Led Astray: Ascidian Larvae Preferentially Settle on Low-quality Sites with Resident Conspecifics

Yu Kai Tan[1,2,3] and Michael Montgomery[1,4]

1. Friday Harbor Laboratories. University of Washington, Friday Harbor, WA 98250.

2. Department of Ecology and Evolutionary Biology. University of Michigan, Ann Arbor, MI 48109.

3. University of Michigan Museum of Zoology Mollusk Division. University of Michigan, Ann Arbor, MI 48109.

4. University of California Santa Barbara

Abstract

Colonial ascidians are abundant encrusting organisms on boat hulls, dock pilings and marinas worldwide. In the Salish Sea of northern Washington, U.S.A. and British Columbia, Canada, *Botrylloides violaceus* is an invasive, seasonally abundant ascidian that threatens regional ecosystem health with recurrent fouling events. Thorough characterisation of its recruitment patterns and population demographics may inform mitigation efforts. Using a factorial laboratory experiment, we determined that the positive density-dependent gregarious settling behaviour of the species' tadpole larvae may be responsible for *B. violaceus*'s locally patchy distribution and rapid population growth. In an all-else-equal scenario, pre-settlement by conspecifics on a substratum otherwise perceived as low-quality or undesirable promotes subsequent larval settling. Our study further identified that *B. violaceus* larvae are able to respond readily to adult chemical cues as a trigger for settlement.

Introduction

Ascidians, or sea squirts, are well-documented as successful and rapid colonisers of natural and artificial hard substrates such as wharves, pilings and, increasingly, surfaces associated with an expanding aquaculture industry (McKenzie et al., 2016; Lambert, 2007). Over the last three decades, a number of non-native taxa have come to be considered "nuisance species" (Lambert & Lambert, 2023) whose inhibition and removal are the subject of a growing body of literature and a number of symposia (Dias et al., 2019).

One of the most widespread and problematic invasive ascidians is *Botrylloides violaceus* Oka 1927 (=*Botryllus aurantius*), the violet or lined compound tunicate (Dijkstra & Harris, 2009). Native to Japan, *B. violaceus* was first recorded from the northeast Pacific in the mid-twentieth century (Cohen & Carlton, 1999) and has now established seasonally abundant populations in suitable subtidal habitats from Baja California to Alaska (Lambert, 2003). Along with the morphologically similar *Botryllus schlosseri*, populations of *B. violaceus* in the Salish Sea of northern Washington, U.S.A. and British Columbia, Canada, appear to tolerate a range of environmental conditions (Epelbaum et al., 2009). In conjunction with the species' protracted breeding season and early production of larvae that are competent upon release (Lambert, 2003), this adaptability has made fouling events by *B. violaceus* difficult to predict and prevent. Future ocean warming, in the Salish Sea and elsewhere, is predicted to further enhance the ecological success of *B. violaceus* at the expense of native or commercially important filter-feeding taxa (Stachowicz et al., 2002; Wing & Gardell, 2022).

The adverse impacts of fouling organisms have inspired research on the distinctive features of marine environments that select and maintain traits specific to their dispersal. One such attribute, propagule broadcasting, has long been thought to confer adaptation by reducing

competition amongst conspecifics in sympatry (Janzen, 1970; Connell, 1971; Hovestadt et al., 2001). Marine organisms in high-occupancy environments such as fouling communities are known to shift along the competition-facilitation spectrum depending on density, size and functional diversity of their neighbours, with consequences for the relative costs and benefits of pelagic larval dispersal (Cameron et al., 2019). If many larvae disperse to the same site, they are likely to compete post-recruitment, reducing inclusive fitness (Burgess et al., 2015). Contrarily, settling among conspecifics may bring facilitative advantages in some sessile marine invertebrates, including, classically, barnacle cyprids (Knight-Jones & Stephenson, 1950), and also in a number of ascidians (Grosberg & Quinn, 1986; Svane et al., 1987). Some studies have suggested that this proclivity for "gregarious" or "aggregative" settling may in fact "predispose" (Rius et al., 2010) colonial ascidians to becoming invasive when introduced outside their native range. One set of experiments on non-botryllid ascidians found support for a potentially negative relationship between recruitment and adult chemical cues, which inhibited larval settlement in three of the six genera studied (Rius et al., 2010).

The present study will determine whether tadpole larvae of *B. violaceus* also follow this pattern, exhibiting a preference for recruitment sites with lower concentrations of conspecifics or conspecific cues, where intraspecific competition is likely lower, or whether they in fact prefer to settle on previously colonised sites, per Hadfield and Paul (2001). We hypothesise that these larvae will preferentially settle away from conspecifics to avoid intraspecific competition. We also hypothesise that tadpole larvae can detect chemical cues released by adult colonies, and extend time to settlement in the presence of such cues by prolonging swimming time to seek recruitment sites with a lower chemical cue concentration.

Previous work on juvenile *B. violaceus* has demonstrated density-dependent size variation. After 10 days of growth, zooids growing in the vicinity of established adult colonies are typically smaller than those growing in isolation, though the mechanism by which the larvae detect nearby conspecifics is unclear and hypothesised to be either chemical cues or depletion of food (Marshall et al., 2006). Adult *B. violaceus* have been shown to produce a metabolite, urochordamine A, that promotes settlement in other colonial ascidians (Tsukamoto et al., 1993). In our study, we test whether adult-derived, dissolved organic compounds—presumably analogous to the urochordamine A noted in Japanese populations—can produce a differential settling response in *B. violaceus* tadpole larvae from the San Juan Archipelago.

Materials & Methods

Collection of Botrylloides violaceus

We collected approximately 1 kg of adult *B. violaceus* colonies from the marina at Snug Harbor Resort, San Juan Island, WA in mid-June 2023, per Wing and Gardell (2022), and maintained them in a flow-through natural seawater system. The evening following collection, a subset of colonies slated for spawning were transferred to a light-tight container (a bucket wrapped in aluminium foil) with an airstone to ensure adequate oxygenation. After 12 h of darkness, we exposed that subset of colonies to bright LED light at approximately 10 a.m. to induce synchronous spawning (modified from Yamaguchi, 1975; Saito et al., 1981).

Establishing Factorial Treatments of Conspecific Densities and Conspecific Cues

Uniform settlement plates were cut from acrylic sheets into 16-cm^2 squares and frosted using 80-grit sandpaper. Plates were submerged in flowing, unfiltered seawater for four days prior to the

experiment to promote development of a biofilm conducive to larval settlement (Hadfield & Paul, 2001). Four-hundred-and-five *B. violaceus* larvae were then collected with a 3-ml transfer pipette over 7 h and evenly distributed into 15 glass custard dishes, each containing one settlement plate and 100 ml of seawater filtered through a 1-μm filter. The remaining five dishes were kept settler-free as a zero-density negative control (see below). As individuals settled and entered early metamorphosis, often on the sides and base of the glass dishes, we gently moved them onto the acrylic plates to encourage high-density settlement amenable to manipulation. All tadpole larvae had settled and metamorphosed by the next morning.

To test hypotheses of density-dependent recruitment, our experimental design included four treatments with quintuple replication: zero-density negative control (n = 0), medium-density (n = 6), high density (n = 12) and medium density with conspecific cues (n = 6). We culled both initial recruits from settlement plates to achieve the target densities and all individuals that recruited on the glass bowl interior (Fig. 1). All dishes were kept for the duration of the experiment at approximately 10°C to approximate *in situ* temperatures.

All dishes were maintained at equal volumes (100 ml) of regular filtered seawater, with the exception of the conspecific "adult cue" treatment, in which each replicate contained 100 ml of filtered seawater collected from the "hatchery" containing adult *B. violaceus*. Removing food particulates through filtration allowed us to test the effects of known dissolved botryllid cues, such as urochordamine A (Tsukamoto et al., 1993; Hadfield & Paul, 2001), as conspecific settlement inducers, independently of resident density.

Experiment and Data Collection

The day after establishing treatments, 240 *B. violaceus* larvae of a single cohort were collected during the morning spawning cycle, beginning at 10 a.m., and promptly distributed across the 20 experimental sets. All larvae for the settling assay were collected within 1 h of introducing the light trigger, and are assumed to be ontogenetically equivalent. As larvae were spawned from a large number of adult colonies (n > 50), effects of intraspecific variation and parentage are assumed insignificant. To minimise random block effects, we randomised the positions of the treatment replicates. Settlement rates were quantified by counting the number of new settlers on the plates and glass each hour. Counts were continued for 12 h, by which time only two free-swimming tadpole larvae remained across all dishes. Larvae were considered settled when they exhibited a clear reluctance to move and their tails showed signs of resorption (Fig. 2). We photographed treatment dishes at 2-h intervals.
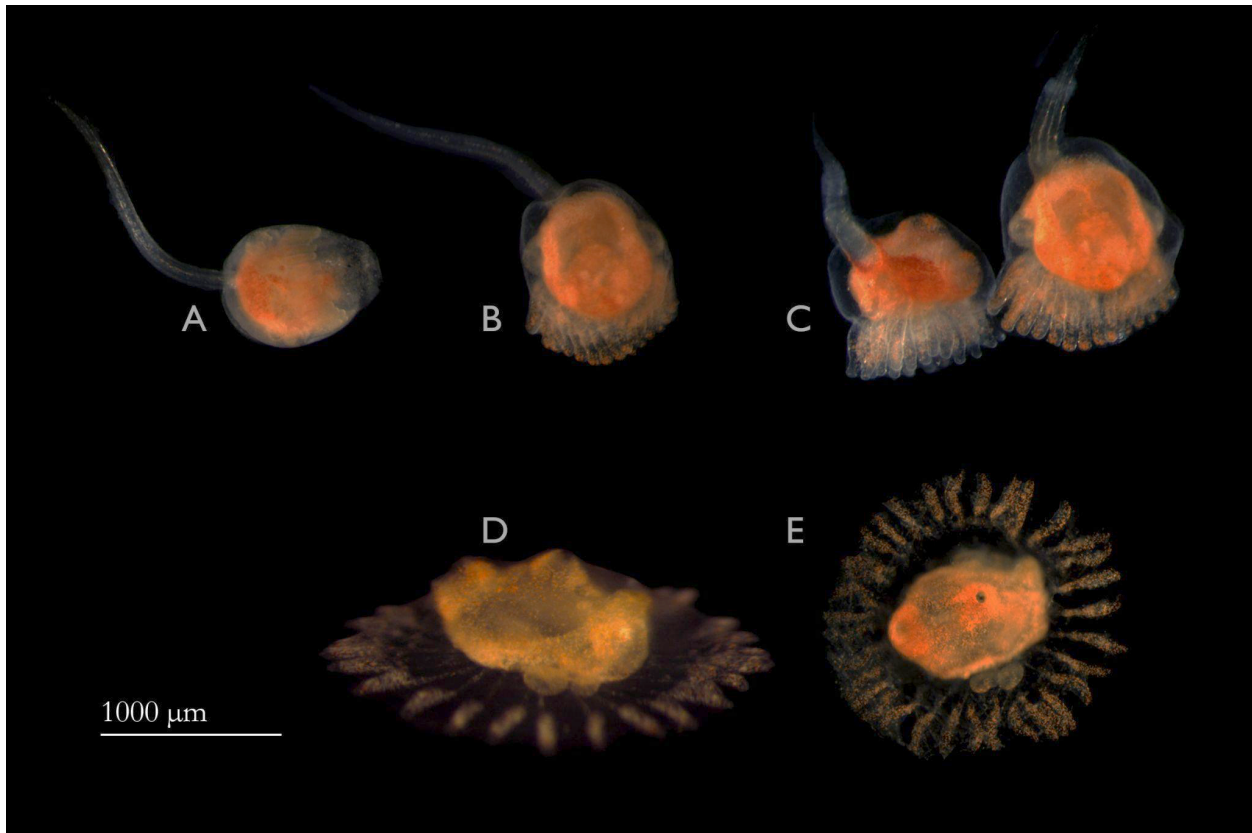
Fig. 2. Developmental stages of competent tadpole larvae of *B. violaceus.* A. Swimming larval tadpole. B. Recently settled larva extending ampullae or adhesive papillae for anchoring. C. Settled larvae showing tail resorption. We consider larvae settled once they have reached this stage, and are less likely to crawl or swim from their settling site. D. Lateral view of metamorphosed juvenile. E. Top view of metamorphosed juvenile showing siphons and extended ampullae.

Settling Substratum Preference Assessment

Free-swimming larvae collected more than 1 h after introduction of the spawning cue were placed in a separate custard dish with filtered seawater and a settlement plate without residents. After 48 h, we counted the number of recruits on each square centimetre of this settlement plate and 16 randomly selected 1-cm^2 plots on the adjacent glass base, excluding the settlers on the walls due to their different orientation. The density of settlers on these two potential substrata was

aggregated to assess substratum preference under severely space-limited conditions. This observation of settlement in single dish was done in retrospect and therefore lacked replication.
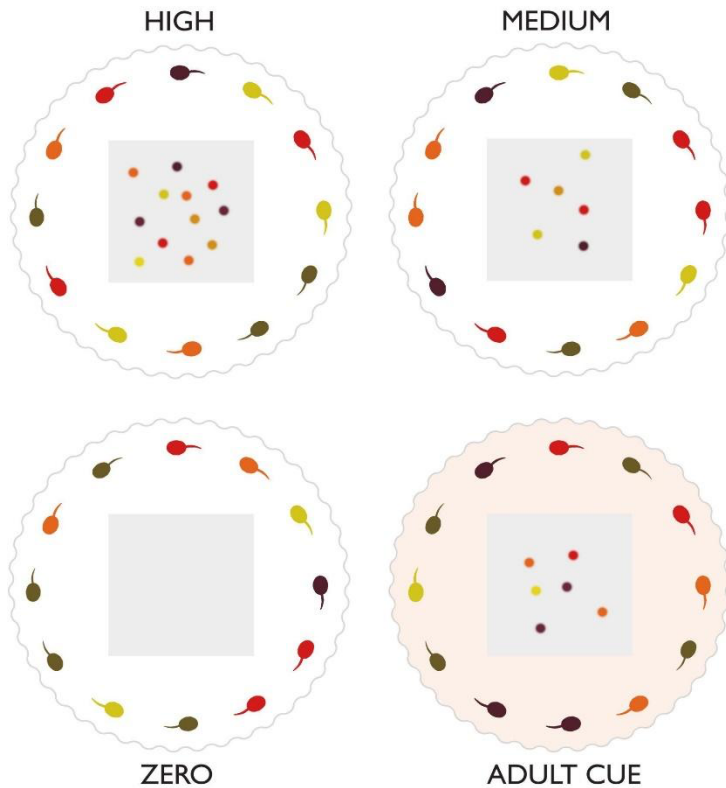

Fig. 1. Schematic of four experimental treatments, each containing 12 free-swimming tadpole larvae but variable levels of incumbent recruitment. Grey squares represent 16-cm^2 settlement plates whereas dots on the squares represent resident recruits. Orange shading represents colony-conditioned, adult-steeped seawater. Each treatment was replicated five times.

Data Analysis

All analyses were performed in R 4.3.1. Using aggregated data across replicates, we produced settling curves for all treatments. To test for effect sizes of our treatments of density and chemical cue, we constructed linear regression models with packages *lme4* and *lmerTest* to determine effect

sizes of fixed effects, including density of residential recruits and adult cues on subsequent recruitment rate.

A generalised linear mixed model (GLMM1) was fitted via Laplace's approximation with time since spawning, four experimental treatments and settlement substrata (glass vs. acrylic settlement plate) as fixed effects. To test for the independent effect size of treatments on larvae recruiting on settlement plates, we constructed a linear mixed model (LM1) fitted with REML using fixed effects of time since spawning and number of settlers across four treatments. We tested the model with Satterthwaite's t-test for differential settling patterns. Equations to the models are given below. Acrylic settlement plate is given as P, time since spawning as t, and treatment names as high density (D), medium density (M), zero density (Z) and adult cue (C).

GLMM1

y = 0.8420t+0.7422P+1.179Pt+15.87618+random intercepts

LM1

Proportion Settled = 0.00394t + 0.0556C + 0.006242D - 0.0268M + 0.00452Ct + 0.00711t(D) + 0.001457Mt-0.000316 +random intercept

Results

Contrary to previous observations by Saito et al. (1981) that botryllid larvae swim for 4–10 h before settling, some of our larvae settled 20 min post-spawning. Except for several outliers, all were settled after 12 h.

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Tadpole larvae strongly prefer glass to settlement plates

Overwhelmingly disproportionate numbers of larvae settled and metamorphosed on the interior glass surfaces of custard dishes (90.7%) relative to the acrylic settlement plates (9.3%) across all treatments (Fig. 3). Settling substrata—glass vs. settlement plate—is the strongest predictor of settlement outcome (Table 1; $p = 0.0014$). The mixed effects of time since spawning and settling substrata predict 93% of settlement outcome. The separate unreplicated substrata assessment assay showed a far higher density of settlers on glass over the same total area (Fig. 4), with larvae appearing to greatly prefer glass over acrylic for attachment and metamorphosis. Median settler density is more than two-fold higher on glass.
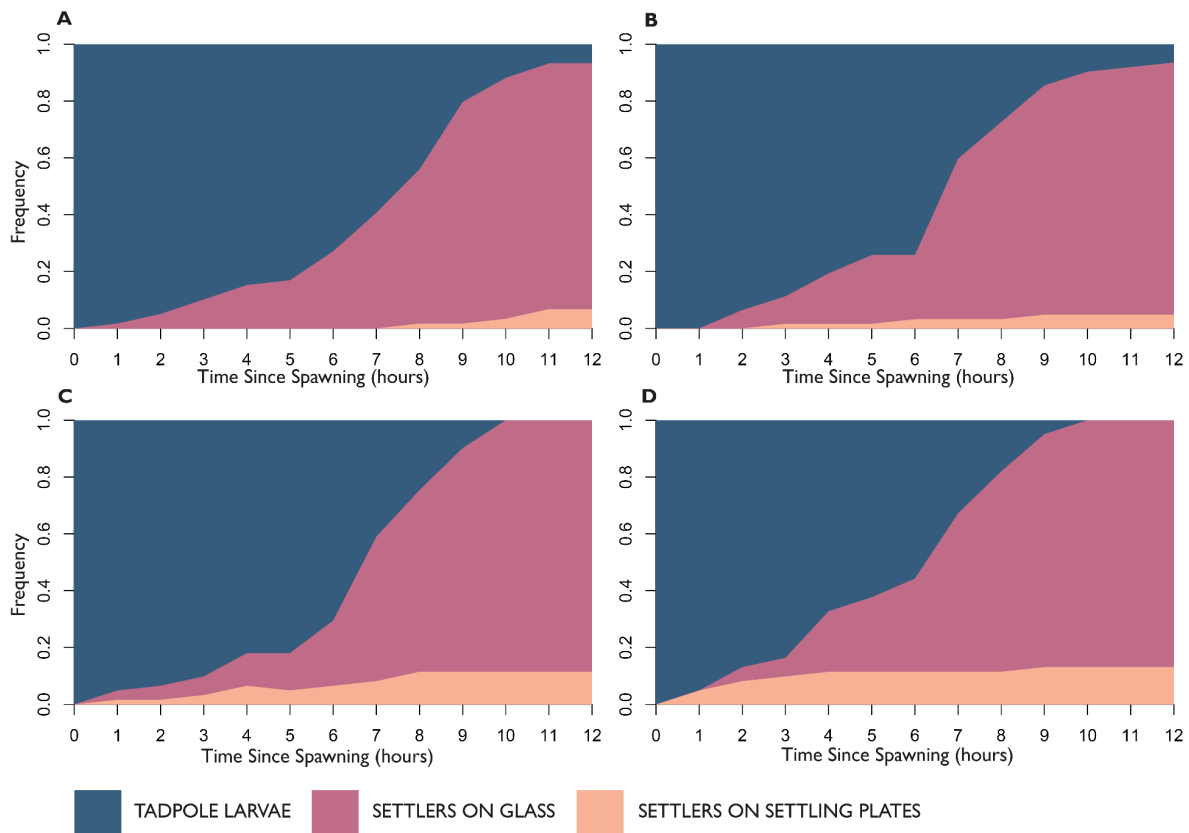
Fig. 3. Comparison of mean proportion of larvae or recruits at each time point across the four treatments. A. Zero-density negative control (n = 0). B. Medium density (n = 6). C. High density (n = 12). D. Medium-density adult-cued seawater (n = 6).

Fixed Effects	Estimate	Std. Error	z-score	Pr(>|z|)
Time Since Spawning	-0.1709	0.01413	-12.09	$<2\times10^{-16}$ ***
Glass *vs.* Settling Plate	-0.3179	0.09971	-3.188	0.00143 **
Time : Glass *vs.* Settling Plate	0.1618	0.01732	9.338	$<2\times10^{-16}$ ***

Table 1. Summary statistics for GLMM1 showing fixed effects with significant differences. Significant differences are denoted as **, $Pr < 0.01$, and ***, $Pr < 0.001$.

Tadpole larvae settling preference shows positive density-dependence

Tadpole larvae seem to prefer settling near and among conspecifics. Despite overall low preference for recruiting on settlement plates, a significantly higher number of larvae recruited on settlement plates with a high density of recent resident recruits, indicating an aggregative settling tendency in *B. violaceus* (Fig. 5). After isolating the settling rate on plates, the high-density and adult-cue treatments show significantly higher recruitment over time relative to the zero- and medium-density treatments (Table 2; $p = 0.0017$; $p = 0.016$). The adult-cue treatment not only promoted earlier settlement, but also encouraged more settlement among conspecifics. Whether these differential settlement outcomes are due to the compound effects of medium-density residents and adult cues or the independent effect of adult cues is not explicitly tested in our

experiment. As such, we are not able to disentangle the effect of these two treatments on settling preference.

Adult chemical cues prompt earlier settlement

Contrary to our expectations, significantly more larvae settled on plates with residential recruits in the adult-cue treatment, and they did so more rapidly than the other treatments (Fig. 5). Swimming larvae were expected to postpone settlement in the presence of adult cues, as Marshall et al. (2006) reported stronger competitive effects and mortality when recruits develop near established colonies. Mixed effects of time and high-density treatment showed a significant difference from the control and medium-density treatments ($p = 0.0002$). Mixed effects of time and adult-cue treatment (with medium density of resident recruits) were also significantly different from the control and medium-density treatments ($p = 0.0161$).
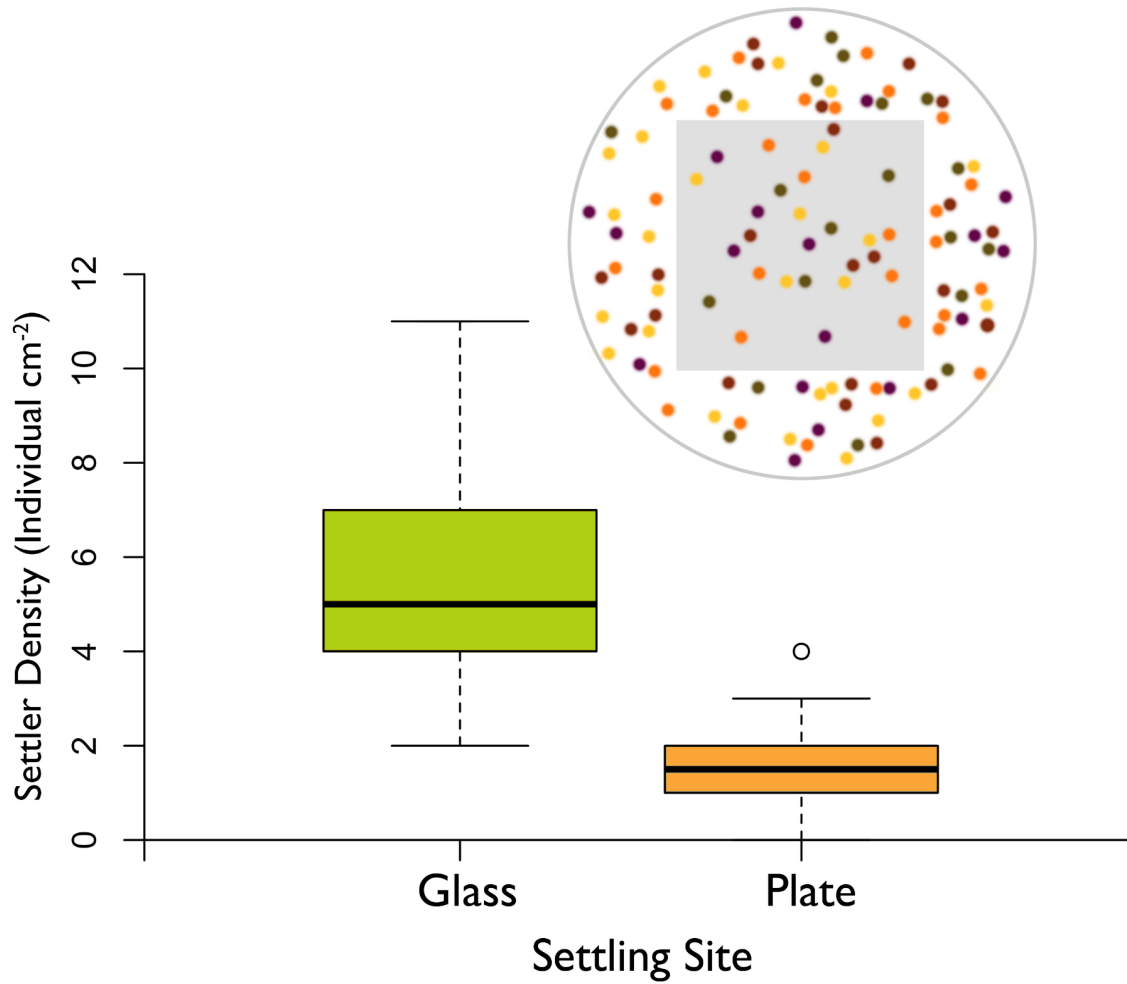
Fig. 4. Settler density per cm^2 on glass and on settlement plates in severely space-limited conditions. Top right circle maps the distribution of settlers in the non-replicated dish, and grey box represents area covered by the settlement plate.
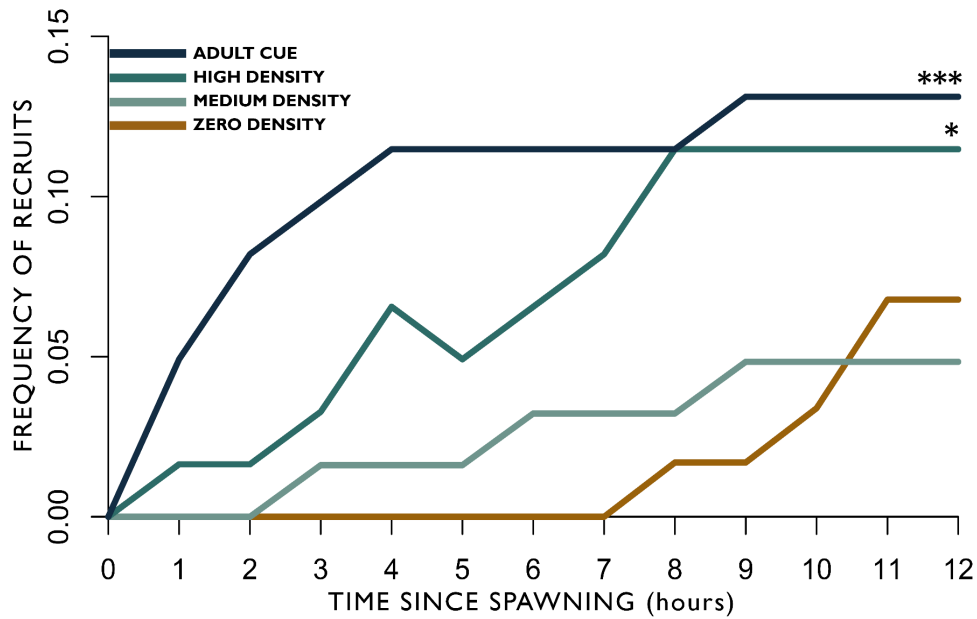
Fig. 5. Mean settling of recruits on settling plates. These settling curves show the mean frequency of recruits over the 12-h study period. Asterisks indicate statistical significance for deviations from the negative control (*** = p < 0.001, * = p < 0.01).

Fixed Effects	Estimate	Std. Error	df	Pr(>|z|)
Time Since Spawning	4.615×10^{-2}	1.570×10^{-2}	2.360×10^{2}	0.00362**
Adult Cue	6.462×10^{-1}	4.116×10^{-1}	1.986	0.1367
High Density	6.154×10^{-2}	4.116×10^{-1}	1.986	0.8840
Medium Density	-3.297×10^{-2}	4.116×10^{-1}	1.986	0.9377
Time: Adult Cue	-4.116×10^{-1}	2.221×10^{-2}	2.360×10^{2}	0.0161*
Time: High Density	8.462×10^{-2}	2.221×10^{-2}	2.360×10^{2}	0.0002***
Time: Medium Density	1.319×10^{-2}	2.221×10^{-2}	2.360×10^{2}	0.5532

Table 2. Summary of LM1 showing effect sizes of fixed effects, with zero-density negative control as a reference. Significant differences are denoted as * = Pr < 0.1, ** = Pr < 0.01 and *** = Pr < 0.001.

Discussion

Synchronously spawned larvae with short pelagic durations, as in *B. violaceus* and other colonial ascidians, tend to quickly settle near their natal site, thus furnishing an illusion of gregarious settlement (Osman & Whitlatch, 1995; Burke, 1986). The positive density-dependence in settling that we observed in *B. violaceus* shows that larvae tend to settle out earlier when the environment indicates high conspecific density, demonstrating that gregarious settlement in this species is likely not due to larval desperation, but rather concerted behaviour of settling larvae (Toonen & Pawlik, 2001). Our findings instead support the premise that the influence of phylogenetically conserved life histories and parental effects notwithstanding, habitat choice is largely determined by larval behaviour (Botello & Krug, 2006; Kingsford et al., 2002).

Given the unbalanced ratio of settlers on glass vs. plate, we inferred that the acrylic plates are likely much less preferred as a settlement substratum. We found it noteworthy that, despite strong substratum aversion, larvae settled more frequently on acrylic plates with resident recruits than on those without, further attesting to their gregarious tendencies. Aggregation on an undesirable substratum bolsters our inference that positive density-dependence in settling is not an artefact of short pelagic duration. Gregarious settlement requires a number of founding individuals to settle at a previously uninhabited site, which in turn primes other aggregator larvae to settle amongst the founders. Rather than desperate founders settling at undesirable sites to avoid conspecifics, 'founders' may be intrinsically distinct from other conspecific 'aggregator' larvae in

their habitat choice (Toonen & Pawlik, 1994). As posited by "the silver-spoon effect" on habitat selection, low-quality and presumably smaller larvae are more likely to settle on low-quality habitats that are avoided by higher-quality larvae (Stamps, 2006). This provides a mechanism by which maternal provisioning might result in individual larvae bring differentially distributed along the founder-aggregator spectrum. The trade-off between risky dispersal and safe local recruitment could be resource or season-dependent, and warrants further inquiry. A propensity to settle on previously unoccupied sites should increase the potential for niche exploration and dispersibility in a species whose larvae have only a short pelagic duration, and would otherwise show high rates of local retention. However, as our results indicate that *B. violaceus* larvae in fact prefer settling near conspecific neighbours, gregarious settling around a founder site allows rapid colonisation and population growth, therefore buffering against high local extirpation risk due to small population size.

Gregarious settlement is frequently invoked to explain colonial ascidians' tendency to become invasive since it facilitates rapid establishment of large, stable populations in novel environments (Rius et al., 2010). The positive density-dependence we detected suggests that populations now established in the Salish Sea produce larvae that prefer to settle among conspecifics, which may elucidate the mechanism behind their successful biological invasion in the region.

Our findings contradict those of a parallel experiment by Osman and Whitlatch (1995), who used varying cover of adult colonies as treatments and found that larval settlement is postponed or inhibited by high substratum coverage by conspecifics as well as by functionally similar heterospecifics. Other experiments have found support for adult chemical cues as settlement inhibitors, contrary to our results (Rius et al., 2010). Field experiments on numerous

sessile marine invertebrates, including botryllid ascidians, have also shown no effect of residential recruits on subsequent settling (Keough, 1998), though that may be due to confounding factors from heterospecific cues and site-specific random effects in the field.

We posit that positive density-dependence in larval settlement may be context-dependent. Recent recruits in our treatment groups likely pose far lower competitive pressure on settling larvae compared to adult colonies. Moreover, field study on barnacles has demonstrated that the same chemical cue may act as an attractant or deterrent to settlement-seeking larvae depending on concentration (Noda et al., 1998). Repeating this experiment with higher densities of resident recruits and higher concentration of adult cues may reveal an upper threshold at which a high conspecific density signals a competitive environment, thus discouraging settlement.

Conclusion

The presence of resident recruits compounded with chemical cues from resident adults likely enhances the appeal of otherwise unattractive recruitment substrata, promoting faster and more gregarious settling of *B. violaceus* tadpole larvae. This settlement strategy is hypothesised to be enforced by facilitative effects from neighbouring conspecifics or risk evasion by settling on a site with known prior recruitment success. Our study demonstrated that *B. violaceus* larvae likely depend on settling cues from nearby conspecifics. Preferential recruitment at sites with a high density of resident conspecifics could explain the species' patchy regional distribution and high local abundances in the heterogeneous distribution of waterways and suitable, often anthropogenic, habitats of the San Juan Archipelago. Physical removal of adult colonies when they first occur at low densities may therefore be a viable method for preventing rapid population growth and geographic expansion of this invasive, introduced ascidian.

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Appendix A

Prompted by an interest in performing settling experiments on colonial sessile marine invertebrates, we initially endeavoured to spawn *Bugula pacifica in vivo* to limited success despite repeated attempts and receipt of ample advice from experienced researchers. We document here our methods lest further futile attempts be made.

Bugula sp. is a common arborescent bryozoan in the Salish Sea. Like many members of the genus, they are particularly amenable to experimentation on larval settlement due to synchronous and rapid release of competent larvae when cued (Wendt, 2000). In addition, ample evidence supports plastic optimisation of life history strategies in *B. neritina*, including a tendency to produce more highly dispersant larvae when parental colony density is high (Allen *et al.*, 2008). *B. neritina* also maintains strong intraspecific competition in harsh environments (Hart *&* Marshall, 2013), contradicting predictions of the stress gradient hypothesis (Callaway, 2007). Our observations at the Friday Harbor Laboratories docks suggested that *Bugula* sp. is typically found in association with other bryozoan taxa (e.g., *Tricellaria* sp.; see Materials and Methods). As such, we set out to examine whether *Bugula* larvae actively minimise competition by avoiding sites occupied by other bryozoans (conspecifics or heterospecifics), or whether they in fact prefer to settle on previously colonised sites. Our experiment was reliant on our success in triggering mass synchronous spawning of *Bugula* in the laboratory.

Materials & Methods

Collection of Specimens

Gravid adults of *Bugula* sp. were collected from tyres off the docks at Friday Harbor Laboratories, San Juan Island, Washington, U.S.A., during mid-June 2023. We separated colonies into two

morphospecies based on colour, branch thickness and number of branches: *Bugula* 'Peach,' an orange, thick branch morphotype; and *Bugula* 'Rembrandt' a pale, delicate, frilly morphotype. Colonies were maintained in glass jars in sea tables with a constant flow of natural seawater to approximate *in situ* temperatures of 10°C. Jars were covered with a 'light-tight' beaker (bucket wrapped in aluminium foil).

Evaluating Phenology and Maturity of Bugula *Colonies*

We randomly sampled five colonies for evaluation. We examined the colonies under a dissecting microscope for ovicells and assessed whether they were swollen and ready to spawn.

Triggering Larval Release with Dark-light Cycle

We exposed colonies to light at varying intervals through our repeated spawning trials (6 hours, 18 hours, 24 hours and 40 hours, not in chronological order) after commencing the dark treatment to induce release of coronate larvae (*vid.* Strathmann, 1987, also Wendt, 1998).

Dismal Results and Dismayed Discussion

All colonies examined were deemed ready to spawn and likely of high fecundity. Ovicells appeared large and swollen except for those on the most apical whorls.

Our first spawning trial took place on June 16th, 2023, the day before a new moon. Three mature colonies of *B. cf. pacifica* measuring on average 4 cm long provided us with >12 coronate larvae. We left the larvae with the adult colonies and observed settlement of at least four larvae within 48 hours. Coronate larvae are visible without magnification and are active swimmers, greatly aiding in their identification. Interestingly, the coronate larvae of *B. pacifica* that we

spawned were not positively phototactic upon release, unlike those of *B. neritina* in previous studies (Allen *et al.,* 2008).

Bolstered by this moderate success, we collected approximately 100 mature colonies on June 20th and separated them into three 1-litre jars in the seatable, placed under the light-tight container. In hopes of "shocking" colonies into more synchronous spawning, we maintained them in the dark for 40 hours. To our great disappointment, the high density at which colonies were kept and the lack of aeration led to attrition and mortality of all of the colonies.

The following morning we placed an airstone into a newly collected batch of approximately 60 mature colonies in a 5-litre bucket wrapped in light-tight aluminium foil. Our initial method followed that described in Strathmann (1987), taking advantage of a five-to-eight-hour light-dark cycle to trigger spawning. Seven hours later, we observed no noticeable spawning. The colonies were placed once more in darkness for a full 13 hours. When triggered the next morning with light, the colonies released only eight coronate larvae over two hours, a disproportionately low outcome compared to our successful first trial with far fewer colonies.

Course instructor Karen Chan had previously successfully spawned *B. neritina* in the western tropical Pacific. Strikingly, application of similar methods to *Bugula* sp. populations in the temperate northeast Pacific, early in the summer breeding season, resulted in a near-total failure to induce spawning in the laboratory. Course instructor Richard Emlet postulated that local *B. pacifica* may be responding to other, yet-uncharacterized cues, perhaps coincident with the lunar cycle–an intriguing possibility that remains to be verified by vigorous experimentation.