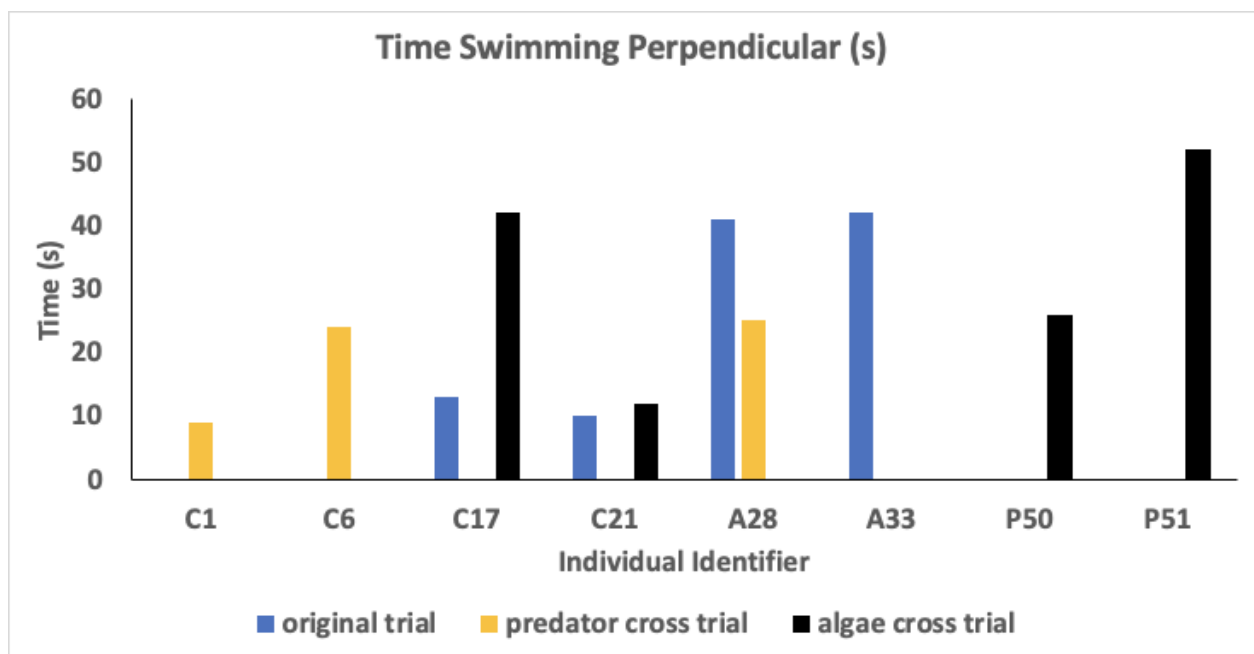
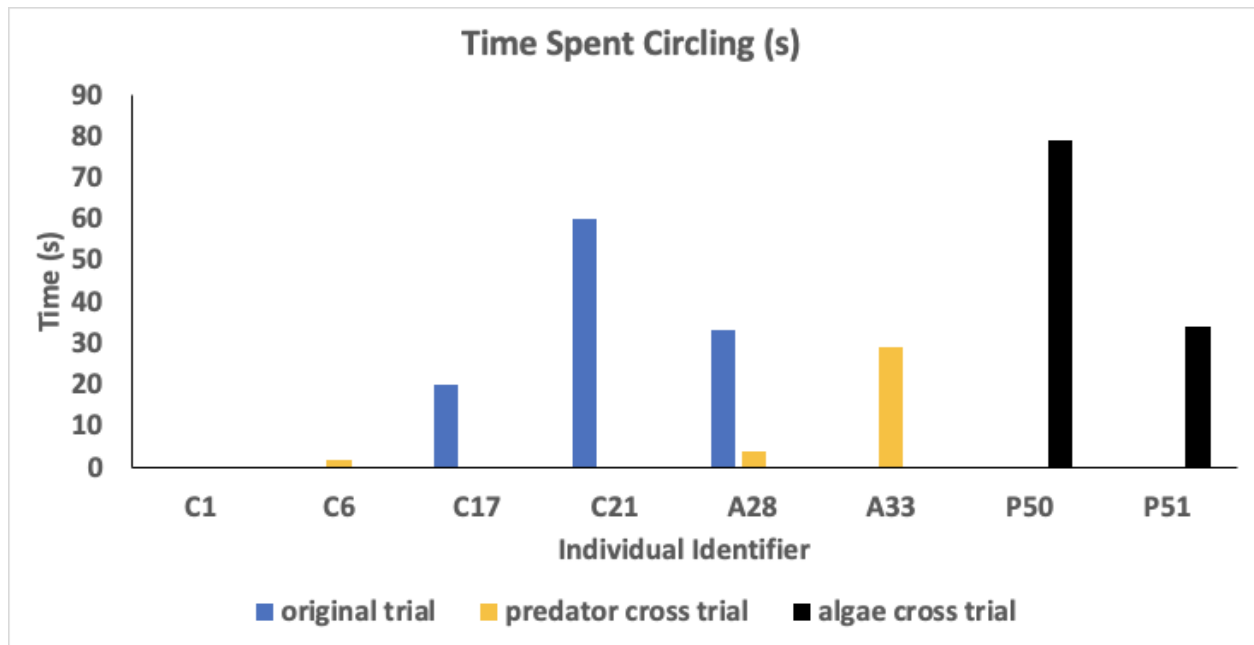
Figure 3: Box plots for the three behavioral types for which significant differences were found between treatments. Trial group A refers to the algal cue treatment, trial group C refers to the control treatment, and trial group P refers to the predator cue treatment. Time is in seconds.

Trial Category	Time perpendicular swimming	Time Away from stimulus	Time circling
Control-Alga	0.00290**	0.211	0.0445*
Control-Predator	0.883	0.732	0.941
Predator-Alga	0.0101*	0.0469*	0.0273*

Table 1: *p*-values for pairwise Tukey comparison of means. Values less than 0.05 are considered to be significant. A * denotes a result where $p < 0.05$, and a ** denotes a result where $p < 0.005$

Phase Two: Cross Trials

When assessing behavior the results for our cross trials were mixed in terms of consistency to our phase one results. The three significant continuous variables from phase one trials were assessed in the cross trials. Larvae did not behave the same way regardless of cue, in fact they appeared to react differently to their phase one cue than they did to their phase two cue from most trials. So, members from the algal group reacted similarly to predator cue as members of the predator group did. The large time differences in behavior for most of the cross trials indicate that our results are not an artifact of reaction bias within each of the groups, since members of each group respond differently to different stimuli (Figure 3). This finding suggests that the differences observed between groups in the individual experiments may be a result of the different cues the larvae encountered.



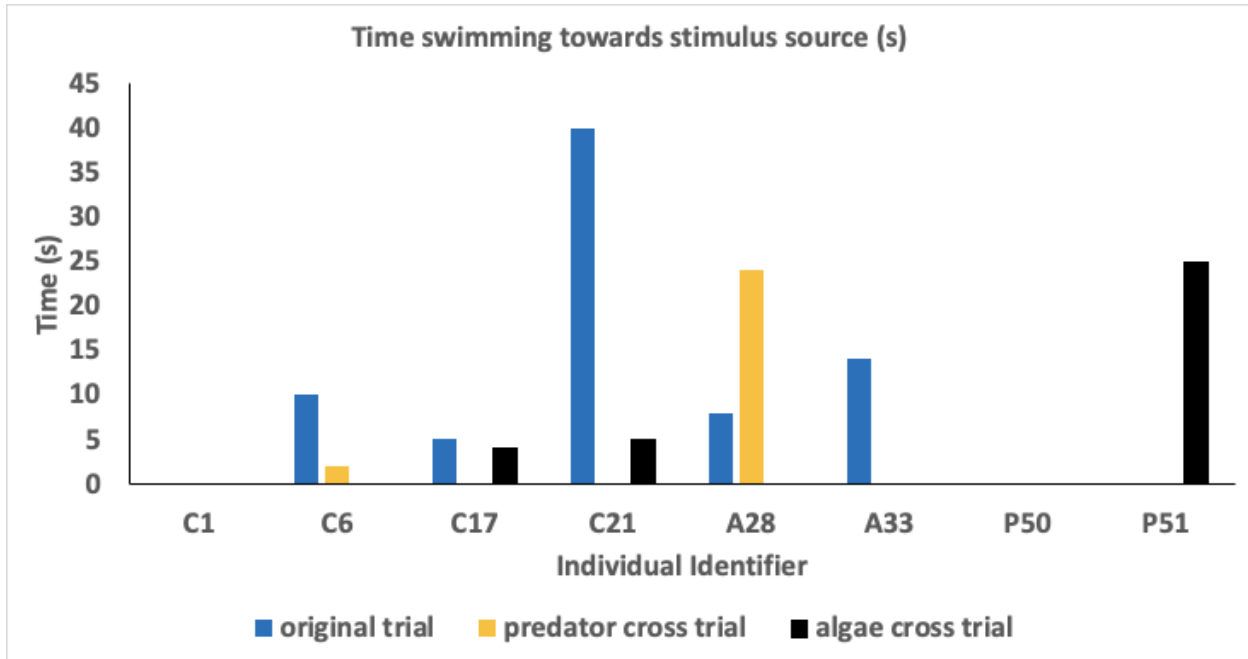


Figure 3: Results of cross-trial studies. The x-axis denotes individuals used in phase one trials that were re-run through phase two trials. Groups starting with A refer to individuals that were exposed to the algal cue during phase one trials, those starting with C refer to phase one exposure to the control cue, and those starting with P refer to phase one exposure to the predator cue. The blue bar depicts the response during the phase one trial, and the response during the cross trial is depicted by either a black bar (meaning their phase two cue was algae) or yellow bar (meaning their phase two cue was predator). Time is in seconds.

Discussion

The goal of this study was to assess the locomotory behavioral responses of cyphonautes larvae to various chemical cues - namely, a suspected positive algal cue and a suspected negative predator cue. We hypothesized that if cyphonautes larvae were able to detect a positive or negative cue, they would swim primarily towards the source of a positive stimulus, like algae, and away

from the source of a negative stimulus, like predator of the adult *Membranipora*. After looking at the results, it is clear that there are indeed behavioral differences in cyphonautes in response to different cue plumes in flow.

It is worth noting that movement away from the source of stimulus was not significantly different between any of the three groups - in each, there was a similar amount of time spent swimming and/or passively drifting downstream. Given the video quality, it was difficult to distinguish active swimming downstream from passive drifting downstream. However, upstream swimming towards the stimulus source was significantly different for each group, with the larvae exposed to algal cues spending more time swimming upstream towards the source of the stimulus than those in other treatment groups. Both the control group and the predator group swam upstream in negligible amounts (Figure 1, Table 1). This result suggests that chemosensation plays a role in detection of algal cues for settling and that cyphonautes larvae are able to assess at least in part where the cue is coming from. This could explain how competent larvae are able to locate algae on which to settle. Many of the larvae that swam upstream also displayed crawling on the bottom of the tank - a behavior previously described as an exploration tactic on suitable settlement substrates (Matson et al. 2010). We interpreted this behavior as a response to algae cues, since only algae-exposed cyphonautes exhibited it except for one cross treatment using a predator cue.

Other behaviors that differed significantly in the three groups are time spent swimming perpendicular to flow, and time spent swimming in a circular pattern. For these behaviors, cyphonautes exposed to algal cues spent the most time doing each. A new cyphonautes exploratory behavior while settling may have been detected in this study, which we have characterised here as “circular swimming”. Circular swimming was observed in multiple individual larvae, primarily when exposed to the positive algae cue. Additionally, this behavior appear in phase two trials in

individuals P50 and P51 when they were exposed to algal cue and were not present in their phase one trials when exposed to predator cue (Figure 3). This circular swimming behavior appears to expose the sensory pyriform organ that is used to sense settlement substratum (Strathmann et al. 2008) systematically to the water column and may help the individual detect the more concentrated end of a dispersing cue plume. Circular and perpendicular swimming may be exploratory mechanisms deployed by larvae ready to settle. No significant differences were found in time spent swimming away from the cue in any of the three treatments. One possible interpretation is that cyphonautes may not be able to recognize the scent of predators. Alternatively, larvae may sense but not respond to the predator of adult *Membranipora* that we assessed here. This may be because the nudibranch *D. steinbergae* is a widespread, mobile predator and therefore predator avoidance while settling may prove to be too costly of an endeavor for Cyphonautes.

Cross trials consisted of subjecting members of each group to different stimuli - select members of the algal group were exposed to the predator cue, select members of the predator group were exposed to the algal cue, and select members of the control group were exposed to both the algal cue and the predator cue. The cross trials suggest that there are minimal behavioral biases in each group - it appears that larvae initially exposed to the predator cue respond similarly to the algae cue as individuals that were initially exposed to the algae cue, and vice-versa for predator cues. The control group responses were consistent with our hypotheses given the observed responses to different cues during our individual trials. However, the number of cross trials that could be performed was limited by time and the number of individuals that were recovered after phase one trials, so these results are largely descriptive and no statistical analysis could be applied. For future study, it could be useful to further understand the role that individual behavioral variability has on the results. It would be interesting to run entire cohorts through cross trials at

once to see if group behavior differs largely from individual behavior.

It is also important to acknowledge the caveats of this study. Firstly, during most trials, we noticed an accumulating amount of cue (revealed by Fluorescein) that pooled on the floor near the outflow of the tank. This was likely due to the low flow speed of water in the tank and the higher density of SW with Fluorescein compared to seawater. The behaviors exhibited by the cyphonautes may have been affected by the pooling cue on the tank floor. Additionally, our trials were relatively short due to the time constraints of this project. Ideally, we could have extended the trials to twenty minutes instead of using five minutes of observation as our benchmark. Vertical movement through the column was difficult to assess with our camera perspective, as was behavior along the walls of the tank so important information may have been lost.

Interesting future directions of study could be to test behavioral responses to cues from other species of algae and other species of adult-form predators. Another interesting investigation would be to understand why circling behavior and swimming perpendicular to flow are responses to algal cues. Perhaps these behavioral responses aid in finding substrate to settle on, just as following a scent plume to its source would. Additionally, investigating behavioral responses of cyphonautes to different flow regimes could provide more information as to how the larvae mediate their responses to scent cues. Meaningful results may also be gained from exposing large groups of cyphonautes to our experimental design. Vertical movement in response to cues would be another valuable variable to consider, as larvae do not exist in a merely two-dimensional world. Assessing the speed at which the larvae swim would allow for determination of the tradeoffs between taking the time to explore algal blades or leaving a blade before exploring further.

References

- Atkins D (1955). The ciliary feeding mechanism of the cyphonautes larvae (*Polyzoa Ectoprocta*). *Journal of the Marine Biological Association of the United Kingdom* 34: 451–456.
- Cardé, R. T., Cardé, A. M. and Girling, R. D. (2012), Observations on the flight paths of the day flying moth *Virbia lamae* during periods of mate location: do males have a strategy for contacting the pheromone plume?. *Journal of Animal Ecology*, 81: 268-276. doi:10.1111/j.1365-2656.2011.01887.x
- Denley D, Fuchs, H. L., Gerbi, G. P., Hunter, E. J., Christman, A. J., & Diez, F. J. (2015). Hydrodynamic sensing and behavior by oyster larvae in turbulence and waves. *Journal of Experimental Biology*, 218(9), 1419–1432. <https://doi.org/10.1242/jeb.118562>
- Fuchs, H. L., Gerbi, G. P., Hunter, E. J., Christman, A. J., & Diez, F. J. (2015). Hydrodynamic sensing and behavior by oyster larvae in turbulence and waves. *Journal of Experimental Biology*, 218(9), 1419–1432. <https://doi.org/10.1242/jeb.118562>.
- Hadfield, M. G., & Koehl, M. A. R. (2004). Rapid behavioral responses of an invertebrate larva to dissolved settlement cue. *Biological Bulletin*, 207(1), 28–43. <https://doi.org/10.2307/1543626>.
- Harvell CD (1998) Genetic variation and polymorphism in the inducible spines of a marine bryozoan. *Evolution* 52:80–86.
- Iyengar, E. V., & Harvell, C. D. (2002). Specificity of cues inducing defensive spines in the bryozoan *Membranipora membranacea*. *Marine Ecology Progress Series*, 225(Harvell 1984), 205–218. <https://doi.org/10.3354/meps225205>

- Keough, M., & Downes, B. (1982). Recruitment of Marine Invertebrates: The Role of Active Larval Choices and Early Mortality. *Oecologia*, 54(3), 348-352. Retrieved from <http://www.jstor.org/stable/4216774>.
- Metaxas A, Short J (2014). Selective settlement by larvae of *Membranipora membranacea* and *Electra pilosa* (Ectoprocta) along algae blades in Nova Scotia, Canada. *Aquatic Biology* 21:47-56. doi: 10.3354/ab00569.
- Matson PG, Steffen BT, Allen RM (2010.) Settlement behavior of cyphonautes larvae of the bryozoan *Membranipora membranacea* in response to two algal substrata. *Invert Biol* 129:277–283.
- McEdward L & Strathmann R (1987). The Body Plan of the Cyphonautes Larva of Bryozoans Prevents High Clearance Rates: Comparison with the Pluteus and a Growth Model. *Biological Bulletin* 172(1):30-45. doi:10.2307/1541604.
- Morello S.L. and P.O. Yund (2016). Response of Competent Blue Mussel (*Mytilus edulis*) Larvae to Positive and Negative Settlement Cues. *Journal of Experimental Marine Biology* 480:8-16.
- Nielsen C & Worsaae K (2010). Structure and occurrence of cyphonautes larvae (bryozoa, ectoprocta). *Journal of Morphology* 271:1094-1109. doi:10.1002/jmor.10856.
- Pasternak Z, Bernd Blasius, Avigdor Abelson (2004). Host location by larvae of a parasitic barnacle: larval chemotaxis and plume tracking in flow. *Journal of Plankton Research*, 26(4): 487–493, <https://doi.org/10.1093/plankt/fbh040>.
- RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA.
- Salinas-de-León P, Jones T, Bell JJ (2012) Successful Determination of Larval Dispersal Distances and Subsequent Settlement for Long-Lived Pelagic Larvae. *PLOS ONE* 7(3):

e32788. <https://doi.org/10.1371/journal.pone.0032788>.

Strathmann, R. R., Foley, G. P. and Hysert, A. N. (2008), Loss and gain of the juvenile rudiment and metamorphic competence during starvation and feeding of bryozoan larvae.

Evolution & Development, 10: 731-736. doi:10.1111/j.1525-142X.2008.00287.x

Stricker SA, Reed CG, Zimmer RL (1988). The cyphonautes larva of the marine bryozoan

Membranipora membranacea. I. General morphology, body wall, and gut. *Canadian Journal of Zoology* 66:368– 383.

Wilson D P (1952). The influence of the nature of the substratum on the metamorphosis of the

larvae of marine animals, especially the larvae of *Ophelia bicornis* Savigny. *Ann Inst Oceanogr* 27: 49-156.

Woodin S.A., S. M. Lindsay, and D. S. Wethey (1995). Process-specific recruitment cues in marine sedimentary systems. *Biol. Bull.* 189(1):49-58.

Yohe L., Philipp Brand, (2018). Evolutionary ecology of chemosensation and its role in sensory drive, *Current Zoology*, 64(4):525–533, <https://doi.org/10.1093/cz/zoy048>.

Yoshioka, P. (1986). Competitive coexistence of the dorid nudibranchs *Doridella steinbergae* and *Corambe pacifica*. *Marine Ecology Progress Series*, 33(1), 81-88. Retrieved from <http://www.jstor.org/stable/24825617>.