Research Article

Life history variation of an invasive species *Botrylloides violaceus* (Oka, 1927) between novel coastal habitats in the Gulf of Maine

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Abstract

Species invasions are characterized by range expansions during which invasive species respond ecologically and evolutionarily to new environmental conditions. Responses to novel environments often involve shifts in life history traits, which may be due to phenotypic plasticity, local adaptation, or both. Identifying how species vary across novel habitats can help determine how invaders integrate themselves into local ecosystems, which is a poorly understood aspect of marine ecology and management. In the Gulf of Maine, the invasive ascidian *Botrylloides violaceus* (Oka, 1927) is found on man-made substrates and has also entered natural subtidal habitats. To investigate if the life history characteristics of *B. violaceus* vary across habitats, colonies were grown on polycarbonate plates in floating dock, eelgrass bed, and rocky subtidal habitats, and life history traits were quantified from settlement until death from June 2012 to July 2013. This was replicated at three sites along the Massachusetts coast. Settlement density differed among habitats and was highest in floating dock, lower in eelgrass bed, and least in rocky subtidal habitats. Terminal age was not different among habitats. Terminal size and maximum growth rates were higher in floating dock and eelgrass bed habitats than in the rocky subtidal habitats. The duration of colony regression did not differ among habitats. In floating dock habitats, distinct, seasonal cohorts were observed. These results suggest that *B. violaceus* is most successful in man-made versus natural habitats. Its integration into natural habitats may, however, be in an early phase; with *B. violaceus* still posing a threat to native species and ecosystems.

Key words: ascidians, floating docks, eelgrass beds, rocky subtidal, life history traits

Introduction

Organisms have been expanding their ranges for millennia (Vermeij 1991; Webb 1991), but the rate at which humans are transporting species today far exceeds their natural spread (Drake et al. 1989). Second only to land use changes, species invasions are thought to be one of the greatest causes of modern species extinctions (D'Antonio and Vitousek 1992; Vitousek et al. 1997). Additional impacts of invasive species include transport of pathogens and disease, rapid and extensive hybridization between invaders and native species, and unknown effects on native species and ecosystems (Grosholz 2002). While invasions of animals and plants on land are well characterized (Elton 2000), less is known about them in marine systems (Steneck and Carlton 2001). How invaders integrate themselves into marine communities and the scale of their impact, for example, is an important but poorly understood aspect of marine ecology and resource management (Whitlatch et al. 1995).

In the Gulf of Maine, a highly degraded ocean region (Halpern et al. 2008), invasive species are an increasing problem (Pederson et al. 2005; Dijkstra et al. 2007). In benthic habitats, the invasive colonial ascidian *Botrylloides violaceus* (Oka, 1927) is a major occupier of space (Dijkstra et al. 2007) and poses problems for native diversity (Dijkstra and Harris 2009) and the aquaculture industry (Arenas et al. 2011). Invasive ascidians are transported around the world on boat hulls, from which they can colonize artificial structures such as floating docks and subsequently expand into natural habitats (Glasby and Connell 1999; Lambert and Lambert 2003; Ruiz et al. 2009; Simkanin et al. 2012). Indeed, in the Gulf of Maine, *B. violaceus* can be found in floating-dock (Pederson et al. 2005; Dijkstra et al. 2007; Dijkstra and Harris 2009), rocky-subtidal (Sebens et al. 1997; Miller and Etter 2008, 2011), and eelgrass

(Berman et al. 1992) habitats. Once in a new range or habitat, invasive species may then exhibit variation in life history traits in response to new environmental conditions (Hanfling and Kollmann 2002). Such variation can be a result of phenotypic plasticity, local adaptation, or both (Grosholz 2001). Worldwide, invasive marine invertebrates in general (Glasby et al. 2007; Ruiz et al. 2009; Dafforn et al. 2012), and invasive ascidians more specifically (Lambert 2002; Marins et al. 2010; Simkanin et al. 2012), occur in greater numbers in man-made versus natural habitats. Differences in settlement, growth, and survival among habitats may account for this pattern.

To test if life history traits of invasive ascidians differed between habitat types, polycarbonate plates were deployed in floating dock, rocky subtidal, and eelgrass bed habitats. Life history traits of *B. violaceus* were monitored from settlement until senescence with the expectation of finding pronounced differences between habitats. Identifying any differences in life histories would elucidate how this species changes as it invades new habitats. This can, in turn, provide insights into the selective regimes acting on individual invaders as well as evolutionary processes that may be key in determining their success in novel ecosystems and regions. By quantifying these processes, we can better predict the impacts of invasive species on native species diversity and ecosystem structure and functioning and seek to mitigate potential ecological and economic costs.

Methods

Study species

Botrylloides violaceus is a colonial tunicate that forms sheet-like colonies composed of zooids embedded in a gelatinous, transparent tunic. Zooids are 2 to 4 mm in size, distributed in long irregular rows around a common exhalent siphon, and are connected by a common vascular network that extends throughout the colony (Carver et al. 2006). Different color morphs exist including white, yellow, orange, red, purple, and black. *Botrylloides violaceus* is invasive to the Gulf of Maine and may have been introduced with oyster aquaculture in the Damariscotta River, Maine, in the early 1970s (Dijkstra et al. 2007). Believed to have originated in Japan (Saito et al. 1981), *B. violaceus* can now be found along the east coast of North America from the Canadian Maritimes to Virginia; the west coast from Alaska to Ensenada, Mexico; and in Europe (Lambert and Lambert 2003; Karlson and Osman 2012).

Botrylloides violaceus is very similar in colony development to *Botryllus schlosseri* (Pallas, 1766),

a well-studied confamilial species (Berrill 1947; Oka and Watanabe 1959). *Botryllus schlosseri* colonies are hermaphrodites and reproduce both asexually and sexually (Milkman 1967). A new colony forms when a sexually produced larva settles and metamorphoses into an oozoid (Grave and Woodbridge 1924). The colony then grows exponentially through several asexual cycles in which adult zooids give rise to, and are replaced by, more adult zooids (Milkman 1967). After about five to ten cycles, sexual reproduction begins (Grosberg 1988), which often coincides with terminal size (Harvell and Grosberg 1988). The sexual cycle is also synchronized and is locked in phase with the asexual cycle (Harvell and Grosberg 1988). The asexual cycles maintain the size of the colony and the sexual cycles result in the synchronized release of brooded larvae (Milkman 1967). After about ten of these cycles (Grosberg 1988), colonies senesce and regress (Chadwick-Furman and Weissman 1995). Little is known about colony regression in *B. violaceus* but, in *B. schlosseri,* colony regression proceeds through four distinct phases and results in the death of all zooids (Chadwick-Furman and Weissman 1995).

Experimental design

Settlement density

To record settlement density of *B. violaceus* in different habitats, polycarbonate plates (20×20 cm, 0.5 cm thick) were deployed in floating dock, rocky subtidal, and eelgrass bed habitats. This was repeated at three locations or sites along the Massachusetts coast, in Gloucester, Beverly, and Sandwich (Figure 1). In total, 36 plates were deployed (three habitats × three locations × four replicate plates in each habitat-site combination). This was a two-factor experimental design with habitat type as a fixed factor with three levels (floating docks, rocky subtidal, and eelgrass beds), and location as a random factor (Gloucester, Beverly, and Sandwich).

Plates were attached to the undersides of floating docks using ropes tied to cleats on either side of the dock. Sufficient rope was left coiled at one end to allow a pulley system to operate (Supplementary information Figure S1). In rocky subtidal habitats, patches of rock were scrubbed bare with a scraper and wire brush. A small mound of under-water epoxy putty (A-788 Splash Zone Compound; Kop-Coat, Rockaway, New Jersey, USA) was then attached with a dry wall anchor embedded. The putty was allowed to dry for three days, after which plates were attached by passing a stainless steel screw with a polycarbonate cap through the plate and then securing

Figure 1. A) Map of Massachusetts Bay showing location of three study locations (sites). B-D) Maps of sites showing locations of floating docks, rocky subtidal, and eelgrass bed habitats. In Gloucester (B), floating dock habitats (42.612715°N; 70.652762°W) were located at the State Fish Pier, and rocky subtidal (42.598323°N; 70.656900°N) and eelgrass bed (42.598394°N; 70.657418°N) habitats were located off Niles Beach. In Beverly (C), floating dock habitats (42.544391°N; 70.860191°W) were located at Beverly Port Marina, and rocky subtidal (42.544138°N; 70.860374°N) and eelgrass bed (42.543403°N; 70.860277°N) habitats were located off Lynch Park. In Sandwich (D), floating dock habitats (41.770187°N; 70.503685°W) were located at Sandwich Marina, and rocky subtidal (41.771951°N; 70.485725°N) and eelgrass bed (41.772147°N; 70.486852°N) habitats were located off Town Neck Beach.
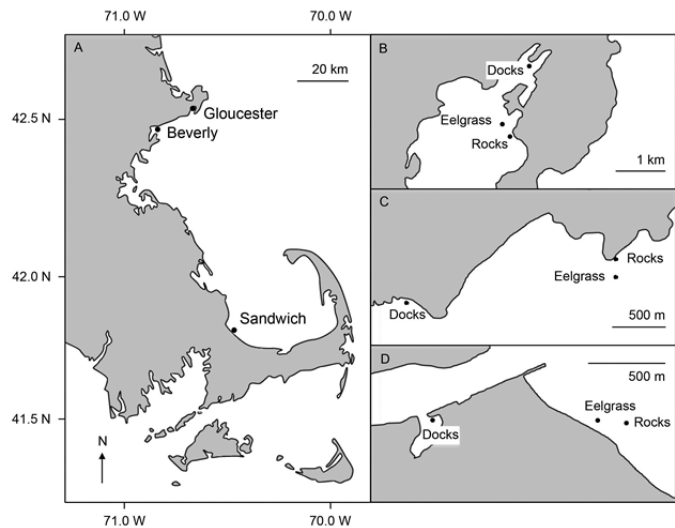
into the anchor (Figure S1). In eelgrass bed habitats, plates were suspended in the water column attached to a float with cinder blocks as anchors (Figure S1). In both rocky subtidal and eelgrass bed habitats plates were fixed and suspended, respectively, approximately 4 m below the mean low water level. Polycarbonate plates were used instead of natural substrate to standardize settlement area, as substrate size is known to affect colony size in *B. schlosseri* (Harvell and Grosberg 1988). This does, however, remove the influence of substrate type.

Settlement density was recorded from June 2012, after first settlement was observed, and continued through December 2012. In the summer months (June, July, and August) settlement density was recorded on weekly basis, and thereafter every two weeks. At each sampling date, plates were photographed with an Olympus Stylus Tough 8010 camera. All habitats in each site were sampled on the same day. Gloucester and Beverly were sampled at the same time but Sandwich, being further away, was sampled on a different day. When weather on a scheduled sampling day did not permit diving, underwater habitats were sampled at the next opportunity. In floating dock habitats, plates were photographed by pulling them up onto docks. In eelgrass bed and rocky subtidal habitats, plates were photographed underwater with by divers using SCUBA. On each sampling date, plates were also scraped clean to allow room for additional settlement. From photographs, the number of settlers on each plate (400 cm^2) was counted on each sampling date and, at the end of the season, the cumulative number of settlers was calculated per plate. Plates were sampled once a week.

Life history traits

To record terminal age, terminal size, maximum growth rate, and the duration of colony regression, colonies of *B. violaceus* were followed on a different set of polycarbonate plates (20 × 20 cm, 0.5 cm thick). These plates were also deployed in floating dock, rocky subtidal, and eelgrass bed habitats and repeated at the same three sites along the Massachusetts coast (Figure 1). For this study 72 plates were used (three habitats × three sites × eight replicate plates in each habitat-site combination). This was also a two-factor experimental design with habitat type as a fixed factor with three levels (floating docks, rocky subtidal, and eelgrass beds), and sites as a random factor (Gloucester, Beverly, and Sandwich). Plates were secured in each habitat as above.

Life history traits were recorded from June 2012 after first settlement was observed and continued through October 2012 when the last *B. violaceus* colony had disappeared. After settlement, a *B. violaceus* settler close to the center of each plate was selected for detailed examination and, at this time, all other settlers were removed to allow for colony growth in the absence of competition. On each sampling date, colonies on plates were photographed with an Olympus Stylus Tough 8010 camera. Sampling was conducted on a weekly basis in summer (June, July, and August), every two weeks in fall and spring (September, October, November, March, April, and May) and once a month in winter (December, January, and February). Photographs were taken as above, i.e. in floating dock habitats, plates were photographed by pulling them up onto docks

and, in eelgrass and rocky subtidal habitats, plates were photographed underwater by divers. Again, on each sampling date, all new settlers were removed to allow for continued colony growth in the absence of competition. Colonies were monitored by photography until no tissue remained.

Terminal age, terminal size, maximum growth rate, and the duration of colony regression were calculated from photographs (Figure S2). Terminal age was recorded as the number of days between the date of settlement (estimated using Bullard and Whitlatch 2004) and the date at which the colony was last observed to be intact, i.e., before the onset of senescence. Terminal size was estimated, using Image J (Schneider et al. 2012), as the area attained on the date at which the colony was last observed to be intact before the onset of senescence. Maximum growth rate was calculated as the change in colony area (area calculated using Image J) between sampling dates during the exponential phase of growth. The duration of colony regression was measured as the number of days taken for the colony to disappear after the onset of senescence.

Cohorts

In floating dock habitats, distinct seasonal cohorts were observed whereby the original cohort of eight settlers produced a new generation of colonies before senescence, and so on. These cohorts were further monitored by photography until July 2013, i.e., for a full year, to further identify potential differences between habitats. During this time eelgrass bed and rocky subtidal habitats were also monitored. During winter months when growth was slow, the sampling interval was reduced to a bimonthly schedule. Cohorts were monitored on different sets of polycarbonate plates.

Statistical analyses

To test for differences in settlement density among habitats, the cumulative number of settlers was analyzed using a two-factor, balanced ANOVA with habitat as a fixed factor with three levels (floating docks, rocky subtidal, eelgrass beds) and site as a random factor with three levels (Gloucester, Beverly, Sandwich). This analysis was conducted using the aov function in R version 3.0.2 (R Core Team 2013). A post-hoc Tukey's HSD test was used to test for differences between habitat means.

To test for differences in terminal age, terminal size, maximum growth rate, and duration of colony regression among habitats, two-factor, unbalanced (unbalanced due to differential settlement among
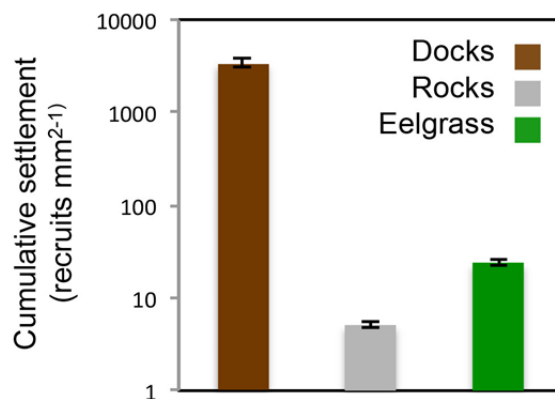

Figure 2. Cumulative settlement of *B. violaceus* in the 2012 season. Settlement was different among habitats and lower case letters denote significantly different means (P < 0.05) based on Tukey's HSD test.

habitats and therefore different numbers of adult colonies, see results) ANOVAs were employed, with habitat as a fixed factor with three levels (floating docks, rocky subtidal, eelgrass beds) and site as a random factor with three levels (Gloucester, Beverly, Sandwich). In these ANOVAs, a Bonferroni-corrected significance level of 0.0125 was used. Models were fit using the lmer function in the lme4 package (Bates et al. 2014) and the difference between group means assessed using the Anova function, with the default type II sums of squares (Langsrud 2003), in the car package (Fox and Weisberg 2011) in R version 3.0.2 (R Core Team 2013). Model parameters were estimated using residual (or restricted) maximum likelihood (REML).

Following all ANOVAs, Tukey's HSD was employed to test for the differences between habitat means. Settlement density, terminal size and maximum growth rate were log transformed. Terminal age and duration of colony regression were not transformed. Normality and homogeneity of variances were visualized at the replicate and group level using Q-Q plots and plots of residual versus fitted values respectively. Patterns of cohort dynamics are described.

Results

Settlement density

Mean settlement density was statistically different between habitats, and all habitats were different from each other (Table 1, Figure 2). The site and interaction terms were not significant. Settlement was highest in floating dock, lower in eelgrass bed, and least in rocky subtidal habitats.

Figure 3. Life history traits of *B. violaceus* colonies in floating dock, rocky subtidal, and eelgrass bed habitats for A) terminal age, B) terminal size, C) maximum growth rate, and D) duration of colony regression. Terminal age and the duration of colony regression did not differ among habitats but terminal size and maximum growth rate were different among habitats. Lower case letters in plots B) and C) denote the different significantly different means revealed by Tukey's HSD test.
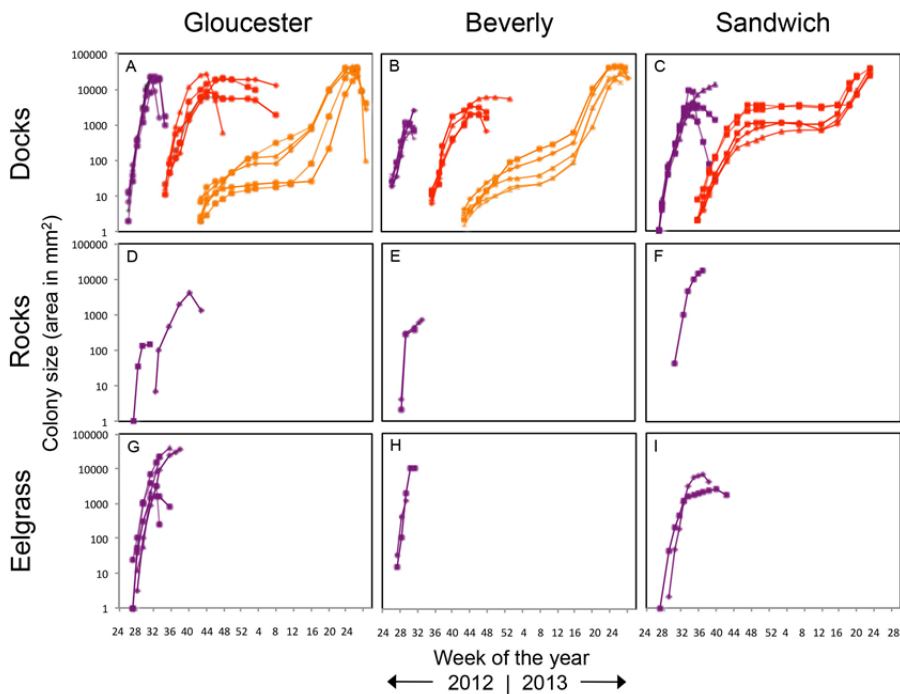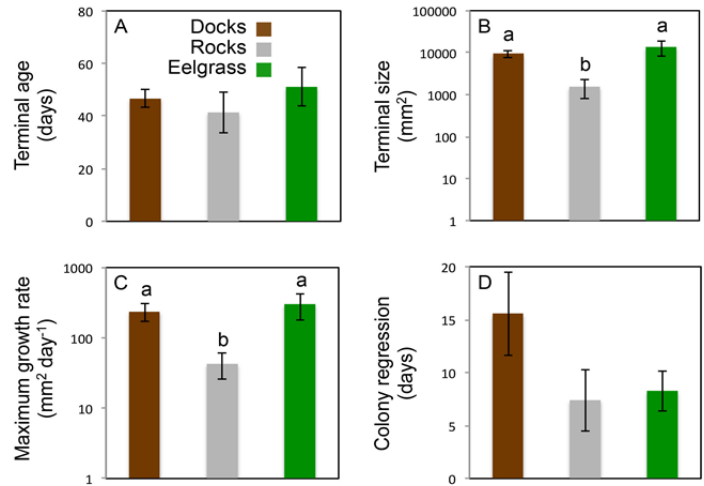

Figure 4. Growth curves for *B. violaceus* colonies in all habitats at all sites. Each individual curve represents a colony. Note the presence of three distinct seasonal cohorts in dock habitats. With the exception of Gloucester eelgrass no other cohorts were identified in rocky subtidal and eelgrass bed habitats. The few colonies observed in these habitats were found at the start of summer only with no observations in late summer or overwinter. Different colors represent different cohorts.

Life history traits

For all tests, the site term and interaction term were not significant (Table 2). Average terminal age did not differ among habitats (Table 2, Figure 3A) but terminal size did differ (Table 2, Figure 3B). Colonies in floating dock and eelgrass bed habitats were larger than colonies in rocky subtidal habitats. The average maximum growth rate was different between habitats (Table 2, Figure 3C) and again, colonies in floating dock and eelgrass bed habitats grew faster than those in rocky subtidal ones. The duration of colony regression was not different between habitats (Table 2, Figure 3D).

Table 1. Results of ANOVA, testing for a difference in cumulative settlement density among habitats, with site as a random factor. Settlement data was log transformed. Significant p-values are shown in bold.

Source	df	SS	F	P
Habitat	2	55.05	70.04	**< 0.001**
Site	2	0.67	2.16	0.135
Habitat X Site	4	1.57	2.51	0.065
Residuals	27	4.22		

Table 2. Results of univariate, habitat X site ANOVAs for terminal age, terminal size, maximum growth rate, and duration of colony regression. As models had a random effect (site) and were unbalanced, parameters were estimated with REML. The outputs are Analysis of Deviance tables and Wald χ^2 test statistics. Terminal size and maximum growth rate were log transformed. Significant p-values, with a Bonferroni-corrected alpha of 0.0125, are shown in bold.

Source	df	χ^2	P
Terminal age			
Habitat	2	2.01	0.366
Site	2	3.15	0.207
Habitat X Site	4	7.87	0.096
Terminal size			
Habitat	2	14.26	**< 0.001**
Site	2	1.83	0.401
Habitat X Site	4	7.01	0.135
Maximum growth rate			
Habitat	2	14.20	**< 0.001**
Site	2	3.89	0.143
Habitat X Site	4	11.18	0.025
Colony regression			
Habitat	2	2.35	0.309
Site	2	0.85	0.653
Habitat X Site	4	3.60	0.464

Cohorts

Distinct seasonal cohorts were only identified in dock habitats (Figure 4). In Gloucester and Beverly, three cohorts were observed: in early summer, late summer, and overwinter (Figure 4A and B). In Sandwich there was one summer cohort followed by an overwintering cohort (Figure 4C). In rocky subtidal and eelgrass bed habitats, only a few colonies were observed and these were at the start of summer (Figure 4D–I).

Discussion

Botrylloides violaceus is a tenacious invader that is expanding its range (Karlson and Osman 2012), and increasing in dominance (Dijkstra et al. 2007). In this study, differences in settlement density and

some life history traits of *B. violaceus* were found between habitats. In general, *B. violaceus* was most successful in the artificial floating dock habitats, where distinct seasonal cohorts were also observed, and least successful in rocky subtidal habitats.

Settlement density

Settlement density differed among habitats and was highest in floating dock, lower in eelgrass bed, and least in rocky subtidal habitats. Differences in settlement may be due to propagule pressure and differential settlement. As *B. violaceus* has a short larval duration of minutes to hours (Saito et al. 1981), propagule pressure will be highest where adult colonies are most abundant. Typically, invasive ascidians occur in greater numbers in man-made versus natural habitats (Lambert 2002; Marins et al. 2010; Simkanin et al. 2012) and in this study, colonies were larger and more abundant in floating dock habitats. Floating docks may also receive new propagules via boat traffic (Carlton and Geller 1993; Ruiz et al. 2000) because invasive ascidians can be transported to these habitats on boat hulls (Lambert 2001; Lambert and Lambert 2003) and in ballast water (Svane and Young 1989; Carlton and Geller 1993). Differences in settlement density could also be explained by larval choice as ascidian larvae are known to select for different light levels and sub-strate angle (Young and Chia 1984; Rius et al. 2010). Invasive ascidians tend to settle in greater numbers on surfaces close to the water's surface (Glasby et al. 2007; Dafforn et al. 2009) such as those of floating dock habitats, and in greater numbers on floating versus fixed substrates (Glasby et al. 2007; Dafforn et al. 2009; Simkanin et al. 2012). The observations in the present study are consistent with the published studies as there was greater settlement in floating dock habitats, intermediate settlement in eelgrass bed habitats, and lowest settlement in rocky subtidal habitats.

Life history traits

Colonies of *B. violaceus* grew faster and attained larger sizes in floating dock and eelgrass bed habitats than in the rocky subtidal. Interestingly, despite differences in settlement between floating dock and eelgrass bed habitats, size and growth rates of *B. violaceus* colonies were comparable between these habitats. Differences in colony growth between the two floating habitats and the fixed rocky subtidal could be due to abiotic and biotic factors altered by substrate movement and suspension above the benthos. Indeed, invasive species generally are more abundant on floating versus fixed substrates (Glasby et al. 2007; Dafforn et al. 2009; Simkanin et al.

2012). Invertebrate success on floating substrates may be enhanced by increased water flow (Glasby 2001; Perkol-Finkel et al. 2008), which can increase food availability (Sebens et al. 1998) and decrease sedimentation (Irving and Connell 2002). Substrate angle also affects invertebrates (Glasby and Connell 2001; Miller and Etter 2011; Dafforn et al. 2012) by changing sedimentation (Irving and Connell 2002), water flow (Leichter and Witman 1997), and light levels (Irving and Connell 2002; Miller and Etter 2008) and thus may have influenced results. Some of the success of *B. violaceus* in floating dock habitats may be due to the downward facing orientation of the plates, which would further minimize, for example, sediment accumulation.

Floating docks may provide a partial refuge from predation as they are not contiguous with the benthos. This contrasts to rocky subtidal habitats, which would provide unimpeded access for a variety of benthic predators (Dumont et al. 2011). In rocky subtidal habitats in British Colombia, Canada, and in Chile, predation negatively affected the recruits and adults of *B. violaceus* (Simkanin et al. 2013) and *Ciona intestinalis* (Linnaeus, 1767) (Dumont et al. 2011), respectively. Furthermore, in Chile, predation was not observed in floating dock habitats (Dumont et al. 2011). In the present study, a small gastropod *Mitrella lunata* (Say, 1826), which is known to prey on ascidian recruits (Osman and Whitlatch 1995, 2004), was observed in eelgrass bed habitats. It was found in greater densities on eelgrass plates in Beverly and Sandwich than in Gloucester (an average of 13, 7, and 4 snails per plate, respectively), which could explain why more colonies of *B. violaceus* were observed in Gloucester. In Sandwich, low numbers of the snail were also found on rocky subtidal plates (an average of 2 per plate), which could explain why only one colony was observed at this site. No predation of adult colonies was observed.

Terminal age (life span) and colony regression (duration of senescence) did not vary among habitats and these traits could be heritable. A heritable aspect to life span was found in lab-raised populations of *B. schlosseri* (Rinkevich et al. 1992) and the duration of colony regression appeared to be genetically programmed in both lab (Rinkevich et al. 1992) and field populations (Brunetti 1974; Chadwick-Furman and Weissman 1995). Similar experiments could reveal if life span and duration of senescence are also heritable in *B. violaceus*.

Cohorts

Distinct seasonal cohorts were observed only in floating dock habitats. The absence of seasonal cohorts in the two natural habitats is not surprising given low settlement densities. In the rocky subtidal habitat, this can also be accounted for by slow growth rates and small colony size. In eelgrass habitats, the absence of year-round cohorts might be explained by the seasonal loss of the actual substrate, and hence seasonal reduction in the number of propagules. In winter, for instance, eelgrass fronds die back to the rhizome and shoots then reemerge the following spring resetting available space (Osman et al. 2010).

Conclusions

In general, *B. violaceus* was most successful in floating dock habitats where settlement density and growth rates were high, colony sizes large, and distinct seasonal cohorts observed. Factors unique to man-made floating docks might promote invader success (Glasby et al. 2007; Dafforn et al. 2009, 2012; Simkanin et al. 2012) such as greater adult density and hence propagule pressure, floating substrates are close to the water surface and provide a partial refuge from predation, and boat traffic may provide an external source of new propagules. Although growth rates and colony size were also high and large, respectively, in eelgrass beds, possibly due to the floating nature of the substrate, this habitat may be protected from a high level of invasion due to its distance from a propagule source, and substrate loss over winter. Invasion resistance in the rocky subtidal might be conferred by the fixed nature of the substrate, and susceptibility to predation by a suite of benthic predators. Identifying these differences in life history traits, as well as the factors that might influence them, represents the first step in elucidating how *B. violaceus* responds to, and integrates into, new habitats and how this species might continue to spread.

As floating docks likely served as initial entry points for invasions (Glasby and Connell 1999; Lambert and Lambert 2003; Arenas et al. 2006; Ruiz et al. 2009; Simkanin et al. 2012), invasive species may also have had longer to establish in these systems. It should not, therefore, be assumed that natural habitats are somewhat protected from invasive species and, instead, these habitats should be also be monitored. The mechanisms that contribute to invader success in different habitats should be investigated further, as well as the factors that confer invasion resistance in the rocky subtidal zone. This could provide us with the information necessary to protect natural subtidal habitats and to appropriately manage man-made ones.

Acknowledgements

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The following supplementary material is available for this article:

Figure S1. Experimental set-up and example plates.

Figure S2. Colony growth from settlement until terminal size.

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