

Avoiding low-oxygen environments: oxytaxis as a mechanism of habitat selection in a marine invertebrate

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ABSTRACT: Oxygen-poor habitats are increasingly common in aquatic environments. Human activities are accelerating the spread of oxygen-poor environments, yet the way in which larvae avoid low-oxygen conditions remains poorly resolved. For organisms with a sessile or sedentary adult phase, habitat selection is crucial, and many organisms show sophisticated responses to various habitat cues during colonization. Whether oxygen availability serves as such a cue is unknown, yet increasingly, it seems that oxygen is an essential limiting resource in some systems. In a series of experiments, we manipulated oxygen levels during dispersal and colonization in larvae of the model marine invertebrate *Bugula neritina* in the laboratory. We found that, in the presence of lower oxygen levels, larvae reduce the time spent in habitat exploration and that they delay settlement. We also found that larvae avoid hypoxic water (positive oxytaxis) — the first such demonstration for marine larvae. All of these behaviors may decrease the likelihood of colonizing low-oxygen habitats in nature. Our results suggest that marine invertebrate larvae, in this species at least, can use oxygen availability as an initial cue for habitat selection but that additional factors (e.g. biofilms) determine settlement patterns.

KEY WORDS: *Bugula neritina* · Colonization · Dispersal · Hypoxia · Larval behavior

INTRODUCTION

Oxygen is a critical resource for most organisms. If oxygen falls below a minimum threshold of tolerance, physiological and behavioral responses are induced that can determine the distribution and abundance of organisms at many spatial scales (Diaz & Rosenberg 1995, Stillman & Somero 1996, Gray et al. 2002, Jacobsen 2008, Vaquer-Sunyer & Duarte 2008, Vargas et al. 2010, Long et al. 2014). At smaller spatial scales, for example, oxygen availability can affect the physiology and morphology of intertidal organisms and therefore limit their vertical distribution (Burggren & McMahan 1981, Stillman & Somero 1996, Lagos et al. 2011, Lucon-Xiccato et al. 2014). At larger scales, species richness can show altitudinal

clines with decreasing partial pressures of oxygen (Jacobsen 2008, DuBay & Witt 2014).

Increasingly, it appears that many human activities are causing the proliferation of low-oxygen environments in aquatic systems. Eutrophication is generating extended hypoxic environments that can lead to the formation of 'dead zones' (Diaz 2001). At large scales, dead zones represent a collapse of aquatic ecosystems, with mass mortality events that can extirpate local communities and the ecosystem services they provide (Altieri & Witman 2006, Diaz & Rosenberg 2008). While naturally occurring regions of low oxygen are common, anthropogenic influences have caused an increase in prevalence and size of low-oxygen regions for over 50 years, and hypoxic environments now represent a major threat to marine

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systems worldwide (Diaz & Rosenberg 1995, Diaz 2001). An understanding of how organisms cope with or avoid low-oxygen environments has therefore taken on importance and urgency.

Human activities can cause low-oxygen environments over larger spatial scales (e.g. km: Joyce 2000, Diaz & Rosenberg 2008, Rabalais et al. 2010), but they can also influence smaller-scale variation in oxygen availability. Habitat modifications by humans can result in low-oxygen environments in marine coastal areas at the small spatial scale where larval settlement and habitat selection occurs (Ferguson et al. 2013, Wilding 2014). Structures such as piers, marinas and docks are designed to reduce water movement so that boats can be docked and boarded safely. These human-made structures are typically colonized by a specialized guild of sessile marine invertebrates known sometimes as a 'fouling community'. Such assemblages can be dense, occupying all of the available human-made surfaces, and wet biomasses can exceed 1 kg per square meter of area. Fouling communities are often topographically complex and can increase the thickness of the boundary layer as well as inducing skimming flow, which further reduces the flow of water adjacent to the community (Vogel 1994). The combination of exceedingly low-flow environments and the metabolic demands of dense fouling communities mean that oxygen levels adjacent to these communities often fall to less than half that of those in freely streaming water, and can sometimes even be zero (Vogel 1994, Ferguson et al. 2013). In effect, docks, marinas and piers, which increasingly make up a significant proportion of the world's coastlines (Duarte et al. 2013), are generating oxygen-limited microenvironments that are otherwise rare in unmodified habitats with higher flows.

In natural habitats, low-flow conditions are commonly associated with soft-bottom communities. In the absence of scouring flows, most upward-facing hard surfaces will accumulate a coating of sediment. However, human-made structures are unusual in that they provide both low-flow conditions and hard surfaces that face sideways and downwards, thereby avoiding sedimentation (Glasby et al. 2007, Ruiz et al. 2009, Duarte et al. 2013, Ferguson et al. 2013). The oxygen-limited habitat associated with human-made structures presents a significant challenge to the organisms that live there: species must either cope with or avoid these low-oxygen conditions.

Many species either avoid low-oxygen conditions or express traits that allow them to cope with such conditions. Mobile animals, such as fishes and zoo-

planktonic organisms have the opportunity to swim vertically in the water column to find more oxygen-rich environments (Breitburg 2002, Craig et al. 2005, Ludsin et al. 2009, Palomares-Garcia et al. 2013, Teuber et al. 2013, Switzer et al. 2015); however, sessile organisms are less able to avoid the stressful conditions of low-oxygen environments, as adults live permanently attached to the substrate or have limited displacement. Several species cope with low-oxygen environments by expressing phenotypes that either reduce oxygen demand or increase oxygen uptake. Some species reduce their metabolic rate, others increase gill surface areas, blood pigment concentrations and oxygen affinities (Truchot 1980, Lallier & Truchot 1989, Dahlhoff et al. 2002, Sollid et al. 2003, Nilsson et al. 2012). The mechanism used will depend in part on oxygen demands of the organism, habitat characteristics, and the frequency and magnitude of the hypoxic conditions (Stillman & Somero 1996, Altieri & Witman 2006). However, all of these coping strategies have their limits when oxygen levels fall below some minimum level, and organisms will still experience the negative effects of hypoxia when oxygen levels are sufficiently low.

For sessile organisms, the mobile larval phase may be the only chance that individuals have to avoid low-oxygen environments; in this way, habitat selection is critical for their fitness. There is extensive literature on the factors affecting larval habitat selection, including the presence of competitors (Grosberg 1981, Bullard et al. 2004), pollution (Hunte & Wittenberg 1992, Knott et al. 2009), light intensity and quality (Mundy & Babcock 1998), chemical cues (Thiyagarajan 2010), biofilm composition (Wieczorek & Todd 1997, Cheung et al. 2014, Whalan & Webster 2014), the presence of conspecific adults (Jensen 1989, Gebauer et al. 2011) and properties of the substratum (Roberts et al. 1991, Tapella et al. 2012). Larvae may use such cues as indicators of habitat quality in order to maximize their chance of surviving to post-settlement (Raimondi & Keough 1990, Donahue 2006). Oxygen availability would seem to be another important cue for habitat selection. Several studies have examined how hypoxic conditions affect larval development and behavior including escape responses (Baker & Mann 1992, Leung et al. 2013, Shin et al. 2013); but to our knowledge, no study has examined whether marine invertebrate larvae avoid settling in low-oxygen microhabitats.

In a series of laboratory experiments, we examined larval responses to differences in the dissolved oxygen content of water. Specifically, we measured the effect of oxygen content on the proportion of time

that larvae spent swimming versus exploring surfaces and the time taken to settle, as well as the oxygen preference of larvae and settlers in water under a gradient of oxygen content. We focused on the larvae of the bryozoan *Bugula neritina* (Linnaeus, 1758), as it is a model for studies of marine larval behavior and this species is known to have a relatively poor tolerance for water with low-oxygen content (Ferguson et al. 2013). Our aim is to understand how oxygen contents within the water column influence behavior and habitat-selection patterns.

MATERIALS AND METHODS

Study species and general methods

Bugula neritina (hereafter *Bugula*) is a ubiquitous arborescent bryozoan that inhabits submerged hard substrates at shallow depths. It occurs on artificial structures around the world and is a common member of the epibenthic community on human-made structures in Australia. *Bugula* release non-feeding larvae that are competent to settle immediately upon release. These characteristics have long made it a model species for studying larval settlement behavior (Doyle 1974). We collected mature colonies of *Bugula* from floating piers located at Royal Brighton Yacht Club, Port Phillip Bay, Victoria, Australia (37° 54' 25" S, 144° 58' 52" E) during the austral spring of 2013. We used standard methods (Strathmann 1987) to collect larvae. Briefly, colonies were maintained in constant darkness for 2 d in aquaria with constantly aerated seawater maintained at a stable temperature (~19.5°C). To collect larvae, we exposed 10 colonies to bright light in each experimental run for up to 1 h. Colonies began to release larvae within 10 min. Larvae were pipetted from the aquaria and placed in the experimental chamber assigned to each experiment. Because *Bugula* larvae prefer to settle on a roughened surface, all of the acetate sheets, petri dishes, and centrifuge tubes used as experimental settlement surfaces were sanded before use (Marshall & Keough 2003). To further encourage settlement, surfaces were maintained in aquaria with constantly aerated seawater for 1 wk prior to the experiment to develop a biofilm (Wieczorek & Todd 1997, Rius et al. 2009). In each experiment we used 0.10 µm-filtered seawater at a temperature of ~19.5°C. The oxygen content of the water (mg l⁻¹) was manipulated by bubbling nitrogen gas and monitored using fiber optical sensors connected to a Firesting O₂ fiber optic oxygen meter.

Swimming and exploring behavior

We tested the effect of oxygen content on larval behavior using a standard behavioral assay (Burgess et al. 2009). Briefly, *Bugula* larvae show clearly recognizable behaviors that can be characterized as 'swimming' and 'exploring' (Burgess et al. 2009). Swimming behavior is identified as erratic and quick movement through the water column, and exploring behavior represents a fine-scale searching behavior that occurs prior to settlement, identified by adoption of a stationary position or slow movement on the settlement surface, and includes characteristic spinning and crawling behavior (Walters et al. 1999). We randomly selected individual larvae from the pool of 10 colonies, and pipetted individuals into a petri dish (3.5 cm diameter, 1.1 cm depth) containing 10 ml of seawater with an oxygen content of either 7.5 mg l⁻¹ (the 'high-oxygen' treatment) or 1.8 mg l⁻¹ (the 'low-oxygen' treatment). These levels were established prior to the addition of the larvae to the petri dish. During the experiments we did not add air or nitrogen to avoid any effect of the bubbles on larvae; however, a preliminary test showed that the water oxygen content can vary a small amount (up to 0.2 mg l⁻¹) during the time necessary for our experiments. Immediately after placing the larvae in the experimental treatment, individual larvae were observed under a microscope for 5 min, and the amount of time spent exploring settlement surfaces and the amount of time spent swimming in the water column were recorded. Previous studies have shown that larval age affects larval behavior (Burgess et al. 2009), so we also tested the effects of larval age, using larvae that were 0 and 60 min old. In total, we measured the behavior of 40 larvae (10 per age class and seawater treatment combination) across 3 experimental runs.

Settlement time

To measure the settlement time of *Bugula* in water with different oxygen contents, randomly selected individual larvae were collected from a pool of 10 colonies and then pipetted into centrifuge tubes filled with seawater (1.5 ml). Again, the oxygen content of the seawater in the centrifuge tubes was manipulated to be either high or low (1.8 or 7.5 mg l⁻¹, as in the previous experiment) prior to the addition of a larva. Then, we monitored individual tubes continuously and recorded the time until the settlement occurred in each larva. Settlement in *Bugula* is straightforward to observe as larvae attach perma-

nently to the substrate. We measured 10 larvae per treatment across 2 experimental runs.

Habitat selection: association and settlement

We created a larval settlement choice chamber that contained a longitudinal oxygen gradient. For these experiments, we used 2 acrylic chambers, 80 × 15 × 15 cm, filled with 0.10 µm-filtered seawater at ~19.5°C. At one end of the chamber we bubbled compressed air (as a control), and at the other end we bubbled nitrogen gas. The turbulence created by the bubbles at both ends was attenuated by screens of 100 µm mesh, located at a distance of 10 cm from each end of the chamber. A stable oxygen gradient formed within 1 h of the commencement of bubbling, with typical oxygen contents between 1.8 and 5.9 mg l⁻¹ (see Fig. 1 for an example of the experimental oxygen gradient). Ten experimental runs were conducted in each of the follow experiments, 2 each day for 5 d, alternating the oxygen-nitrogen orientation in each chamber and, as *Bugula* larvae exhibit phototaxis, covering the chambers to keep them in constant darkness to exclude potentially confounding effects of light (Wendt & Woollacott 1999).

To measure larval association with different levels of oxygen, we pipetted larvae across 5 locations (20 larvae per location, collected immediately after spawning) at 10 cm intervals along the chamber (100 larvae in total). Thirty min after larvae had been introduced, acrylic separators were introduced into the chamber 10 cm apart to isolate the larvae and

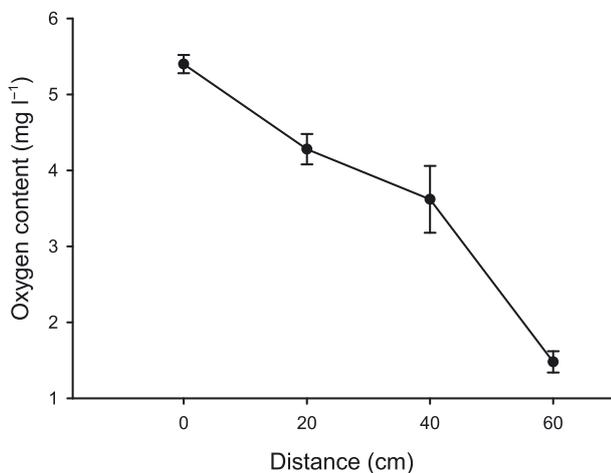


Fig. 1. Measured mean (\pm SE) oxygen content (in our test chambers used for examining the effect of oxygen availability on the distribution and settlement of *Bugula neritina* larvae (n = 10)

keep them in the position they were located; after this, larvae were collected using funnels and counted. The number of larvae that were in each section was then recorded.

To measure settlement, we repeated the same protocol as above but also covered the inner surface of the chamber with a contiguous piece of acetate sheet that had been pre-roughened and then pipetted larvae into the chamber as described above. Thirty min after larvae had been introduced, the acetate sheet was retrieved and the position of each settled larvae was measured. We then repeated this experiment with a longer duration, measuring the larval settlement 60 min after release into the chamber.

Statistical analyses

The effects of oxygen content and larval age on larval behavior and larval settlement time were tested with a mixed-model generalized linear model (GLM). Oxygen content and age were fixed factors; experimental run (day) was a random factor. Non-significant interactions including the random factor were eliminated from the model (Quinn & Keough 2002). All ANOVA were done using the statistical software Systat ver.13. Association and settlement selection data were analyzed with χ^2 tests using Microsoft excel v.14.1.0. Alpha value was set at 0.05 in each test.

RESULTS

Oxygen content affected the behavior of *Bugula neritina* larvae. In the swimming and exploring behavior experiment, the larvae explored potential settlement surfaces significantly less when oxygen levels were low (Fig. 2). The effect of oxygen content was consistent regardless of larval age and experimental run (Table 1). Also, we found significant differences in the settlement time experiment (Table 1); here, *Bugula* larvae delayed their settlement in the presence of low oxygen (Fig. 3).

In the habitat-selection experiment we found that *Bugula neritina* larvae tended to avoid swimming in water with low-oxygen content ($\chi^2 = 4.55$, $p = 0.03$). Unsettled larvae were strongly associated with high oxygen content (3.7–4.8 and 4.8–5.9 mg l⁻¹; Fig. 4). The oxygen preference of swimming larvae was not reflected in their settlement. We detected no settlement preference between low- and high-oxygen contents. This result was consistent when settlement

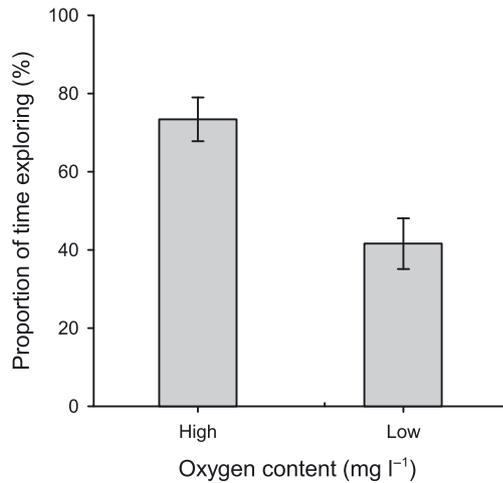


Fig. 2. Exploring time of *Bugula neritina* larvae at low and high levels of oxygen content (1.8 and 7.5 mg l⁻¹). Bars represent means (\pm SE) of the percentage of time used exploring

Table 1. ANOVA for effect of oxygen content, day, age and factor interactions on exploring time and settlement time of *Bugula neritina* larvae. Both models were edited and reduced after testing for nonsignificant interactions between random and fixed factors

Source	df	MS	F	p
Exploring time				
Oxygen content	1	10 115.58	13.49	0.001
Day	2	1217.50	1.62	0.212
Age	1	6.97	0.01	0.924
Age \times Oxygen content	1	557.26	0.74	0.394
Error	34	749.52		
Settlement time				
Oxygen content	1	351.649	33.59	<0.001
Day	1	80.089	7.65	0.009
Day \times Oxygen content	1	58.564	6.00	0.247
Error	36	10.468		

was assessed both 30 and 60 min after larvae were placed in the experimental chambers (30 min: $\chi^2 = 0.16$, $p = 0.68$; 60 min: $\chi^2 = 0.32$, $p = 0.56$).

DISCUSSION

Low-oxygen conditions have always been a feature of aquatic systems, but they are increasing in prevalence and severity (Diaz 2001, Diaz & Rosenberg 2008). We found evidence that settling larvae show a variety of behaviors to avoid low oxygen during colonization. *Bugula* larvae delayed settlement and spent more time swimming than exploring settlement sur-

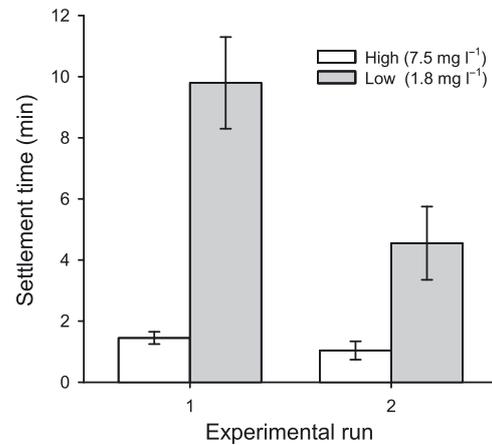


Fig. 3. Settlement time of *Bugula neritina* larvae on 2 experimental days at low-oxygen content levels. Bars represent means (\pm SE)

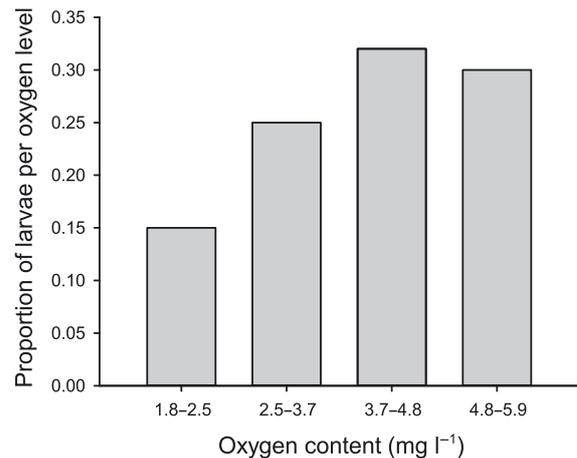


Fig. 4. Association of *Bugula neritina* larvae with water across an oxygen gradient. Bars show the proportion of larvae in each oxygen category

faces in low-oxygen conditions, both behaviors that are likely to increase the chance that larvae escape low-oxygen conditions. We also found evidence for oxytaxis—larvae preferentially associated with higher oxygen conditions compared with low-oxygen conditions when given a choice. To our knowledge, to date no studies have demonstrated oxytaxis in larvae of sessile species, although it is common in unicellular organism such as bacteria and microalgae (Hillesdon & Pedley 1996, Porterfield 1997, Lorz 2010, Yazdi & Ardekani 2012, Broszeit et al. 2013).

During dispersal and habitat selection, marine invertebrate larvae are neither entirely passive nor completely in control of their movement and destination; instead, they exert important, though limited, influence on both (Walters et al. 1999). By changing

their position in the water column and accepting or rejecting habitat, marine larvae settle non-randomly and even counter to prevailing hydrodynamics (Vogel 1994). In our study, in the presence of low-oxygen larvae swam upwards, engaged in less exploratory behavior on the benthos and avoided settlement. In the field, these behaviors would increase the probability that larvae settle in higher oxygen conditions. Previous studies have shown that oxygen availability varies over very small spatial scales (Ferguson et al. 2013); even slight water movement might therefore be sufficient to move a larva the necessary short distance to a new location with possibly higher oxygen conditions if the larva refuses to settle when encountering low oxygen and instead swims upward.

It could be argued that changes in larval behavior and settlement patterns could be the results of a direct effect of low oxygen on metabolism, causing reductions of metabolic rate. It is important to consider that *Bugula* larvae are negatively buoyant, and any metabolic depression and decrease in activity would be likely evidenced as more exploring behavior; however, we observed the opposite effect in the behavior trials. Our results show more activity in low-oxygen conditions, with larvae swimming rather than exploring; therefore, metabolic depression is not an explanation. If metabolic rates are maintained at low-oxygen levels, the differences in behavior and settlement rates are likely to be mediated by processes such as oxygen sensing, rather than metabolic rate per se.

The avoidance of low-oxygen conditions, while presumably beneficial, is not without costs. In species with nonfeeding larvae such as *Bugula*, delaying settlement carries significant fitness costs—energy expended on swimming cannot be used for post-metamorphic growth, and even brief extensions of the larval period can therefore reduce post-metamorphic performance (Wendt & Woollacott 1999, Marshall et al. 2003, Elkin & Marshall 2007, Burgess et al. 2009). Furthermore, while estimates of planktonic mortality are rare, it seems likely that these rates are high and delaying settlement will increase the risk of mortality. If behavioral avoidance of low-oxygen conditions is adaptive, then the costs imposed by delaying settlement must be balanced by other benefits, presumably a higher likelihood of settling in conditions that are more conducive to post-metamorphic success.

There are 2 broad classes of likely benefits for sessile species in avoiding low-oxygen conditions. First, aerobic respiration produces a large quantity of energy per mole of substrate and produces less toxic byproducts than anaerobic respiration. Low-oxygen environments cannot supply the high amount of oxy-

gen required to maintain aerobic metabolism. The low-oxygen levels we used in our experiments (1.8 mg l^{-1}) exceeded the minimum level recorded for this species (Ferguson et al. 2013). Metabolic functions can still be more costly at this level, as oxygen levels below the organism requirements can have a negative effect on enzymatic activity, feeding activity and changes in metabolic rate that can lead to behavioral changes in organisms (Stillman & Somero 1996, Haye & Ojeda 1998, Dahloff et al. 2002, Lagos et al. 2011).

Second, locally low-oxygen levels may act as a proxy for the intensity of local competition and the availability of food at a given settlement location. Such cues are vital because the larval stage is the last and only chance to avoid unsuitable locations for sessile species. In the habitats that *Bugula* occupies, in the water column and on settlement surfaces that are not adjacent to the benthic community, the oxygen availability exceeds 7.1 mg l^{-1} (Ferguson et al. 2013). Oxygen levels fall to low levels adjacent to respiring organisms and under exceptionally low flows within the boundary layer of a topographically complex benthic community—producing a low-quality micro-environment. Marine larvae must make rapid decisions about the long-term suitability of settlement sites using limited sensory capabilities. Studies have shown that larvae can avoid settling near residents that are superior competitors, though no study has determined the mechanism by which species recognize residents (Grosberg 1981, Young & Chia 1981, Rius et al. 2009).

The idea that a larva can recognize a whole host of different species and classify each according to its competitive ability seems unlikely. Therefore, the idea that larvae could use oxygen as a proxy for the presence of competitors becomes more plausible, given the changes in behavior we found. It has been shown that superior spatial competitors cause a greater reduction in local oxygen levels than inferior spatial competitors (Ferguson et al. 2013); therefore, the reduction of oxygen at a micro-scale level may be a reliable indicator of the presence of competitors, and the high availability of oxygen indicates the absence of strong competitors. To test this hypothesis, an experiment that orthogonally manipulates competitor presence and absence and oxygen availability is required: if larvae settle in the presence of a superior competitor under high-oxygen conditions, then our hypothesis is supported. Regardless of why larvae avoid low oxygen, it is clear that oxygen availability should be added to the canon of cues that marine larvae use to select settlement sites.

Despite finding evidence for the avoidance of low oxygen during the larval phase, we found no differences in the number of larvae that settled in high- and low-oxygen environments. This apparent paradox may be due to the nature of our experiment. In our study, *Bugula* larvae have the tendency to swim selectively to be associated to high-oxygen water. However the attachment to the substrate is a mechanism regulated by several factors, some of them unknown. In our settlement choice assays, the settlement surfaces that we offered larvae were not allowed to accumulate a biofilm under the different oxygen conditions. A recent publication indicates that biofilm can be modified by the oxygen content in the water, altering the recruitment patterns of larvae (though whether these recruitment patterns reflect settlement remains unclear: Cheung et al. 2014). Specifically for *Bugula*, preliminary work (M. E. Lagos unpubl. data) indicates that different biofilms generated under high- and low-oxygen conditions affect larvae settlement. As such, the settlement behavior we observed may have been driven by the fact that we created an artificial scenario where water oxygen levels were not reflected by biofilms on the settlement surfaces.

Alternatively, the low overall settlement rates in the chamber experiment may explain the lack of an effect of oxygen on settlement. Only about 35% of larvae settled; it is therefore possible that the settlement surfaces in this experiment were so unappealing that only the most 'desperate' subset of larvae settled and that they settled indiscriminately. By the end of the experiment the larvae were 60 min old, and as has been described in previous works, 'old' *Bugula* larvae tend to settle indiscriminately and settlement inhibitors are less effective (Marshall & Keough 2003, Gribben et al. 2006).

Considering that low-oxygen environments are spreading throughout the world, our research may be important for invasion ecology. Recent literature suggests that human-made structures may be creating habitats that are more conducive to invasive species that have close associations with low-oxygen environments and artificial structures (Glasby et al. 2007, Dafforn et al. 2009, Ruiz et al. 2009, van Kleunen et al. 2010). We focused on *Bugula neritina* because, in the literature, it has long been associated with low-flow environments, increasing the likelihood that it has evolved responses to low-oxygen conditions. An important next step will be to explore whether these larval behaviors are present in native species that may be less commonly associated with low oxygen environments.

In conclusion, it is clear that *B. neritina* detect and react to variations in the oxygen content of water. Larvae delay settlement under lower oxygen conditions and associate with higher oxygen conditions and we suggest that *Bugula* larvae use oxytaxis to maximize their chances of encountering favorable habitats.

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