Living on the edge: tolerance to environmental stressors and reproductive dynamics of the gastropod *Crepidula fornicata* across the intertidal-subtidal boundary

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Abstract

Species' ranges are generally studied on a latitudinal scale, and species are often limited by their tolerance and responsiveness to physical stressors. Many organisms, though, live in the transitional area between terrestrial and marine environments, the intertidal zone. Most of the species that occupy the intertidal zone are of marine descent and many can also be found deep into the subtidal zone where they live their entire lives covered by seawater. Thus, the intertidal zone represents a range boundary for many marine organisms, beyond which they cannot survive. For these organisms, the intertidal range edge is characterized by a suite of stressors associated with aerial exposure that may limit their upper distribution and can ultimately cause them negative physiological impacts; such stressors include desiccation, high aerial temperature, and reduced feeding time, and they are absent only meters away, in the subtidal zone. Comparing particular aspects (e.g. phenotypic traits, fecundity) of adjacent intertidal and subtidal organisms of a single species can provide insight into which factors control the upper distribution of that species in the intertidal zone, the ability of organisms to adapt to stressful intertidal conditions, and the healthiness of the populations that reside there. I investigated some basic biological aspects (thermal tolerance, desiccation tolerance, feeding physiology) of the sessile suspension-feeding gastropod, *Crepidula fornicata* at Bissel Cove in Narragansett Bay, Rhode Island, USA in order to better understand the factors that limit the upper distribution of this species and also the effects of intertidal stressors on the reproductive capacity of these organisms.

I found that high summer temperatures are probably the most important factor limiting the upper distribution of this species, but desiccation stress can be particularly troublesome for very small individuals; reduced available feeding time is probably not a major limiting factor. Intertidal and subtidal *C. fornicata* at Bissel Cove had similar upper thermal tolerances,

desiccation tolerances, and feeding rates. However, they differed both in their behavior when exposed to the air and in their gill morphology: when emersed, intertidal individuals spent less time with their tissues exposed to the air than did subtidal conspecifics, and intertidal individuals also had relatively larger gills. The stresses associated with intertidal life had no immediate effects on their ability to successfully reproduce. In fact, the breeding season was slightly longer for intertidal individuals of *C. fornicata* and their fecundities higher when compared to those of subtidal conspecifics.

Taken together my results suggest that *C. fornicata* has had a long association with the intertidal zone, and though desiccation and high temperatures are likely keeping them from living high up in the intertidal, the individuals that recruit to the mid/low intertidal are not particularly stressed there as adults. Their high tolerance to environmental stressors and their adaptability may be partially responsible for their extreme success as an invasive species. As global temperatures rise, this species will likely be relegated to the subtidal zone only, but will probably continue to colonize new locations.

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Chapter 1

General Introduction

For centuries, humans have been interested in learning where different organisms live, why they only live in certain areas, and why they are able to survive in those places but not others. Of this interest was born ecology: the study of the distribution and abundance of organisms (Andrewartha and Birch, 1954) and their interactions with the environment (Haeckel, 1866). As scientific investigation has become more sophisticated, the field of ecology has concurrently expanded to include many disciplines that in some way seek to inform our understanding of both organismal interactions – biotic and abiotic – and organismal distributional patterns (Krebs, 2001). These disciplines are now extremely wide ranging, and each adds a different perspective and useful information to our current understanding of ecology. For example, while a community ecologist may discover that predation is the chief driving force shaping the distribution of the mussel *Mytilus californianus* in the intertidal zone in California (Paine, 1974), an ecophysiologist working in the same system can show us that its cosmopolitan distribution would not be possible without its efficient energetic strategies (Matzelle et al., 2014). Many of these ecological disciplines overlap, rely on each other, or contain each other, and many ecologists are working simultaneously in several disciplines to answer interesting and important questions about our planet and the organisms that occupy it.

The driving force behind all of this ecological research is the simple fact that all species are spatially restricted to some degree, though the amount of physical area that different species occupy is extremely variable. But why are species spatially restricted? And what can the organisms at or near the edges of their range tell us about the ecology and evolution of that species? Recent studies on organisms at or near the limits of their ranges have provided vital information on the factors that limit species' distributions (e.g. Fawcett, 1984; Zacherl et al., 2003; Lima et al., 2007) and their adaptations to those limiting factors (e.g. Joshi et al., 2001;

Etterson, 2004; Geber and Eckhart, 2005), which can offer insight into the evolution of niches (Holt and Gomulkiewicz, 1997; Sexton et al., 2009).

To study the factors that limit species' distributions, investigators often compare some aspect of a species (e.g. abundance, fecundity, phenotypic traits) from different populations within the range of that species. There are countless variables that may limit the distribution of a species, but those associated with climate have been the most widely studied and acknowledged as important for limiting species' ranges (reviewed in Sexton et al., 2009). Climatic variables have been proposed as the major determining factors that limit the distributions of organisms ranging from marine snails (Lima et al., 2007) to pine trees (Iverson et al., 1999) to African ticks (Cumming, 2002) to marine mussels (Helmuth et al., 2002) to butterflies (Crozier and Dwyer, 2006) to lizards (Parker and Andrews, 2007) to European blueberries (Coudun and Gégout, 2007), and many others. Correlative studies of species' ranges with particular climatic variables (e.g. temperature, rainfall) (e.g. Pigott and Huntley, 1981; Arundel, 2005), and more in-depth studies of physiological tolerances (e.g. Neilson and Wullstein, 1985; Stillman and Somero, 2000) and energy budgets (e.g. Sarà et al., 2013; Woodin et al., 2013) have proved important in demonstrating the pervasive effects of climate as a limiting factor for many species. However important climatic variables are for determining species' distributions, there are indeed many other variables to consider when investigating limiting factors. In many cases, factors such as food availablity (e.g. Hersteinsson and Macdonald, 1992), competition (e.g. Connell, 1961), predation (e.g. Paine, 1974), parasitism (e.g. Settle and Wilson, 1990), habitat availability (e.g. Hill et al., 1999), and physical barriers (e.g. Arntzen and Themudo, 2008) have also been demonstrated to be important limiting factors. Furthermore, many factors may act in concert to limit species' distributions (e.g. Settle and Wilson, 1990; Hill et al., 1999; Firth and Williams,

2009; Williams et al., 2011; Iacarella and Helmuth, 2012), and these limiting factors may change over time. For example, Jump et al. (2006) found that beech trees (*Fagus sylvatica*) in Europe have been limited at their southern range boundary by drought for years, but that recent climatic changes have shifted their major limiting factor to high temperatures. Taken together, these considerations further complicate the situation and make detailed investigations into the effects of particular potential limiting factors on organisms especially valuable.

Specific conditions in particular areas may be detrimental enough to exclude some organisms from those areas. For example, though climate change has allowed for the range expansion of skipper butterflies (*Atalopedes campestris*) in North America, individuals of this species are definitively restricted by low winter temperatures at their northern range edge, beyond which they cannot survive (Crozier, 2003). If conditions are poor enough in some areas, individuals there may actually be part of a sink population that requires frequent immigration to prevent local extinction (Pulliam, 1988). However, if selection is strong enough, some organisms possess the trait variability necessarry to adapt to those factors that limit their distribution (Holt and Gomulkiewicz, 1997), and studying the factors that limit the distibutions of organisms can inform us about the evolution of niches (Holt and Gomulkiewicz, 1997; Gomulkiewicz et al., 1999).

Studies comparing populations of organisms within their range have also revealed phenotypic differences among those organisms, usually because organisms have adapted to cope with stressful conditions in particular areas, often at a range edge (e.g. Tellería and Carbonell, 1999; Pérez-Tris et al., 2000; Pfenninger et al., 2007). For example, Jenkins and Hoffmann (1999) found that the fruit fly *Drosophila serrata* was limited by cold temperatures at its southern range edge in Australia; however, post-winter collections there showed that members of

a range edge population had higher physiological tolerance to cold stress than members of central populations. Conversely, understanding the reasons why some organisms don't adapt to those factors that may limit their distribution has become an increasingly popular topic of study (e.g. Holt and Gomulkiewicz, 1997; Angert et al., 2008).

Ultimately, the fate of a species in any given area should be expressed in its ability to survive, grow, and reproduce there. For example, it is commonly believed that organisms have lower fitness at their range boundaries, probably due to reduced resource availability and increased energy expenditure to cope with stressful conditions (Gaston, 2009). This reduction in fitness at the range edge has been demonstrated in many species, but is certainly not a universal finding. For example, Lester et al. (2007) found that reproductive output of the intertidal sea urchin *Strongylocentrotus purpuratus* was actually highest at its southernmost range edge, and that reproductive output was not correlated with latitude. Others have found similar results (reviewed in Gaston, 2009, Sexton et al., 2009), suggesting that some species may not be particularly stressed in areas previously thought to be stressful, but that measuring reproductive dynamics is a useful practice to assess the healthiness of particular populations (e.g. Lewis 1986; Herbert et al. 2003; Ling et al. 2008; Pecorino et al. 2013).

The intertidal zone – the area of coastline between the highest high and lowest low tides of the year – represents a very clear range boundary for many marine organisms (Holt and Keitt, 2005). Most ocean-dwelling species are confined to the subtidal zone where they are constantly surrounded by water, but many others live a life characterized by periodic (daily) exposure to the air – in the intertidal zone. In fact, nearly all of the organisms that occupy the intertidal zone are of marine descent (Colman, 1933; Stephenson and Stephenson, 1949). Though many organisms that occupy the intertidal zone are found only there (e.g. Stephenson and Stephenson, 1949;

Connell, 1961; Connell, 1972; Perez et al., 2009; Bourdeau, 2011), a host of species have distributions extending from the subtidal zone at least partly up into the intertidal zone (e.g. Dame, 1972; Palmer, 1980; Fletcher, 1984; Page, 1984; Matta and Chapman, 1991; Saier, 2002; Stenseng et al., 2005; Altieri, 2006; Muths et al., 2006; Schaffmeister et al., 2006; Todd et al., 2009; Bird et al., 2011; Dong et al., 2011). For those species, members of both subpopulations experience nearly identical conditions at high tide; only the well-established suite of conditions associated with aerial exposure should differ between intertidal and subtidal individuals. We can thus study intertidal and subtidal subpopulations of a species in order to learn about the factors that limit the distribution of those species in the intertidal zone, the potential ability of intertidal organisms to adapt to stressful conditions, and the healthiness of intertidal animals as compared to subtidal conspecifics that may live only meters away.

Additionally, we can learn about the evolutionary history of organisms by studying particular aspects of a species' physiology, behavior, and morphology in the intertidal zone. Because intertidal organisms often require specialized adaptations to be able to survive the harsh conditions associated with aerial exposure, comparisons of species that thrive both intertidally and subtidally with those normally found only in the intertidal zone can inform us about the degree to which a species is actually adapted to live intertidally, or if individuals of that intertidal-subtidal species may find themselves in the intertidal zone but are just tolerating the harsh conditions they encounter there. For example, organisms adapted for intertidal life are often highly tolerant of desiccation (e.g. Marshall and Mcquaid, 1992), have the ability to endure high temperatures for long periods of time (e.g. Tomanek and Somero, 1999), and perform "gaping" or "lifting" behaviors in part to aerobically respire in the air (e.g. Lent, 1968). Detailed investigations in an intertidal-subtidal species of aspects like these can allow investigators to

speculate on the evolutionary history of any given species and the degree to which they are adapted to live intertidally or subtidally.

Though intertidal and subtidal subpopulations of species are not often explicitly compared, a number of researchers have attempted to uncover the major limiting factors for organisms that live in the intertidal zone, though often for those species that are said to occupy only a thin portion of the intertidal zone (e.g. Connell, 1961; Foster, 1971; Connell, 1972; Wolcott, 1973; McMahon, 1990; Stillman and Somero, 1996; Wethey, 2002; Dong and Williams, 2011). The old paradigm in intertidal ecology was that biotic interactions (e.g. competition, predation) were responsible for defining the lower limits of many intertidal organisms, while abiotic factors (e.g. temperature, desiccation) were responsible for defining the upper limits of species, made famous by Connell's (1961) classic study on barnacle zonation patterns. Often the situation is more nuanced, though, and the fact that many species living in the intertidal zone have broad distributions that extend deep into the subtidal zone demonstrates that the forces (biotic or abiotic) that may be important for shaping the distribution of one species may not matter at all for another.

Even so, it is hard to deny the importance of some environmental factors for limiting the distribution of organisms in the intertidal zone. Stresses from high and low temperatures seem to be particularly important for determining where intertidal organisms can live (e.g. Hofmann and Somero, 1995; Helmuth and Hofmann, 2001; Somero, 2002; Stillman, 2002; Wethey, 2002; Somero, 2005; Helmuth et al., 2006b; Dong and Williams, 2011). Because of the high specific heat of seawater and the vast volumes of ocean basins, temperature changes are relatively slow and minor in most marine habitats. In contrast, temperature changes in terrestrial environments – including the intertidal zone when the tide is out – can be extremely rapid and dramatic. In the

coastal waters of Narragansett Bay, Rhode Island, for example, air temperatures can rise several degrees in only minutes and up to approximately 10°C within an hour while fluctuating between -17°C and 36°C over the course of a year; water temperatures in the same area may only range from 0°C to 27°C degrees in any given year (NOAA National Data Buoy Center, 2004 – 2013 yearly averages). Thus intertidal organisms face a constantly changing and unpredictable thermal regime in the intertidal zone (Helmuth et al., 2002; Helmuth et al., 2006a), which may require special adaptations for survival there. Due to its pervasiveness as a selective force in the intertidal zone, temperature regimes in particular areas and their effects on some species have been well-characterized (e.g. Stillman and Somero, 1996; Stenseng et al., 2005; Dong et al., 2008; Marshall et al., 2011). Though mobile organisms have the ability to seek refuge from intertidal stressors at low tide, species that are predominantly sessile cannot escape changes in temperature by changing their location and instead often have various physiological adaptations to cope with high temperature stress. Upper thermal limits of organisms are mediated by many different factors including the physical structure and concentration of particular enzymes (Somero, 2004), changes in membrane fluidity (Hazel, 1995), and the induction of the heat shock response (Feder and Hofmann, 1999), all of which may be under selection and contribute to defining species' ranges in the intertidal zone.

Desiccation is another important factor that has been clearly implicated in limiting the distribution of marine organisms in the intertidal zone (e.g. Kensler, 1967; Sutherland, 1970; Wolcott, 1973; Branch, 1975; McQuaid, 1982; Jenewein and Gosselin, 2013). Since water is a standard, necessary component for metabolic and cellular processes, the loss of too much tissue water can impair normal functioning or increase solutes to levels that will deter normal cellular activity. When exposed to the air, marine organisms can lose tissue water rapidly (e.g. Branch,

1975; McQuaid 1982); this is especially true for very small organisms (or juveniles) because of their large surface area to volume ratios (e.g. Davies, 1969; Branch, 1975; McQuaid, 1982; Williams et al., 2005; Jenewein and Gosselin, 2013). As with strategies for dealing with high temperatures, avoidance behavior is the most effective strategy for combating desiccation stress, but the limited behaviors of even sessile organisms demonstrate the importance of desiccation stress as a limiting factor in the intertidal zone. Specifically, intertidal mollusks often have simple behaviors ("clamming up", "clamping down") that help them conserve tissue water (e.g. Wolcott 1973; McMahon, 1988; McMahon, 1990), but that may ultimately cause other stressful conditions within their sealed cavities, such as hypoxia, reduced pH, and increases in ammonium concentrations (Williams et al., 2005; Chaparro et al., 2009; Chaparro et al., 2011). Tolerance to tissue water loss is wide-ranging, but most intertidal invertebrates cannot lose much more than 50% of their tissue water without dying, an amount that may take only a few hours of aerial exposure to lose.

Finally, though many other factors (e.g. competition, predation, parasitism) are surely involved in limiting the distribution of intertidal animals, food limitation is often cited as a major deterrent for occupation of the intertidal zone. Though some organisms reduce foraging during low tides to avoid other stressors (e.g. desiccation, high temperature) (e.g. Coleman et al., 2004; Rilov et al., 2005), other organisms just do not have the physical ability to feed when they aren't surrounded by water. In particular, suspension-feeding organisms should be at a distinct energetic disadvantage in the intertidal zone, as their food source (particles suspended in seawater) is not available to them for hours at a time every day while they are exposed to the air. If an intertidal organism cannot obtain enough food while the tide is in to maintain normal metabolic processes it will starve. Furthermore, high aerial temperatures can potentially increase

energy expenditure during periods of exposure, making the intertidal zone even more challenging from an energetic standpoint (Somero, 2002). Even if organisms can attain enough food in the intertidal zone to maintain normal metabolic activities, a reduction in energy intake from food limitation may manifest itself in other ways, such as decreased reproductive output. Changes in resource allocation toward stress resistance and maintence of particular tissues at the expense of growth and reproductive output has been found in some intertidal animals (e.g. ribbed mussels, California mussels, Franz, 1993; Petes et al., 2007; Petes et al., 2008). Additionally, to combat the energetic disadvantages associated with intertidal life, some organisms show modified feeding behavior and have modified feeding structures that help them collect more food while they are covered by seawater (e.g. Morton, 1957; Newell et al., 1971; Franz, 1993; Charles and Newell, 1997). For example, when submerged, the radular (feeding) activity of high intertidal snails (*Littorina littorea*) is approximately three times faster than the radular activity of low intertidal conspecifics (which have more time to feed) from the same shore (Newell et al., 1971). Suspension-feeding organisms may (e.g. Morton, 1957; Newell et al., 1971) or may not (Griffiths and Buffenstein, 1981; Widdows and Shick, 1985; Bayne et al., 1988) be able to compensate for reduced feeding time, but a severe reduction in feeding time is certainly a contributing factor to the distributional limits of many organisms that live intertidally.

The above considerations help to explain why the ranges for many marine organisms end somewhere in the intertidal zone, but nuanced evaluations of the factors that may limit the distribution of many species and the degree to which the individuals of those species can surivive and reproduce in the intertidal zone are still lacking. The calyptraeid gastropod *Crepidula fornicata* is an excellent example of one such species. As adults, *Crepidula fornicata* are sessile, protandrous hermaphrodites – all complete a male phase before transitioning to female – that

generally live atop one another in "stacks" (Coe, 1936) (Fig. 1). The bottom-most member of a stack is attached to some hard substrate, which is usually a rock or empty *C. fornicata* shell but can also be an array of living (e.g. oysters, mussels, horseshoe crabs) or non-living (e.g. boat hulls, docks, empty shells) substrates. Stacks may vary in number of individuals from as few as one (solitary individuals) to upwards of 20 members, though they are generally composed of approximately 3 – 7 individuals, usually with larger females on the bottom, transitional individuals in the middle, and smaller males on the top (Coe, 1936) (Fig. 1).

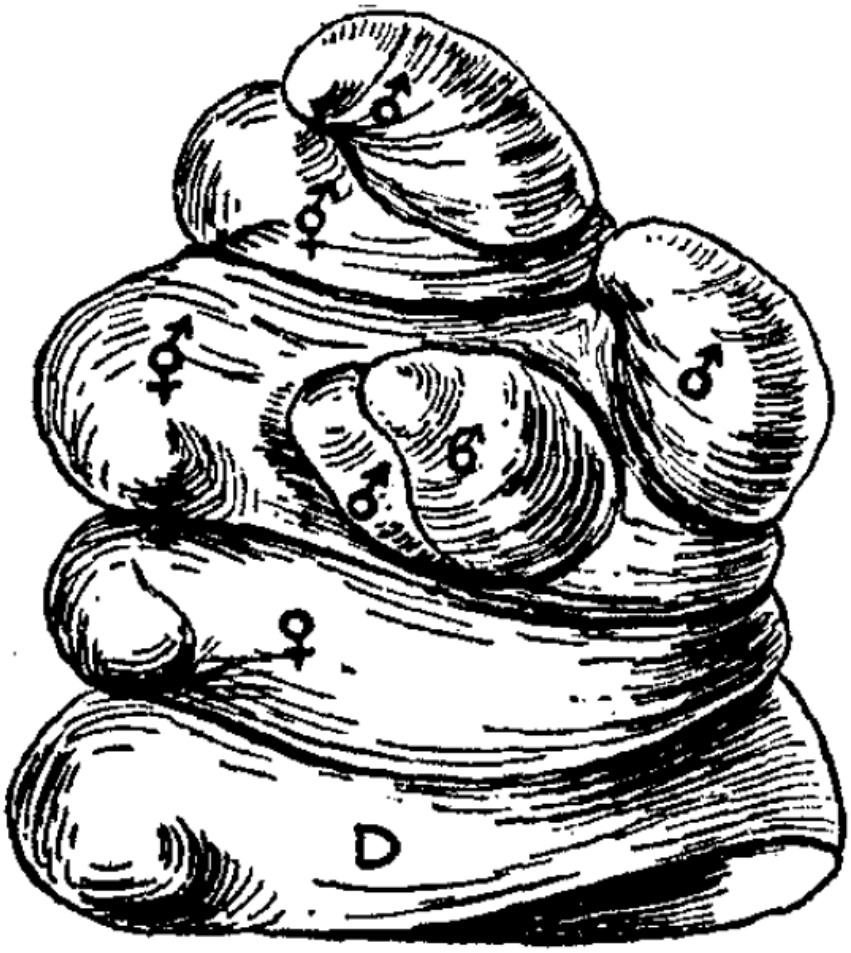

Figure 1.1: From Coe (1936); a stack of *Crepidula fornicata* (7 individuals) on top of an empty shell (D), with a large female at the bottom of the stack and smaller transitional individuals and males toward the top of the stack.

Since adults of this species do not move and fertilization is internal, living in a stack ensures fertilization success (Dupont et al., 2006). Once the eggs are fertilized, females lay down gelatinous egg masses composed of up to approximately 70 small egg capsules (~ 3 mm length, ~3 mm width) that are each filled with embryos. The individual egg capsules are all attached to a central stalk, which the female affixes to the substrate that she is adhered to, and the egg mass is subsequently brooded under the neck of the female in the mantle cavity for several weeks (Conklin, 1897). Inside the egg capsules, non-feeding embryos develop from the morula stage to the ciliated trocophore stage and finally to the veliger stage before they hatch (Chipperfield, 1951). After hatching, feeding veliger larvae remain swimming in the plankton for several weeks before they reach metamorphic competency, at which point they will metamorphose if they encounter the proper cue, which is usually associated with conspecifics (Pechenik, 1980; Pechenik and Gee, 1993). Metamorphosis involves resorbtion of the ciliated swimming and feeding organ, the velum, and further development of the gills (which they use to collect food after metamorphosis). Small juveniles are said to be "mobile," though to what degree has not been established; if juveniles encounter a stack of *C. fornicata* individuals or a solitary female they will attach themselves and grow normally but remain in the male phase (Coe, 1936). If they do not find a stack they will quickly pass through the male phase and become female; a large male that lives in a stack in which the female dies will also quickly transition to the female phase (Coe, 1936).

Aside from copulation, adult *C. fornicata* have a very limited behavioral repertoire. Though they spend their adult lives affixed to one particular spot, they have been observed to lift their shell off of the substrate while keeping their foot securely attached and – after being lifted – clamp again firmly to the substrate; they perform this behavior both while submerged and

exposed to the air. While submerged, the shell lifting behavior is necessary for respiratory and feeding purposes while clamping may help them avoid poor external conditions (e.g. low salinity) or predators that wish to pry them from the substrate. The large gill of *C. fornicata* draws oxygenated water under the shell and into the mantle cavity using long lateral gill cilia (Jørgensen, 1984). Water passes through the gill (where oxygen from the water is utilized) and leaves from under the other side of the shell, but small particles that are suspended in the water are trapped on the gill surface. These particles are subsequently transported to one side of the gill, concentrated in a food groove (where they are mixed with mucus), and moved to the mouth where they are ingested (Shumway et al., 2014). The lifting behavior that they perform while exposed to the air, though anecdotally reported several times (Newell and Kofoed, 1977, Hoagland, 1984), has not been thoroughly investigated.

For the purposes of my dissertation, *Crepidula fornicata* was an appealing organism to study because of its distribution (in addition to its relative simplicity and amenability to laboratory experimentation). First, individuals of *C. fornicata* are routinely collected in both intertidal (e.g. Diederich et al., 2011) and subtidal (e.g. Hoch and Cahill, 2012) locations worldwide (e.g. Thieltges et al., 2003; LeCam and Viard, 2011), though this broad vertical distribution had never been explicitly documented for a single population. Because individuals of *C. fornicata* are sessile for most of their lives, this subtidal to intertidal distribution makes *C. fornicata* an excellent organism to study in the context of limiting factors in the intertidal zone. Second, *C. fornicata* is native to the east coast of North America, but has become a highly successful invasive species around the world, most notably in southern UK and French coastal waters (Blanchard, 1997). Its original introduction into European waters came on oyster shipments in the late 19th century, and its populations have since exploded, often reaching

10,000 individuals m^{-2} in some French bays (Blanchard, 1995). Though the invasion of *C. fornicata* has spurred a considerable amount of research in its invaded range (particularly on its possible effects on commercially important species native to European waters: Orton, 1926; Le Pape et al., 2004; Thieltges, 2005; Leloup et al., 2008; Kostecki et al., 2011), there is shockingly little known about many aspects of the basic biology and ecology of *C. fornicata* in its native range.

In this dissertation I aimed to uncover some of those basic aspects of *C. fornicata* at a field site in its native range: Bissel Cove which lies in Narragansett Bay, Rhode Island, United States. By comparing some unstudied (or little-studied) aspect of *C. fornicata* between intertidal and subtidal conspecifics I aimed to learn not only about this species at its intertidal range edge (limiting factors, potential adaptation to intertidal stressors, reproductive consequences in intertidal individuals), but also to provide basic information on particular aspects of its biology that might be useful to other investigators, especially those that are studying invasive populations of *C. fornicata* and wish to compare them to native populations. Additionally, two other species in the same genus that have similar lifestyles (*C. convexa* and *C. plana*) can be found in New England waters, and there are hundreds of species in the calyptraeidae family that have been poorly studied; the work presented here could form the basis of future comparative studies with these organisms.

In the first three data chapters of this dissertation I investigated three specific potential limiting factors for *C. fornicata* in the intertidal zone: high temperature stress, desiccation stress, and food limitation. I first investigated the thermal tolerance of *C. fornicata* individuals at multiple life-history stages (embryos, adults) that were collected from intertidal and subtidal habitats (Chapter 2). In this chapter I also documented the subtidal-to-intertidal distribution of *C.*

fornicata in Bissel Cove, Rhode Island, and I characterized the summer thermal regime to which individuals of this species have been exposed. I then investigated the desiccation tolerance of *C. fornicata* adults from intertidal and subtidal habitats, and the desiccation tolerance, effects of size on rates of water loss, and percentage of water loss that was necessary to kill juvenile *C. fornicata* (Chapter 3). Furthermore, in this chapter I characterized the lifting behavior of intertidal and subtidal adults as well as lab-reared juveniles of this species while also assessing the tradeoff between aerial respiration and water loss when individuals are lifted off of the substrate in the air. Next, because intertidal individuals have reduced time to collect food compared to subtidal conspecifics, I investigated the gill (food collecting organ) morphology and clearance rates (feeding rates) of intertidal and subtidal *C. fornicata* to assess the effects of and potential compensation strategies for food limitation (Chapter 4) in this species. In this chapter I also ran parallel studies on a closely related species, *Crepipatella fecunda*, which has a similar subtidal-to-intertidal distribution, but along the southern coast of Chile. In my last data chapter I thoroughly investigated the reproductive dynamics of *C. fornicata* collected from the intertidal and subtidal zones in Bissel Cove, Rhode Island (Chapter 5). This chapter is the first real examination of *C. fornicata* reproduction in its native range, and the comparison of intertidal and subtidal breeding periods and fecundity, among other variables, provides insight into the degree to which *C. fornicata* are stressed in the intertidal zone. Finally, I end this dissertation with some concluding remarks on not only the limiting factors and reproductive dynamics for *C. fornicata* in the intertidal zone, but also other unexplored limiting factors for this species, other information that can be gleaned from this dissertation, and what the future may hold in terms of distributional patterns for *C. fornicata* (Chapter 6).

Chapter 2

Thermal tolerance of *Crepidula fornicata* (Gastropoda) life history stages from intertidal and subtidal subpopulations

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Abstract

The ability to withstand high summer temperatures is an extremely important determinant of species distributions in the intertidal zone; most marine organisms cannot live intertidally because of the harsh conditions experienced during aerial emersion. The members of some species, though, live both intertidally and subtidally, and their physiological tolerance to stressors may differ depending on their genetic connectivity, acclimatization, and differential post settlement mortality. Furthermore, tolerances of organisms at different life-history stages within both habitats may differ and will play an important role in determining adult distributions. To determine the effect of habitat and life-history stage on physiological tolerance and the likely contribution of upper thermal tolerance limits to the native distribution of an important invasive species, the slippershell snail *Crepidula fornicata*, we compared the thermal tolerance of intertidal and subtidal embryos and adults in relation to conditions that they experience in the field. We found *C. fornicata* living both intertidally and subtidally in Bissel Cove, Rhode Island, where they experienced drastically different thermal conditions. Intertidal individuals achieved body temperatures as high as 42°C, more than 15°C higher than those recorded in subtidal conspecifics. However, subtidal individuals had remarkably high thermal tolerances that were nearly identical to those of intertidal conspecifics (lethal range = 33−37°C following a 3 h exposure). Furthermore, embryos were even more tolerant to high thermal stress than adults. These results are surprising: the few previous studies that have compared thermal tolerances among individuals at different life-history stages have found differences among them, and early stages are generally more sensitive than adults. Interestingly, the markedly higher temperatures that intertidal animals experienced in this study had little effect on their thermal tolerance.

Intertidal (but not subtidal) slippershell snails are now living dangerously close to their upper thermal limits and will almost certainly be relegated to the subtidal as global temperatures rise.

Introduction

The intertidal zone is one of the most stressful environments on the planet. When the tide recedes, intertidal organisms must cope with rapid and dramatic changes in conditions such as temperature, food supply, predation pressure, humidity, and salinity (e.g. Davenport et al. 1980, Ellis et al. 2007, Hunt & Denny 2008, Szathmary et al. 2009, Iacarella & Helmuth 2012). Moving along the range from the subtidal up through the intertidal zone the intensity of these factors in creases, and pressures selecting for traits that confer the ability to live through such changes markedly increase. It is not surprising that this gradient of physical and biological stressors at least partially controls the distribution of species in the intertidal zone, and is thought to play an important role in the evolution and ecology of many marine intertidal species (Connell 1972, Stillman & Somero 1996, Denny & Wethey 2001, Somero 2002, Tomanek & Helmuth 2002, Davenport & Davenport 2005).

Among all of the difficulties associated with living intertidally, temperature plays an extremely important role in determining species distributions (e.g. Somero 2002, Stillman 2002, Wethey 2002, Gilman et al. 2006, Helmuth et al. 2006). Thermal tolerances of closely related species within the intertidal zone often correlate with the magnitude of thermal stress that organisms of those species experience, due to their vertical position on the shoreline (Tomanek & Somero 1999, Stillman & Somero 2000, Stillman 2002, Stenseng et al. 2005). For example, Stillman & Somero (1996) have shown that porcelain crabs living high in the intertidal zone can maintain higher levels of aerial oxygen consumption, have little whole-body lactate accumulation, and have higher thermal tolerances when compared to low-intertidal congeners. Intertidal species require specialized adaptations such as high physiological tolerance to thermal stress, because although they are physiologically capable of living subtidally, they often do not

live there due to intense predation and competition (e.g. Connell 1972, Perez et al. 2009, Bourdeau 2011). Similarly, organisms that can compete subtidally are unlikely to be found in the intertidal zone because it is such a harsh environment.

Though most marine animals can be found either intertidally or subtidally, some species live both intertidally and subtidally (e.g. Dame 1972, Fletcher 1984, Saier 2002, Schaffmeister et al. 2006). Though researchers have investigated phenotypic differences among conspecifics living at different tidal heights within the intertidal zone, relatively few studies have investigated the adaptations of single-species populations that live both intertidally and subtidally. Due to differential mortality, acclimatization, or genetic differentiation, we might expect to see differences among intertidal and subtidal subpopulations, especially when observing physiological tolerances to stressors. In fact, in the few studies available, when intertidal and subtidal conspecifics have been compared, behavioral, physiological, and morphological differences among the 2 groups have commonly been found (e.g. Sharp et al. 1994, Bingham et al. 1997, Altieri 2006, Weihe & Abele 2008). However, though there is increasing evidence for local adaptation in marine invertebrates across a wide range of spatial scales (reviewed by Sanford & Kelly 2011), phenotypic differences among many populations do not exist (e.g. Kuo & Sanford 2009, Sanford & Worth 2010) due to extensive genetic mixing and high degrees of plasticity (reviewed in Sanford & Kelly 2011).

Studies that assess the physiological tolerances of marine species and those that compare tolerances among conspecifics from different populations generally focus on a single life history stage. When multiple life history stages have been compared, differences in physiological tolerances to various stressors have often been found, with early life history stages often more vulnerable to stressors than adults (e.g. Crisp & Ritz 1967, Gosselin & Chia 1995, Qiu et al.

2002, Freitas et al. 2010). Since early life history stages (embryos, hatchlings, juveniles) are often found in the same habitat as adults, the ability of all life history stages to endure physiological stressors is important for an organisms' ability to survive in a particular habitat like the intertidal zone.

The gastropod *Crepidula fornicata* is one species with conspicuous intertidal and subtidal populations; in New England their populations extend from at least 5 m below low tide up into the low/mid intertidal zone (Lindsey et al. 2006, C. M. Diederich pers. obs.). Though *C. fornicata* is widely studied and often collected both intertidally (e.g. Thieltges et al. 2003, Diederich et al. 2011) and subtidally (e.g. Le Cam & Viard 2011, Hoch & Cahill 2012), the upper thermal tolerance of this species and the degree to which its members are adapted to live in either zone has not been reported. The effects of temperature on members of this species may be particularly important since individuals of *C. fornicata* are sessile as adults – and thus cannot rely on behavioral adaptations to avoid thermal stress – and brood developing embryos in the mantle cavity for several weeks (Conklin 1897), exposing those embryos to the same temperature fluctuations experienced by the adults.

Investigating the thermal conditions that *Crepidula fornicata* experience and their tolerances to these conditions in various life-history stages allows us to explore the degree to which the environment shapes the phenotypic variation in a population. Furthermore, understanding the thermal physiology of intertidal and subtidal *C. fornicata* is important because this species is not only a key member of its native communities in the western Atlantic, but is also an important invasive species in many areas – most notably France and the UK (Blanchard 1997). With global temperatures rising, learning how close these organisms are living to their

thermal maximum and how much variation exists in upper thermal tolerance within the population is important for predicting future changes in distribution in response climate change.

In this study, we first aimed to document the subtidal-to-intertidal distribution of *Crepidula fornicata* in a Rhode Island population where it is extremely abundant. In order to learn just how different the conditions are that intertidal and subtidal animals face, we then estimated the temperatures of intertidal and subtidal animals using biomimetic temperature loggers during the summer months in that population. Finally, to determine the effect of environment on thermal tolerance and the role of temperature in controlling the upper distribution of this species, we investigated the tolerances of field-acclimatized embryos and adults as well as laboratory-reared *C. fornicata* juveniles to thermal stress.

Materials and Methods

Population characteristics

All experiments were performed on individuals from a population in Bissel Cove near Wickford, Rhode Island, USA. *Crepidula fornicata* adults are very abundant in the tidal channel leading from the cove to Narragansett Bay, and in the associated intertidal and subtidal areas adjacent to the cove. Surveys of intertidal and subtidal subpopulations were performed in September 2011 by running horizontal transects at different tidal heights (+0.6 m, +0.4 m, +0.2 m and −1.0 m mean low lower water). Hereafter, any mention of tidal height or water level is made in reference to the mean low lower water (MLLW) mark. Five square plots (each 1 m^2) were placed along the transect every 2 m, and all *C. fornicata* in each plot were counted. Average emersion time was determined over the months of June, July and August 2010 and 2011 using verified water level data from a nearby National Data Buoy Center buoy (operated by the

National Oceanic and Atmospheric Administration) located at Quonset Point, Rhode Island (Station QPTR1−8454049). During Hurricane Irene on 27 and 28 August 2011, the predicted water levels were used in computing average emersion times, as the actual water levels on those days were drastically different than those predicted.

Biomimetic temperature loggers

To determine the thermal environment that intertidal and subtidal *Crepidula fornicata* experience, we deployed biomimetic temperature logging devices (hereafter termed 'robosnails') intertidally and subtidally from June to August 2011. Similar devices have previously been successfully constructed and deployed in the field to record approximate tissue temperatures of other mollusks (e.g. Helmuth & Hofmann 2001, Lima & Wethey 2009, Seabra et al. 2011), though this is the first time such a device has been used for *C. fornicata*. A Thermochron® iButton® (Part # DS1921G − Maxim Integrated Products) that was set to record temperature every 15 min was placed into an empty *C. fornicata* shell and the shell was subsequently filled with 3M Scotchcast™ 2130 Flame Retardant Compound, which hardens after mixing and has been shown to mimic tissue temperature very well (Lima & Wethey 2009). We further confirmed the usefulness of the Scotchcast™ compound as a suitable substance for accurate estimates of tissue temperatures by mimicking intertidal conditions in the laboratory and heating (Philips™ 90W heat lamp) adjacent robosnails and live animals in air (live animal vs. robosnail, $r = 0.961$). Due to the size of the loggers, only larger adult *C. fornicata* shells could be used for robosnails, but we found that tissue temperatures (sampled in the field) of animals within a stack of *C. fornicata* varied by only ~1.1°C (range 0.6 – 2.0°C). The robosnail was attached to a small rock with a thin layer of the Scotchcast™ compound. Robosnails were deployed among living *C. fornicata* in the intertidal zone at +0.4 m and subtidally at −1.0 m MLLW and were swapped out

with new robosnails approximately every 15 d due to their finite recording capabilities. The intertidal zone in Bissel Cove is composed mainly of rocks and empty *C. fornicata* shells that do not provide shade or refuge from conditions during aerial exposure, and thus robosnails were placed in areas that experienced conditions that were identical to living *C. fornicata*. Temperature loggers were excised from used robosnails and temperature data was downloaded using OneWire-Viewer software (Maxim Integrated Products).

Thermal stress in adults

Adult *Crepidula fornicata* were collected at Bissel Cove, RI from intertidal (approximately +0.4 m MLLW) and subtidal (−1.0 m MLLW) habitats in June and August 2010 and August 2011. This species is sedentary, and forms stacks of several members (usually 2−10) presumably to ensure reproductive success. Stacks are composed of small, newly metamorphosed males on top and successively larger, older animals on the bottom. *C. fornicata* is a protandrous hermaphrodite; the lowest member of a stack is always female with transitional individuals and smaller males higher in the stack (Coe 1936). Top members of each stack were removed so that only the bottom-most member of the stack remained attached to the substrate (empty *C. fornicata* shell or rock); thus all adults collected were female (size range of 20.0 to 41.4 mm, longest shell dimension). Adults were transferred to the laboratory and all thermal stress experiments were performed within 48 h of collection, to avoid letting the animals acclimate to laboratory conditions (e.g. Widdows & Bayne 1971, Mcmahon & Payne 1980). Animals were measured and separated into size classes, then distributed into treatments prior to thermal stress to ensure homogeneity of size among replicates and treatments. Seawater was filtered to 1 μm and heated to the desired temperature (measured with Control Company Traceable® waterproof digital thermometers) in 5 l plastic aquaria, and placed in incubators

(Percival, Model #I-30BL) to maintain constant temperature throughout the experiments. After seawater in the incubators remained at the desired temperatures for ~1 h, animals were placed into the heated seawater where they remained for 3 h (5 replicates of at least 8 animals for each treatment). A temperature of 23°C was chosen for the control stress (and recovery) temperature because that was the typical subtidal temperature recorded in the field during the time of the experiments, while stress temperatures of 32 to 37°C were chosen because pilot experiments revealed these temperatures (for a 3 h duration) to be realistic on a particularly hot summer day.

Animals were thermally stressed in water, not air, to ensure that their tissues reached the desired temperature and to avoid the additional stress of desiccation. Water temperatures were monitored throughout the experiments and they did not fluctuate more than 0.4°C. At the conclusion of the 3 h thermal stress period, animals were transferred to individual glass dishes with ~75 ml phytoplankton suspension per dish (equal parts *Isochrysis galbana* clone T-ISO and *Dunaliella tertiolecta* clone DUN) at 23°C. Phytoplankton suspensions were changed once after 24 h. Mortality was determined 24 and 48 h after the end of the thermal stress by touching a probe to the head of the animal and monitoring muscular response. At 24 h after the thermal stress, animals that could not easily be pried from the substrate were scored as alive. At 48 h after the thermal stress all animals were pried off their substrate and prodded for muscular response.

Collecting and maintaining animals and thermal stress in juveniles

In order to obtain juveniles for studies of variability in thermal tolerance, adult *Crepidula fornicata* were collected from Bissel Cove, Rhode Island (see above) in June, July and August 2010 and 2011. Animals were maintained at 23°C in glass aquaria of aerated seawater (30 psu). Adults were fed phytoplankton suspensions composed of a mixture of T-ISO and DUN once or

twice daily and water was changed every other day. Larvae released by adults were collected on 120 μm mesh filters, rinsed with seawater, and transferred to glass aquaria in 0.45 μm-filtered seawater. Larvae were fed T-ISO at ~18 × 10^4 cells ml^{-1} (Pechenik & Lima 1984, Pechenik et al. 2002) with water changed every other day. When larvae reached ~900 μm they were exposed to 20 mM excess KCl in seawater for 6 h to induce metamorphosis (Pechenik & Heyman 1987, Pechenik & Gee 1993), which does not affect juvenile growth or survival (Eyster & Pechenik 1988).

Juveniles were then maintained in glass aquaria of aerated seawater (changed daily), and fed T-ISO suspensions twice daily until their shell lengths (longest dimension) reached an average size of ~2.5 mm (reared for ~10 d after metamorphosis). Thus, juveniles from both intertidal and subtidal mothers spent at least 3 wk exposed to identical laboratory conditions before experimentation and were presumed to be fully acclimated to laboratory conditions by then (Bayne 1976, Newell & Kofoed 1977). Thermal tolerance experiments were then performed identical to adults (see above), except that juveniles were placed in 6-well plastic tissue culture plates (1 juvenile per well), and the plates were submerged in the temperature-controlled seawater for the duration of the experiment. After the end of the thermal stress, juveniles were removed from the incubators and the water in each well was replaced with 23°C water containing T-ISO. Phytoplankton suspensions were replaced once after 24 h. Mortality was determined at 24 and 48 h after thermal stress by observing pedal and head movement, monitoring contraction into the shell when stimulated with a stream of water, and observing heart beat (visible through the shell at this stage of development).

Thermal stress in embryos

To determine the effect of environment and life history stage on thermal tolerance, experiments were performed on embryos collected from intertidal and subtidal *Crepidula fornicata* adults from Bissel Cove, Rhode Island. Adults were gently pried from their substrate and egg masses were carefully removed. The color of the egg mass is a good indicator of the stage of development of the embryos contained within; only dark-grey, late-stage egg masses that had been exposed to the typical environmental conditions for an extended period of time (weeks; Conklin 1897) were used for these experiments. To confirm that all embryos were at the same stage of development before experimentation, egg masses were dissected in the laboratory and only late-stage, shelled veliger larvae of approximately 300 μm (longest shell length) were used. Since embryos that are prematurely removed from the protective care of their mothers die soon after removal (Conklin 1897, C. M. Diederich pers. obs.), they were subsequently placed in vials containing 0.22 μm filtered seawater with 50 mg l^{-1} streptomycin sulfate and 40 mg l^{-1} penicillin G (Henry et al. 2006). This antibiotic-treated seawater allows for survival to maturity and was used for all experiments containing embryos.

Thermal tolerance experiments were performed on all embryos within 24 h of collection to avoid laboratory acclimation. Prior to thermal stress, egg masses were sliced open and embryos were emptied into a small glass dish. They were mixed thoroughly and randomly portioned into replicates (25−30 embryos per replicate, 5 replicates per treatment). Embryos were pipetted into small glass dishes containing water at the desired temperatures and maintained at that temperature for 3 h. At the end of the thermal stress, embryos were removed from the heated water and transferred to water at 23°C. Water was changed after 24 h. Mortality was monitored by observing swimming and muscle movement at 24 and 48 h after the end of the stress.

Data analysis

Densities of *Crepidula fornicata* at different tidal heights were compared using an ANOVA with a Bonferroni multiple comparisons test to determine specific differences among snail densities at each tidal height.

In order to meet the assumptions of the statistical tests, all percent survival data were arcsine transformed before specific comparisons were made (GraphPad Prism Software v. 4.03). In previous studies, the amount of time between the end of the stress and the measurement of mortality is highly variable (e.g. Sanders et al. 1991, Tomanek & Somero 1999, Hammond & Hofmann 2010, Sorte et al. 2010, Zippay & Hofmann 2010). Furthermore, in our pilot studies many animals that survived the first 24 h after the stress ended died in the following 24 h. Therefore, we checked mortality of all individuals at both 24 and 48 h after the end of the thermal stress. For each thermal tolerance experiment, differences in mortality between 24 and 48 h after the end of the thermal stress were compared using paired t-tests matched for each replicate. In animals that were kept in the laboratory for longer than 48 h, no additional mortality was observed.

To determine if embryos were more susceptible than adults to death from thermal stress, comparisons were made between the survival of these 2 life history stages using 2-way ANOVAs with temperature and life-history stage as independent variables. Comparisons were only made between embryos and adults collected during the same time of year (to control for seasonal effects on thermal tolerance) and that originated from the same tidal height (to control for the effect that environment may have on thermal tolerance). Experiments performed on intertidal individuals in August 2010 and August 2011 and subtidal individuals in August 2010 fit the requirements of this statistical test.

To determine the effect of tidal height on thermal tolerance, comparisons were made between the survival of individuals using 2-way ANOVAs with temperature and tidal height as independent variables. Comparisons were only made between animals at a particular life-history stage (embryo or adult; to control for potential effects of life history stage on thermal tolerance) collected during the same time of year (to control for seasonal effects on thermal tolerance). Individual thermal tolerance comparisons between intertidal and subtidal animals at a single temperature and recovery time were determined after ANOVA using Bonferroni multiple comparisons tests ($\alpha < 0.05$). Experiments performed on adults in June and August 2010 and embryos in August 2010 and June 2011 fit the requirements of this statistical test.

For thermal tolerance experiments with laboratory-acclimated juveniles, all larvae and juveniles were treated identically before and after thermal stress (see above). This allowed us to pool the results from the 3 experiments in which juveniles came from intertidal mothers, and pool the results of the 3 experiments in which juveniles came from subtidal mothers, to determine if there were differences in thermal tolerance between intertidal and subtidal organisms. Comparisons were made between intertidal and subtidal juveniles using a 2-way ANOVA with temperature and mother's habitat (i.e. intertidal or subtidal population) as independent variables.

Results

Population characteristics

Individuals of *Crepidula fornicata* were abundant both intertidally and subtidally in Bissel Cove, Rhode Island (Fig. 1). Densities (snails m^{-2}) varied significantly along the vertical gradient (1-way ANOVA, $F = 11.55$, $p = 0.0003$), but densities in the low intertidal (+0.2 m) and

subtidal (−1.0 m) were not significantly different (Bonferroni multiple comparison test, $p > 0.05$). At this location, the average high tidal mark in the summers of 2010 and 2011 was +1.38 m. Thus, since *C. fornicata* populations become less dense at approximately +0.4 m and are absent above +0.6 m, they occupy the mid and low intertidal zone and can be found continuously into the subtidal zone (Fig. 1).

Crepidula fornicata occupying the intertidal zone experienced different degrees of aerial exposure depending on their location within the intertidal zone (Fig. 1). Though on average animals at +0.4 m spent 3 h 39 min per tidal cycle (single low tide) exposed to air (Fig. 1), they were exposed for as long as 5 h 41 min during spring tides, but were often not exposed to air during neap tides. Similarly, the range of exposure time for animals at +0.2 m was 0 to 4 h 27 min per tidal cycle in the summer months of 2010 and 2011. Since tides are semidiurnal at this location, intertidal *C. fornicata* at the higher end of their vertical range (+0.4 m) spent on average 30.4% of the time exposed to the air during the summers of 2010 and 2011, while subtidal animals (−1.0 m) were never exposed to air (Fig. 1).

Thermal environment

Intertidal and subtidal animals experienced drastically different thermal environments in the summer months of 2011 (Figs. 2 & 3). The highest temperature that subtidal animals experienced was 26.5°C, while intertidal animals experienced temperatures more than 15°C warmer, reaching a maximum of 42°C in July 2011 (Fig. 3). Intertidal animals did spend most of the time within the range of temperatures experienced by subtidal conspecifics (81.3%), but when they experienced temperatures outside of this range they tended to be warmer (12.0%) rather than cooler (6.7%) (Fig. 3). The temperature changes that intertidal *Crepidula fornicata*

experienced were also often very rapid (Fig. 2), with tissues warming as quickly as 0.4°C min^{-1} and cooling as quickly as 0.5°C min^{-1}.

When intertidal *Crepidula fornicata* experienced high temperatures in the field, they often did so for extended periods of time (Fig. 4). On clear days when low tides occurred during the early afternoon, *C. fornicata* experienced temperatures above 30°C for as long as 5 h 15 min. On one occasion the intertidal robosnail recorded temperatures at or above 37°C for 3 continuous hours (Fig. 4) (temperatures above 30°C for 3 straight hours occurred 22 times), which is the amount of time we chose to thermally stress animals in the laboratory (Figs. 5−7).

Thermal tolerance

When thermally stressed in the laboratory, intertidal and subtidal embryos did not differ in upper thermal tolerance (Fig. 5, Table 1). Some embryos died after being stressed for 3 h at temperatures as low as 33°C in June 2011 (Fig. 5b), and nearly all (Fig. 5a) or all (Fig. 5c) embryos died following a single 3 h exposure to 37°C. Adults collected intertidally and subtidally also did not differ in thermal tolerance in June 2010 (Fig. 6a, Table 1), but intertidal adults were significantly more tolerant of high thermal stress in August 2010 (Fig. 6b, Table 1).

Life history stage had a significant effect on thermal tolerance, as embryos were more tolerant of the thermal stress than adults were (Figs. 5 & 6, Table 2). In August 2010 all embryos collected from intertidal and subtidal adults survived a 35°C stress (3 h) (Fig 5a) while some adults died following thermal stresses of 34 or 35°C (Fig 5b). Similarly, in August 2011 nearly all embryos from intertidal adults survived a thermal stress of 35°C (Fig 5c) while nearly all intertidal adults died after experiencing the same stress (Fig. 6c). However, though thermal tolerances were significantly different between life history stages (Table 2), absolute tolerances

differed only slightly, as nearly all embryos died after being exposed to thermal stresses only 1°C higher than those experienced by adults (Figs. 5 & 6).

We could detect no differences in traits responsible for upper thermal tolerance between intertidal and subtidal animals: laboratory-reared, fully-acclimated (23°C) juveniles originating from intertidal and subtidal mothers had nearly identical upper thermal limits (Fig. 7, Table 3). Variation in upper thermal limits did exist among individuals, but only over a small range of temperatures: in our study every juvenile survived a 3 h stress at 32°C, but nearly all died following a 3 h stress at 35°C (Fig. 7).

Discussion

In Narragansett Bay, Rhode Island, *Crepidula fornicata* is common both intertidally and subtidally (Fig. 1). This is not surprising, as they have long been collected from both habitats in both their native range (e.g. Diederich et al. 2011, Hoch & Cahill 2012) and their invasive range (Thieltges et al. 2003, Viard et al. 2006). However, though their distribution has been anecdotally described by some (e.g. Collin 2001), this is the first time that the distribution of a single population of *C. fornicata* has been formally recorded in both the intertidal and subtidal zones. Although *C. fornicata* is not alone in straddling the low tide mark (e.g. Dame 1972, Palmer 1980, Fletcher 1984, Jensen & Armstrong 1991, Bingham et al. 1997, Saier 2002, Altieri 2006, Schaffmeister et al. 2006), their considerable abundance in both zones is surprising: unlike many intertidal species that are overwhelmed by subtidal predators or subtidal competitors (Stephenson & Stephenson 1949, Paine 1974, Perez et al. 2009), populations of *C. fornicata* do very well subtidally, directly adjacent to thriving populations living in the physically harsh intertidal zone.

Among the challenges associated with living in the intertidal zone (e.g. exposure to periodic desiccation, salinity stress, and starvation), thermal stress can be particularly harsh, as temperature changes upon emersion can be both large and extremely rapid (e.g. Helmuth & Hofmann 2001, Wethey 2002, Lima & Wethey 2009, Szathmary et al. 2009). Similarly, our biomimetic robosnails recorded considerable temperature increases in the intertidal zone upon emersion, with temperatures reaching 15.5°C higher than those of subtidal robosnails in the summer of 2011 (Figs. 2 & 3) and increasing as rapidly as 6°C in 15 min (Fig. 2). In addition to experiencing rapid temperature changes, *C. fornicata* robosnails also remained at high temperatures for many hours at a time (Fig. 4). For example, robosnails reached 35°C (8.5°C higher than subtidal animals ever reached) 19 different times in 3 months during our study, and they remained at or above that temperature for up to 4 h at a time (Fig. 4). These temperature recordings highlight one of the major difficulties in living intertidally: most organisms that normally live subtidally and experience only a relatively small range of temperatures that change very slowly (seasonally) may not be able to cope with the large temperature changes that occur so quickly in the intertidal zone.

Individuals of *Crepidula fornicata*, like many intertidal organisms, are sessile as adults (Conklin 1898) and cannot rely on behavioral adaptations (e.g. movement subtidally or into shaded, damp crevices) to reduce high thermal stress during low tide. Thus, these organisms must rely entirely on high physiological tolerance to high temperatures in order to occupy the intertidal zone. In some species, intertidal organisms may have higher thermal tolerance limits than subtidal organisms due to environmentally induced plasticity (e.g. via the heat-shock response, Feder & Hofmann 1999) upon experiencing high temperatures in the field. Indeed, tolerances to environmental stressors across the intertidal–subtidal boundary have been shown to

be inducible in other organisms including mussels (Altieri 2006), and the upper thermal limits of many organisms are effected by prior acclimation temperatures (e.g. Cuculescu et al. 1998, Beitinger & Bennett 2000, Stillman & Somero 2000). Additionally, selective post-settlement mortality of individuals with low thermal tolerance in the intertidal zone can result in phenotypic differences among subpopulations, even when those subpopulations are extensively mixed. Schmidt & Rand (1999) for example, have shown that barnacles occupying different microhabitats in the intertidal zone undergo post-settlement mortality that favors different genotypes in different microhabitats. Though newly-settled barnacles are genetically homogeneous across microhabitats, this differential mortality would be expected to yield adult subpopulations with different tolerances to intertidal stressors (e.g. temperature and desiccation) (Schmidt & Rand 2001). Finally, local adaptation, even among species with long-lived larvae, is surprisingly prevalent in marine invertebrates (reviewed by Sanford & Kelly 2011). Fine-scale population structure has since been found in species with long-lived larvae (Hoffman et al. 2012) and even between intertidal and subtidal conspecifics with differing stress responses (Weihe & Abele 2008, de Aranzamendi et al. 2008, but see Hoffman et al. 2010). In these cases, intertidal organisms would be expected to have higher average thermal tolerance limits when compared with subtidal conspecifics that do not experience such high temperatures; but that is not what we found for this population of *C. fornicata*.

In our experiments, the upper thermal tolerance limits of intertidal and subtidal *Crepidula fornicata* were nearly identical (Figs. 5 & 6). On only one occasion in our study were intertidal individuals more thermally tolerant than subtidal individuals, and that was only by about 1°C (Fig. 6b). However, even in that experiment, subtidal animals had remarkably high thermal

tolerances considering the relatively low temperatures that they consistently experience in the field. Furthermore, the upper thermal tolerance limits of juvenile *C. fornicata* reared entirely in the laboratory from larvae obtained from intertidal and subtidal parents had identical thermal tolerance limits (Fig. 7), indicating that there are no phenotypic differences in thermal tolerance between individuals in these 2 subpopulations.

Though many species' upper thermal tolerance limits have been shown to be positively correlated with the temperatures that they experience, on both local and more broad geographic scales (e.g. Sharp et al. 1994, Bingham et al. 1997, Kuo & Sanford 2009), this is not always the case. For example, though Kuo & Sanford (2009) found differences in thermal tolerance among *Nucella canaliculata* from different populations over a broad geographic range (100s of km), many populations within their study had nearly identical upper thermal limits, despite differences in midday exposure time (and presumably temperature). In our study, subtidal *Crepidula fornicata* may have obtained relatively high upper thermal limits by 'seeding' (i.e. genetic mixing) from warm-adapted intertidal populations (Somero 2010). *C. fornicata* have long-lived, highly dispersive larvae which may allow for extensive genetic mixing of intertidal and subtidal subpopulations. If there are no costs to maintaining this high thermal tolerance in the subtidal zone, extensive mixing could yield similar physiological tolerances among these subpopulations.

In addition, warm-adapted subtidal *Crepidula fornicata* may be the source from which intertidal populations arise. This species has lived for millions of years in southern climates (Hoagland 1977) and living intertidally is apparently derived within the genus (Hoagland 1977). Collin (2001) has shown that clades of *C. fornicata* in the western Atlantic (sampled from New Brunswick to Florida) were not based on geography (little population structure exists). She also noted that *C. fornicata* does not form intertidal populations in southern habitats (Collin 2001).

Thus, subtidal *C. fornicata* may have obtained their relatively high thermal tolerance limits from warm-adapted ancestors or southern populations, and intertidal organisms may be part of a sink population that are living under suboptimal conditions. Indeed, in our study, individuals of *C. fornicata* from intertidal populations died following thermal stresses that they occasionally experience in the field, and so they may not be optimally adapted to intertidal conditions. However, we cannot rule out the possibility that there may still be slight differences in thermal tolerance among members of intertidal and subtidal subpopulations. When stressing the animals in our experiments, we chose controlled and relevant stress temperatures and durations, but did not mimic intertidal conditions completely. Further investigation including, for example, twice daily exposures to high temperature for several days, might produce slight differences in stress tolerance between animals of the 2 subpopulations.

Most previous studies on thermal tolerance of marine organisms have been conducted with a single life-history stage. However, since adults of *Crepidula fornicata* brood embryos for several weeks (Conklin 1897) (exposing embryos to about the same environmental conditions experienced by adults), the ability of this species to live intertidally is also contingent upon high thermal tolerance limits for all of its life history stages. Indeed, we found that both intertidal and subtidal *C. fornicata* embryos were at least as tolerant of thermal stress as were adults (Fig. 5). This is not the case for many species, as early life history stages are often less tolerant of many environmental stressors than later stages of development (Crisp & Ritz 1967, Kinne 1970, Gosselin & Chia 1995, Freitas et al. 2010, but see Miller et al. 2013). For example, Gosselin & Chia (1995) found that nearly all new hatchlings—but no adults—of *Nucella emarginata* died from an aerial thermal stress of 30°C for 8 h. Thus, species distributions may in some cases be

determined (and limited) by the sensitivity of early life history stages, or gametes, to environmental stressors that adults can tolerate (Andronikov 1975). In the future, multiple life-history stages should be assessed when determining the tolerance of a species to physiological stressors in order to develop a more complete picture of the overall capacity of a species to withstand such stressors. The remarkable ability of *C. fornicata* embryos to withstand temperatures as high as adults can tolerate has apparently allowed these organisms to occupy a substantial portion of the intertidal zone, and to take advantage of a habitat that is too harsh for most other marine species to occupy.

Though members of *Crepidula fornicata* now successfully occupy the intertidal zone in New England, our data suggest that these intertidal populations may be vulnerable to climate change in the coming years. Somero (2010) suggests that though many intertidal organisms have higher thermal tolerances than subtidal organisms have, they are also living close to their upper thermal limits and are thus likely to be the 'losers' as climates warm. For example, Tomanek & Somero (1999) found that although snails of the genus *Tegula* (now *Chlorostoma*) living in the intertidal zone were able to survive much higher temperatures (37°C for 2.5 h) than their subtidal congeners (30°C for 2.5 h), the intertidal individuals experienced field temperatures closer to their thermal maximum (max. temp of 33°C intertidally as opposed to 24°C subtidally). In our experiments, some adult *C. fornicata* died after a single 3 h thermal stress at 34°C in the laboratory, a stress level that intertidal robosnails recorded many times in our study. It is possible that robosnail recordings could slightly overestimate tissue temperatures of animals in the field, because *C. fornicata* may lift up from their substrate and evaporatively cool their tissues. However, this behavior has been shown to cool tissues by only 2°C in limpets (*Cellana grata*) and it was effective for less than 2 h (Williams et al. 2005). Thus, the temperatures that killed

intertidal *C. fornicata* in the laboratory were very close to temperatures that they experience in the field; subtidal animals, on the other hand, did not experience temperatures within 7°C of temperatures that kill them. Furthermore, the one occasion on which intertidal organisms had a slightly higher thermal tolerance than subtidal organisms in our study (Fig. 6b) may have been the result of high mortality of less-tolerant intertidal organisms in the months preceding the study (though differential acclimatization is also possible). These data indicate that intertidal – but not subtidal – *C. fornicata* in our study area are living close to their upper thermal limit. As the anticipated climate change further warms coastal habitats, *C. fornicata* will likely be relegated to the subtidal, where, unlike intertidal specialists, they are not outcompeted or heavily preyed upon. Their disappearance from the intertidal could have interesting community-wide effects, as they presently compete with other intertidal suspension feeders for food (e.g. mussels and oysters, Lesser et al. 1992, Decottignies et al. 2007); are eaten by many other animals including the predatory gastropod *Urosalpinx cinerea*, a number of crab species (*Dyspanopeus sayi*, *Cancer borealis*, *Pagurus longicarpus*, and *Hemigrapsus sanguineus*), and the sea star *Asterias forbesi* (Hoagland 1974, Pratt 1974, Lindsey et al. 2006, Pechenik et al. 2010); and are a host for the ectoparasitic gastropod *Boonea seminuda* (Boss & Merrill 1965, C. M. Diederich pers. obs.) and the boring sponge *Cliona celata* (Hoagland 1974, LeCam & Viard 2011). In addition, their shells provide homes for numerous polychaetes and other invertebrates such as juveniles of the invasive crab, *Hemigrapsus sanguineus* (C. M. Diederich pers. obs.) and hermit crabs (Williams & McDermott 2004) while creating biogenic structures for organisms such as xanthid crabs (Lindsey et al. 2006).

Though we now know that individuals of *Crepidula fornicata* may be killed by summer temperatures presently characterizing the intertidal zone in New England, the specific cause of

thermal death in this species is not clear. Somero (2002) reviews many possible 'weak links' in physiological systems that cause thermal death, including failure of organ systems (especially the heart), action potential generation, mitochondrial respiration, membrane integrity, the heat shock response, and physical stability of enzymes. No doubt the failures of these systems act on different time scales. The fact that in all but one of our experiments (Fig. 5c) most animals survived for at least 24 h after the thermal stress ended indicates that some of the faster acting mechanisms for death (e.g. heart failure, nervous system failure) are probably not the cause of death for *C. fornicata*. It would be interesting to learn the specific cause(s) of death in this and other intertidal species, as the cause of death from high temperatures would be the most likely target of selection for animals living close to their upper thermal limits; the ability of these physiological systems to change will be important in determining the fate of these species in the face of global climate change (Somero 2010).

In our experiments, the fact that few individuals died within the first 24 h after the thermal stress was applied underscores the importance of monitoring organisms for an extended period of time in experiments assessing tolerance to environmental stressors. Though stressors may be applied to organisms in the laboratory depending on specific ecological conditions that those organisms are likely to encounter, the time selected to observe organisms for mortality after the stress ends varies widely (e.g. immediately; Zippay & Hofmann 2010, after 6 h recovery; Kenny 1969, after 12 h recovery; Singletary 1971, after 24 h recovery; Sanders et al. 1991, after 48 h recovery; Sorte et al. 2010, after 30 d recovery; Coles & Jokiel 1978). Our data suggest that monitoring organisms for less than 24 h may produce misleading results.

In summary, *Crepidula fornicata* can be found in considerable numbers both intertidally and subtidally in New England; these populations experience drastically different thermal

environments. However, the temperature differences that they face are not reflected in their physiological tolerance to thermal stress, as both embryos and adults sampled from intertidal and subtidal subpopulations had nearly identical thermal tolerances, as did juveniles reared in the laboratory from intertidal and subtidal mothers. Though they are a major member of both the intertidal and subtidal zones in the temperate Atlantic, intertidal *C. fornicata* are now living very close to their thermal maximum and will likely be lost from the intertidal zone in the coming years as summer maximum air temperatures increase. However, compared with most marine species, *C. fornicata* is relatively eurythermal, a factor that could have contributed to their substantial success as an invader in Europe and elsewhere (Blanchard 1997) and that should allow them to persist subtidally in the face of global climate change.

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Tables

Table 2.1: Two-way ANOVA determining the effects of temperature (Temp) and tidal height (TH) on survival (after 48 h of recovery time) of field-collected *Crepidula fornicata*. **Bold**: significant at $p < 0.01$.

Source	df	MS	*F*	p	cf. Fig.
Aug 2010 – Embryos					
Temp	4	5.700	411.0	**<0.0001**	5a
TH	1	0.02928	2.111	0.1540	
Temp × TH	4	0.01126	0.8120	0.5240	
Residual	40	0.01387			
Jun 2011 – Embryos					
Temp	3	0.4410	28.39	**<0.0001**	5b
TH	1	0.02031	1.308	0.2613	
Temp × TH	3	0.01550	0.9981	0.4063	
Residual	32	0.01553			
Jun 2010 – Adults					
Temp	3	1.744	67.23	**<0.0001**	6a
TH	1	0.04967	1.914	0.1761	
Temp × TH	3	0.03618	1.395	0.2623	
Residual	32	0.02594			
Aug 2010 – Adults					
Temp	3	1.380	40.33	**<0.0001**	6b
TH	1	0.3542	10.36	**<0.0001**	
Temp × TH	3	0.4536	13.26	**0.0030**	
Residual	32	0.03421			

Table 2.2: Two-way ANOVA determining the effects of temperature (Temp) and life history stage (LHS) on survival (after 48 h of recovery time) of field-collected *Crepidula fornicata*. **Bold**: significant at $p < 0.01$.

Source	df	MS	*F*	p	cf. Fig.
Aug 2010 – Intertidal					
Temp	2	0.0817	5.762	**0.0090**	5a & 6b
LHS	1	0.2118	17.93	**0.0007**	
Temp × LHS	2	0.0817	5.762	**0.0090**	
Residual	24	0.0142			
Aug 2010 – Subtidal					
Temp	2	1.102	35.08	**<0.0001**	5a & 6b
LHS	1	1.317	41.91	**<0.0001**	
Temp × LHS	2	1.102	35.08	**<0.0001**	
Residual	24	0.0314			
Aug 2011 – Intertidal					
Temp	3	5.364	314.9	**<0.0001**	5c & 6c
LHS	1	134.7	7.57	**<0.0001**	
Temp × LHS	3	0.9449	55.47	**<0.0001**	
Residual	32	0.0170			

Table 2.3: Two-way ANOVA determining the effects of temperature (Temp) and mother's environment (mother's tidal height, MTH) on survival of laboratory-acclimated juvenile *Crepidula fornicata*. **Bold**: significant at $p < 0.01$.

Source	df	MS	*F*	p	cf. Fig.
Temp	4	8.654	284.85	**<0.0001**	7
MTH	1	0.02144	0.71	0.4027	
Temp × MTH	4	0.03213	1.06	0.3811	
Residual	110	0.03038			

Figures

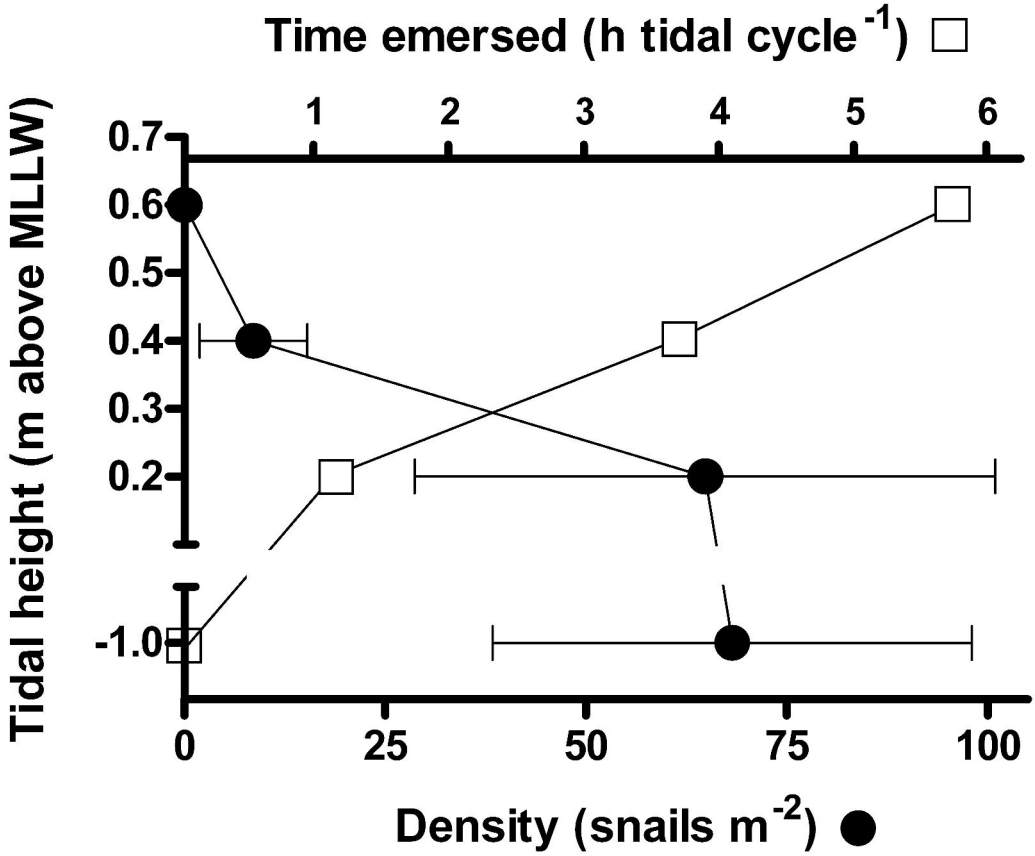

Figure 2.1: *Crepidula fornicata*. Field distribution and average emersion times at different tidal heights in Bissel Cove, Rhode Island. Filled circles = mean (±SD) of 5 quadrats along a horizontal transect at each tidal height. Open squares = the average time an individual at a particular tidal height was exposed to air during 1 tidal cycle from June to August 2010 and 2011. A tidal height of 0 m is defined as the mean low lower water mark (MLLW) as determined by the Quonset Point, RI, buoy operated by the NOAA National Data Buoy Center.

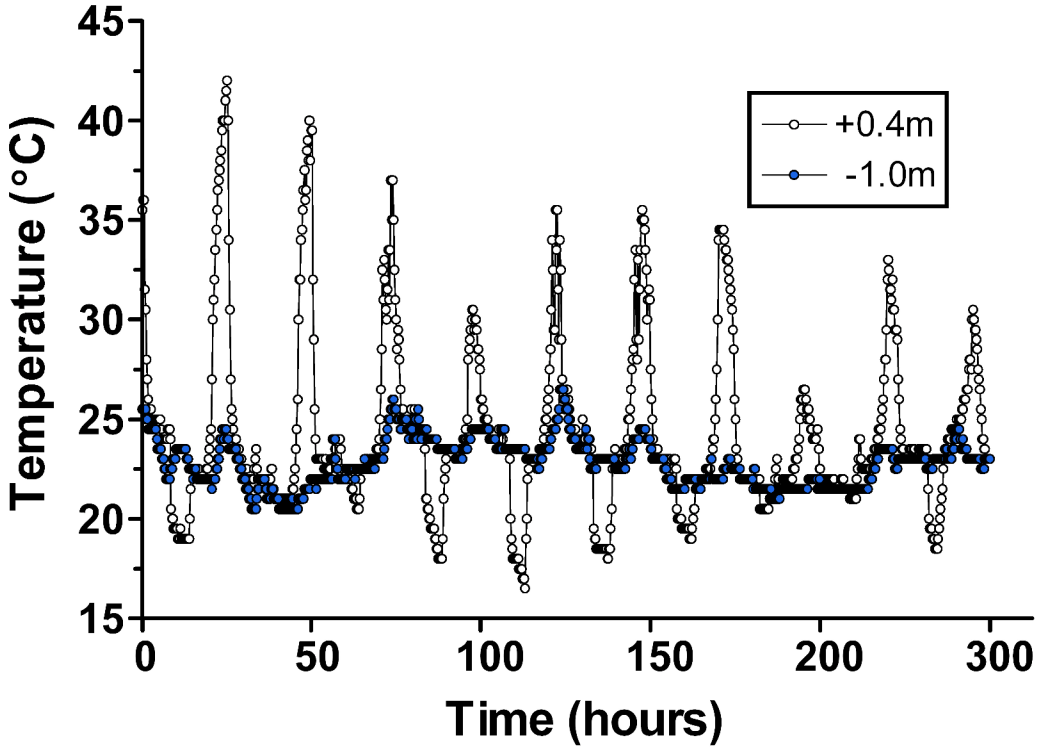

Figure 2.2: Typical thermal environment of intertidal and subtidal *Crepidula fornicata* in Bissel Cove, RI. Temperatures were recorded from biomimetic robosnails placed among living *C. fornicata* in the intertidal zone (+0.4 m mean low lower water [MLLW], open circles) and the subtidal zone (−1.0 m MLLW, filled circles) in June, July and August 2011. Data are from a representative 10 d time period in July 2011; data points are 15 min apart.

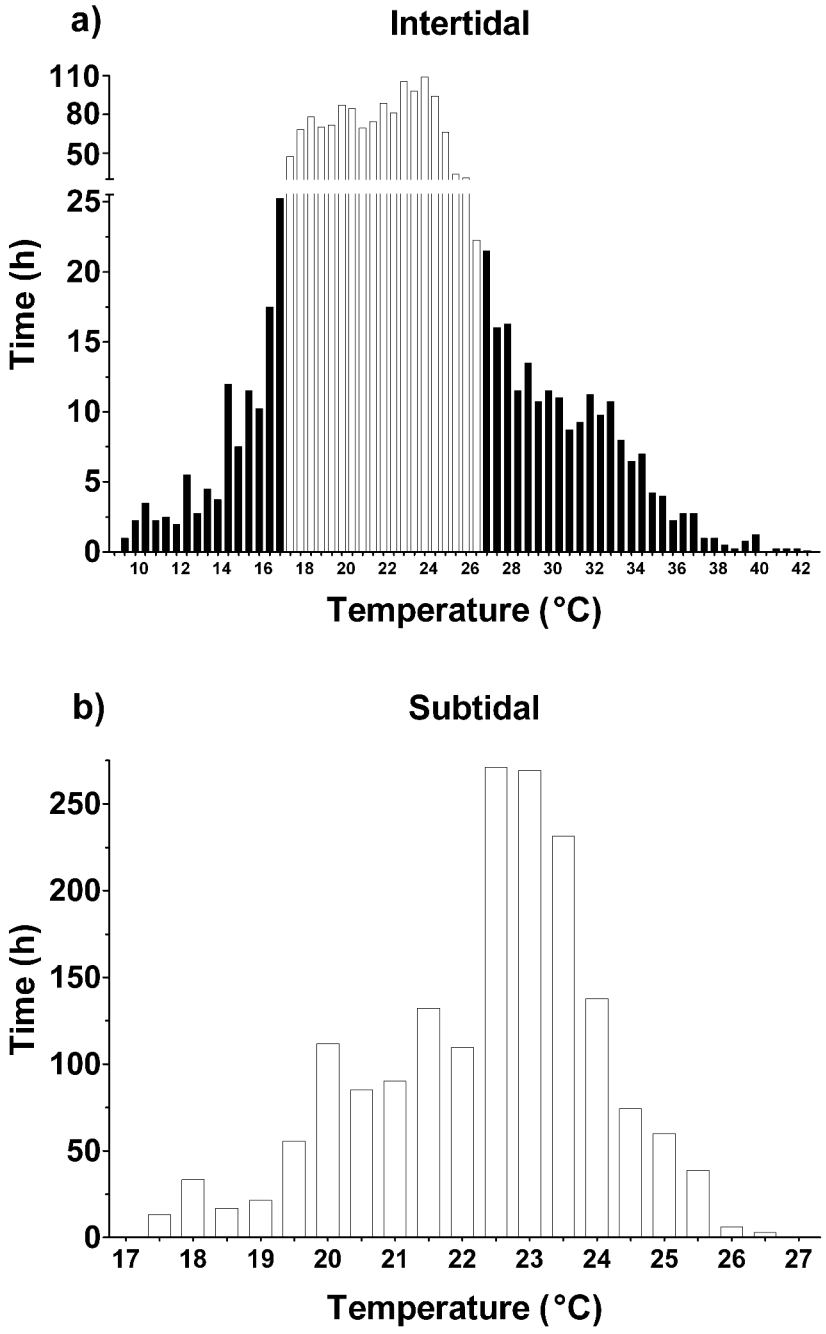

Figure 2.3: Temperatures experienced by intertidal and subtidal *Crepidula fornicata* in the summer of 2011. Continuous recordings from biomimetic robosnails placed among living *C. fornicata* were made in June, July, and August 2011. Recordings were taken every 15 min; therefore, cumulative time was estimated by multiplying the number of recordings at a particular temperature by 15 min. (a) Recordings taken at +0.4 m mean low lower water (MLLW). Black bars = temperatures experienced by intertidal robosnails that were outside the range of temperatures experienced by subtidal robosnails; white bars = within the range of subtidal robosnails. (b) Recordings taken at −1.0 m MLLW.

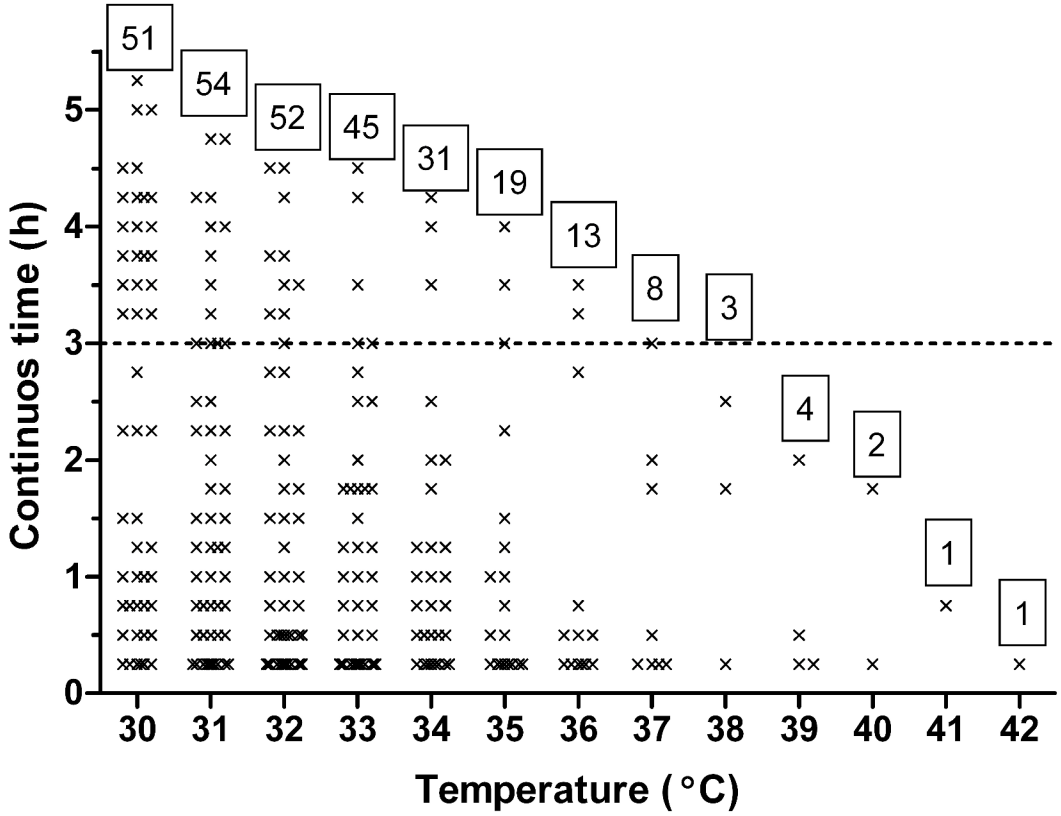

Figure 2.4: Thermally challenging events in the intertidal zone (+0.4 m mean low lower water [MLLW]) at Bissel Cove in Narragansett Bay, RI. Boxed numbers = number of times intertidal robosnails remained at or above the given temperature from June to August 2011. Symbols = amount of time spent at or above the given temperature on each occasion. Horizontal dashed line = amount of time we chose to thermally stress animals in laboratory experiments.

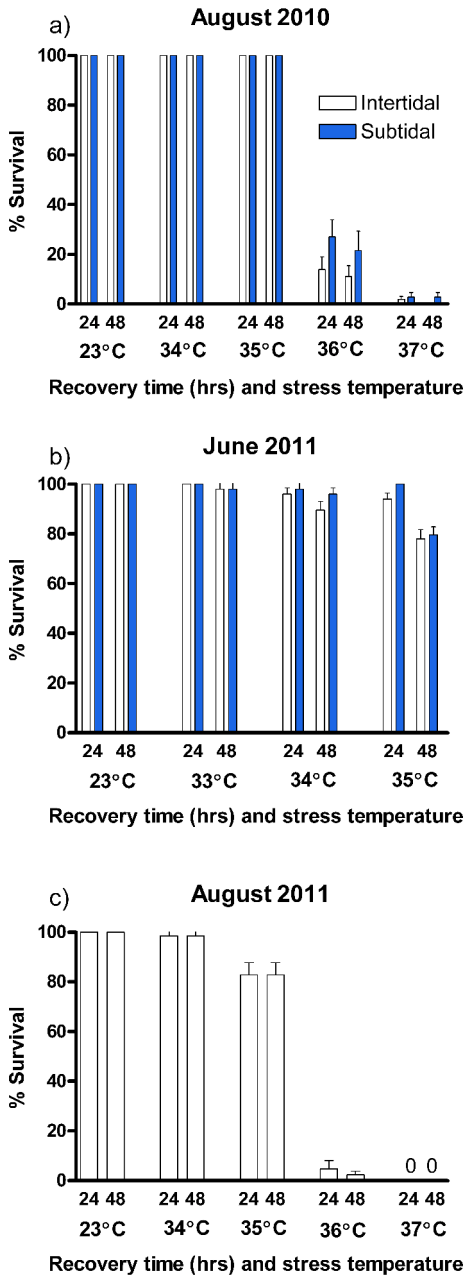

Figure 2.5: *Crepidula fornicata*. Thermal tolerance of intertidal (white bars) and subtidal (blue bars) embryos collected from Bissel Cove, RI in (a) August 2010, (b) June 2011, and (c) August 2011. Embryos were stressed for 3 h at the indicated temperature and mortality was assessed 24 and 48 h later. Bars = mean (+SD) of 5 replicates with 25−30 embryos per replicate. Survival was significantly different (paired t-tests) between 24 and 48 h of recovery time in (a, $p = 0.0070$) and (b, $p < 0.0001$) but not (c, $p = 0.324$). No significant differences between survival of intertidal and subtidal embryos at a particular temperature and recovery time were found (Bonferroni multiple comparisons, all $p > 0.05$). Detailed statistical analyses regarding the effects of temperature, tidal height, and life history stage can be found in Tables 1 & 2.

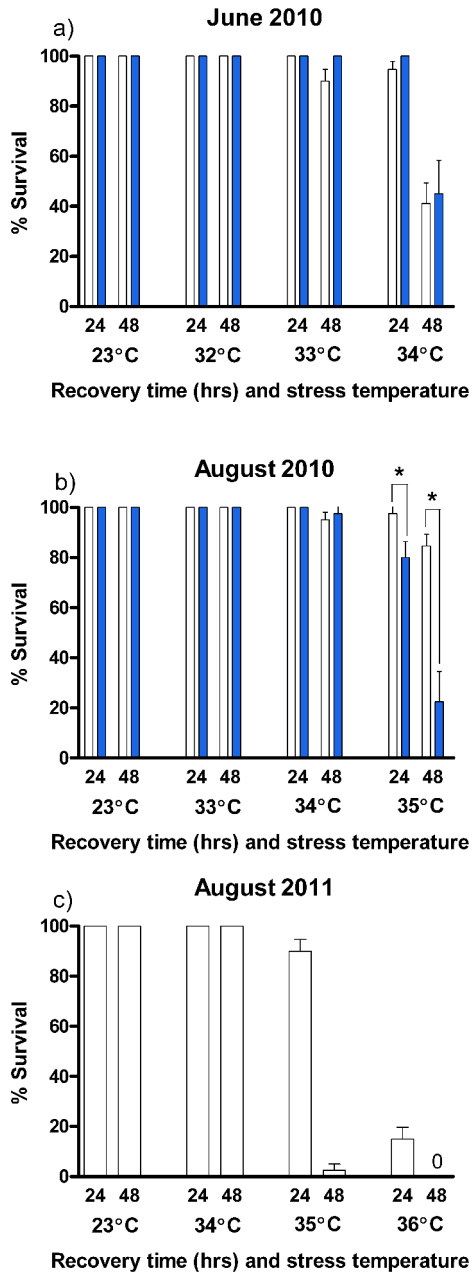

Figure 2.6: *Crepidula fornicata*. Thermal tolerance of intertidal (white bars) and subtidal (blue bars) adults collected from Bissel Cove, RI, in (a) June 2010, (b) August 2010, and (c) August 2011. Adults were stressed for 3 h at the indicated temperature and mortality was assessed 24 and 48 h later. Bars = mean (+SD) of 5 replicates with 8 animals per replicate. Survival was significantly different (paired t-tests) between 24 and 48 h of recovery time in all experiments: (a, $p = 0.0005$); (b, $p = 0.0009$); (c, $p = 0.0043$). Asterisks = significant differences between survival of intertidal and subtidal adults at a particular temperature and recovery time (Bonferroni multiple comparisons, $p < 0.001$). Detailed statistical analysis regarding the effects of temperature, tidal height, and life history stage can be found in Tables 1 & 2.

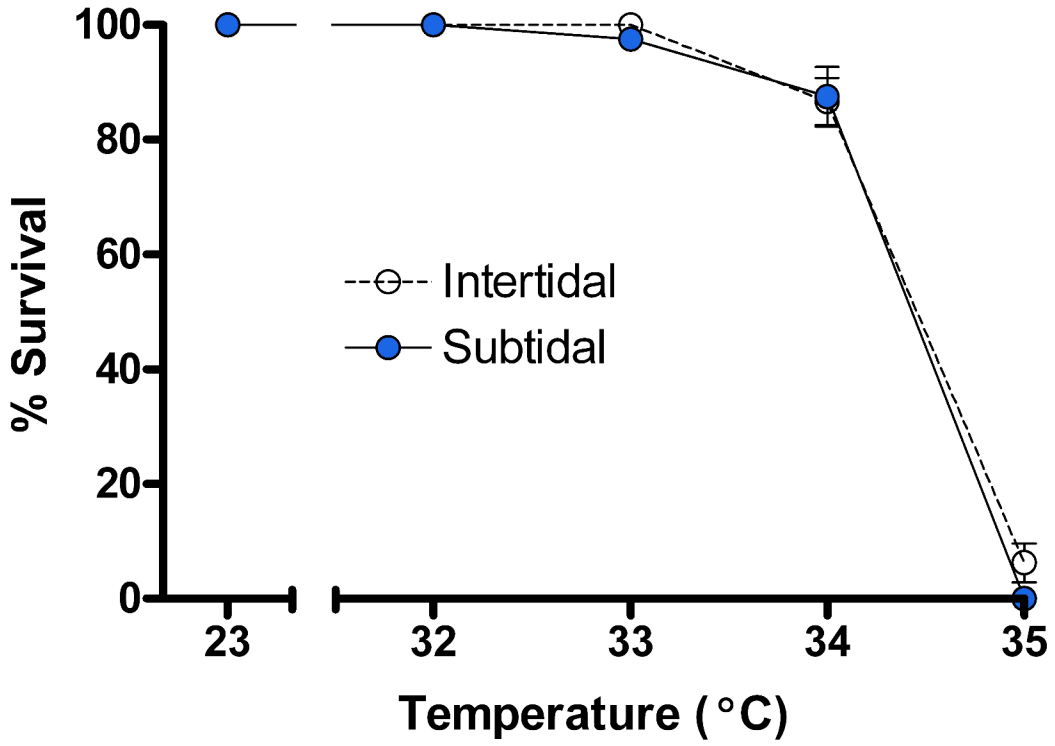

Figure 2.7: *Crepidula fornicata*. Variation in thermal tolerance between laboratory-reared juveniles originating from 3 sets each of intertidal (white circles, dashed line) and subtidal (blue circles, solid line) parents. All juveniles (~2.5 mm shell length) were reared from hatching in the laboratory at 23°C for at least 3 wk before being subjected to a 3 h thermal stress at the indicated temperature. Data points = mean (±SD) survival after 48 h of recovery time. Sample sizes of data points: 23°C, N = 15; 32°C, N = 5; 33°C, N = 15; 34°C, N = 15; 35°C, N = 10 replicates with 8 juveniles per replicate. Thermal tolerances of juveniles from intertidal and subtidal mothers were not significantly different ($p = 0.4027$, see 2-way ANOVA results in Table 3).

Chapter 3

Desiccation tolerance and lifting behavior in *Crepidula fornicata* (Gastropoda) from intertidal and subtidal subpopulations

Note: Submitted to *Marine Ecology Progress Series*

Abstract

Desiccation stress is a major limiting factor for many organisms in the intertidal zone. Sessile mollusks often isolate themselves from the external environment upon aerial exposure to control water loss, which may result in other stressful conditions within their sealed cavity. Interestingly, many intertidal animals willfully expose their tissues to the air when emersed ("gaping", "mushrooming", or "lifting" behavior). This basic behavior – and the desiccation tolerance – of the cosmopolitan gastropod *Crepidula fornicata* have not been thoroughly investigated. We found that individuals of *C. fornicata* perform this lifting behavior in the intertidal zone. When exposed to the air, intertidal adults lifted and clamped with significantly greater frequency and spent less time with their tissues exposed than both subtidal adults and lab-reared juveniles. However, we found no measurable difference in desiccation tolerance between intertidal and subtidal adults: they all survived aerial exposures of up to 10 hr. Most small juvenile *C. fornicata*, though, died following only 2 – 6h of exposure. Juveniles died after losing between 38.5 and 75.5% of their tissue water, and smaller individuals lost water more quickly than did large individuals. Individuals maintained aerobic respiration in the air and aerial CO_2 accumulated in their mantle cavity when they were clamped against the substrate, indicating that lifting behavior is probably for respiratory purposes. Because juveniles lost water so quickly, desiccation stress may be an important limiting factor for *C. fornicata*, unless juveniles can move to wet areas when exposed until they are large enough to withstand long periods of aerial exposure.

Introduction

The intertidal zone is characterized by a suite of environmental stressors that make life there difficult for most marine organisms. It is not surprising, then, that the intertidal zone represents a range boundary for many marine species, beyond which they cannot survive (Holt & Keitt 2005). The study of species at their upper range boundaries is important not only for understanding the factors that limit species distributions, but also for investigating species' adaptations and the evolution of niches (Holt & Gomulkiewicz 1997, Bridle & Vines 2007, Sexton et al. 2009).Though multiple environmental factors may act in concert to stress intertidal organisms (e.g. Firth & Williams 2009, Williams et al. 2011, Iacarella & Helmuth 2012), the ranges of some intertidal species can be limited by the overwhelming negative effects of individual stressors (e.g. desiccation, temperature, hypoxia) (e.g. Kensler 1967, McQuaid 1982, Wethey 1983, Somero 2010). When soft-bodied marine organisms are exposed to the air they can lose tissue water rapidly (e.g. Branch 1975, McQuaid 1982); the percentage of tissue water that organisms can lose before they die is species-specific, but typically ranges from 10 – 70% in mollusks (e.g. Lent 1968, Davies 1969, Branch 1975, Marshall & Mcquaid 1992, Williams & Morritt 1995, Sokolova & Pörtner 2001, Hicks & McMahon 2003). Within a species, since tissue surface-to-volume ratios change as organisms grow larger, small organisms (e.g. juveniles) lose tissue water more rapidly than adults and thus juveniles are often highly susceptible to desiccation stress (e.g. Davies 1969, Branch 1975, McQuaid 1982, Williams et al. 2005, Jenewein & Gosselin 2013). Not surprisingly, desiccation stress is widely reported as a major limiting factor for many marine organisms in the intertidal zone (Kensler 1967, Sutherland 1970, Branch 1975, McQuaid 1982, Jenewein & Gosselin 2013).

While most marine organisms simply cannot tolerate aerial exposure, many have evolved special behavioral (e.g. Chapperon & Seuront 2011, Miller & Denny 2011), physiological (e.g. Somero 2002, Marshall & McQuaid 2011), and morphological (e.g. Harley et al. 2009, Miller & Denny 2011) characteristics that allow them to survive the harsh conditions associated with exposure to the air. As it relates to desiccation, behavioral adaptations of organisms are critical for surviving daily bouts of aerial exposure. Mobile animals may follow the tides as they move up and down the shore (e.g. Robles et al. 1989), take cover under algae or in crevices where desiccation stress is reduced (e.g. Garrity 1984, Menge 1978), or aggregate to reduce water loss (e.g. Rojas et al. 2013). But sessile (or very slow-moving) organisms do not have those luxuries, and so they must isolate their tissues from the external environment to avoid losing water. Bivalve mollusks do this by withdrawing their tissues into their shells and tightly closing their shell valves (e.g. McMahon 1988). Some gastropods (limpets) may tightly clamp to the substrate in order to achieve similar isolation (e.g. Wolcott 1973), while other gastropods may completely withdraw into their shell and use their operculum for isolation from the external environment (McMahon 1990).

However, this isolative behavior carries with it a tradeoff. Though the ability to isolate from the outside environment may reduce water loss, the isolated chamber of bivalves and gastropods (the mantle cavity) may itself become a stressful environment, as oxygen is depleted and metabolic end products accumulate (Williams et al. 2005, Chaparro et al. 2009, Chaparro et al. 2011). When exposed to the air, some animals remain isolated for extended periods of time (McMahon 1990) thus reducing dehydration, while other mollusks perform behaviors that periodically expose their tissues to the air: ‘gaping’ in bivalves (e.g. Lent 1968, Boyden 1972, Byrne & McMahon 1994, Nicastro et al. 2010) and ‘mushrooming’ or shell ‘lifting’ in limpets

(the foot remains attached to the substrate, e.g. Wolcott 1973, Garrity 1984, Williams & Morritt 1995, Williams et al. 2005). Although such exposure to air should increase tissue water loss, it is those mollusk species that have a long evolutionary history with the intertidal zone (or aerial exposure in general) that perform such behaviors routinely (e.g. Lent 1968, Boyden 1972, Widdows et al. 1979, Garrity 1984, Widdows & Shick 1985, Byrne & McMahon 1994, Hicks & McMahon 2003, Dowd & Somero 2013). They may do so for several reasons: to re-supply depleted oxygen for respiratory purposes (e.g. Lent 1969, Boyden 1972, Widdows et al. 1979, Dowd & Somero 2013), to cool tissues through evaporation (e.g. Williams et al. 2005, Nicastro et al. 2012), or to enhance energy utilization (e.g. Widdows & Shick 1985). Though gaping behavior has been compared among congeners (or other closely related species) (e.g. Boyden 1972, Nicastro et al. 2010, Dowd & Somero 2013), the influence of past experience or habitat (living intertidally or subtidally) on this behavior within a species is less well-studied.

The gastropod *Crepidula fornicata* is an important member of both intertidal and subtidal communities along the east coast of North America (Diederich & Pechenik 2013). Juvenile *C. fornicata* are mobile (Coe 1936), but because adults are sessile and fertilization is internal, members of this species live attached to one another in stacks to ensure reproductive success (Richard et al. 2006). Native to the western Atlantic, *C. fornicata* has become an extremely successful invasive species worldwide, but especially along the coastlines of France and the UK (Blanchard 1997). Though this invasion has spurred a considerable amount of research on *C. fornicata*, little is known about many basic aspects of its biology and ecology. In particular, the role of desiccation stress in controlling the upper distribution of this species has never been studied. Additionally, though individuals of *C. fornicata* have been anecdotally observed lifting off of their substrate in the intertidal zone (hereafter "lifting behavior") (Newell & Kofoed 1977,

Hoagland 1984, Diederich & Pechenik 2013), this behavior has never been explicitly studied or quantified in this species. Furthermore, its distribution across the intertidal-subtidal boundary makes it an excellent study species to investigate the role of past experience in shaping future lifting behavior.

In this study we first aimed to discover if both adults and juveniles of *Crepidula fornicata* displayed such lifting behavior. We then characterized the lifting behavior of intertidal adults and subtidal adults, as well as that of lab-reared juveniles that had never experienced aerial exposure. To investigate the degree to which desiccation stress limits the upper distribution of this species, we also determined the desiccation tolerance of *C. fornicata* adults and juveniles. Additionally, we determined the amount of water loss necessary to kill *C. fornicata* juveniles, as well as the effect of animal size on the rate of water loss in juvenile *C. fornicata*. Finally, we measured CO_2 production and water loss in organisms that were free to lift off of the substrate or artificially forced to clamp to the substrate to determine the extent to which lifting behavior imposes a tradeoff between aerial respiration and water loss in this species.

Materials and Methods

Collection of animals and field measurements

All field observations and all animal collections for laboratory experiments were made at Bissel Cove, in Narragansett Bay, Rhode Island. At this site, *C. fornicata* is abundant both intertidally and subtidally (Diederich & Pechenik 2013). Measurements of both ambient humidity and the proportion of *Crepidula fornicata* that were lifted off of the substrate were made here on July 4, August 1, August 16, September 3, October 1, October 14, and December 10, 2012 at low tide. In the intertidal zone, it was possible to get very close to individuals of *C.*

fornicata without disturbing them (Fig. 1A). At approximately +0.2 – 0.4 m above mean low lower water (MLLW), the first 40 individuals of *C. fornicata* that were encountered were scored for being either lifted off of the substrate or clamped tightly to the substrate. Any animal whose shell was lifted off of the substrate so that a visible gap could be discerned between the shell and the substrate was scored as 'lifted.' In the same areas where *C. fornicata* were encountered, 20 humidity and temperature measurements were made using a handheld Extech Instruments RH300 digital psychrometer. Adults were then collected at approximately +0.4 m MLLW (hereafter 'intertidal') and -1.0 m MLLW (hereafter 'subtidal') for desiccation tolerance experiments and to obtain offspring.

Maintenance of laboratory animals

Desiccation tolerance experiments and laboratory lifting behavior observations with intertidal and subtidal *C. fornicata* adults were performed within 48 h of collection, to avoid acclimation to laboratory conditions (Rising & Armitage 1969, Widdows & Bayne 1971). Otherwise, adults were maintained in 3 l glass aquaria with 0.45µm-filtered seawater at ~20ºC and a salinity of 30. Aquaria were constantly aerated and water was changed every other day. Adults were fed phytoplankton suspensions composed of a mixture of *Isochrysis galbana* (clone T-ISO) and *Dunaliella tertiolecta* (clone DUN) twice daily until larvae were released. Larvae hatched from several intertidal and subtidal mothers within 1 day of each other. Those larvae were collected on 120µm mesh filters, rinsed with seawater, and maintained in 3 l glass aquaria filled with 0.45µm-filtered seawater. Larvae from intertidal and subtidal mothers were subsequently mixed to avoid any genetic or maternal effects on subsequent results. Larvae were fed daily with T-ISO at approximately 18 x 10^4 cells ml^{-1} (Pechenik & Lima 1984, Pechenik et al. 2002). Water was changed every other day until larvae were approximately 900µm, at which

point they were exposed to seawater with 20mM excess KCl for 6 h to induce metamorphosis (Pechenik & Heyman 1987, Pechenik & Gee 1993). This method for the induction of metamorphosis does not affect juvenile survival or growth (Eyster & Pechenik 1988). After metamorphosis, juveniles were maintained just as adults were, except that they were initially fed only T-ISO for approximately 2 weeks before switching to a diet of T-ISO and DUN. Thus, juveniles for all subsequent experiments had never experienced aerial exposure prior to experimentation.

Desiccation tolerance

Freshly-collected intertidal and subtidal adults (see above) were brought into the laboratory, and the top-most members of each stack were removed so that each individual was alone on its substrate (small rock or empty shell). Adults used for these experiments ranged in size from 25.3 – 43.2 mm (intertidal) and 24.4 – 44.0 mm (subtidal). For each treatment, 12 – 15 intertidal and subtidal adults each were taken out of their aquaria and were placed into a Percival model I-41VL temperature and humidity controlled incubator at 26ºC and 75 % relative humidity (conditions which were similar to those found at the field site). Different groups (treatments) of animals were removed from the incubator after 1, 2, 3, 4, 5, 6, and 10 h of desiccation stress. Animals were immediately returned to aquaria filled with aerated, 0.45µm-filtered seawater at ~20ºC and a salinity of 30 and fed a mixture of T-ISO and DUN (see above). Mortality was assessed 48 h after the end of the desiccation stress by prying animals from their substrate and testing muscular response by prodding with a small probe.

For experiments with lab-reared juveniles, 275 mL glass containers were used for desiccation stress. In these sealed containers, specific relative humidity levels in the air can be achieved using saturated solutions of various ions (Winston & Bates 1960). The bottom ~2 cm of

each glass container was filled with a saturated solution of a particular ion. Solutions were made according to Winston and Bates (1960) by dissolving solids of each ion into heated distilled water and then adding additional solid as the solution cools, until solid crystals remained undissolved in each container. In this way, at 20ºC relative humidity levels of approximately 100%, 85%, 75%, and 32% were achieved using distilled water alone, and saturated solutions of KCl, NaCl, and $CaCl_2$, respectively. Humidity levels were confirmed using a handheld digital psychrometer. At the time of desiccation stress, 5 – 6 lab-reared juveniles per replicate (3 – 6 replicates per humidity level) were placed on plastic Petri dishes (diameter 40 mm) and quickly suspended over each solution in the sealed containers, so that they remained exposed to each humidity level for the duration of the stress period (1 – 6 hr). Juveniles ranged in size from 1.3 – 4.6 mm and average size of juveniles in each treatment were not statistically different (one-way ANOVA, $F_{31} = 1.265$, $p = 0.17$). After the end of the desiccation stress, juveniles were removed from the containers and placed in individual glass dishes with ~75 ml phytoplankton suspension per dish (equal parts T-ISO and DUN) at 20ºC. Water was changed after 24 h and mortality was assessed 48 h after the end of the stress by observing pedal and head movements and monitoring contraction into the shell when stimulated with a probe (Diederich & Pechenik 2013).

Impact of water loss and effect of animal size

To determine the amount of water loss necessary to kill *C. fornicata* juveniles (2.7 – 5.3 mm shell length), individuals were desiccated (see above) in 32 – 100% relative humidity at 20ºC for up to 5 hr. Before desiccation, the shell of each juvenile was blotted dry before the juvenile was weighed in a Mettler Toledo model AL104 balance. The juveniles were then desiccated and weighed again following desiccation, after which they were transferred to individual dishes containing 0.45µm-filtered seawater and mortality was assessed 18 h later.

Immediately after mortality was assessed all juveniles were dried at 50ºC in a VWR model 133OG drying oven for 48 h to determine final dry weight. Percent water loss was determined following the approach of Sokolova and Pörtner (2001):

$$\%WL = [(W_i - W_e)/(W_i - W_d)] \times 100$$

where W_i is the initial weight, W_e is the weight after the desiccation period, and W_d is the final dry weight. Though some of the organisms died during the 18 h recovery period, their tissues were still intact before determination of final dry weight.

To determine the effect of animal size on the rate at which they lost water, juvenile *C. fornicata* were first measured (2.2 – 5.9 mm shell length), and then blotted dry and weighed. Twelve juveniles each were then desiccated for 10 minutes at either 32, 75, or 85% relative humidity. Weight after desiccation was determined and then all juveniles were immediately transferred to a drying over at 50ºC for 48 hr, after which their final dry weight was determined and percent water loss was calculated (see above) for each juvenile.

Lifting behavior and movement

To determine if typical lifting behavior of *C. fornicata* is affected by prior aerial exposure, behavioral observations of intertidal adults, subtidal adults, and lab-reared juveniles were made. Adults were collected from Bissel Cove, RI and juveniles were reared from larvae in the laboratory (see above). In order to be able to reliably observe their lifting behavior, juveniles were grown in the laboratory for longer than in other experiments (9.5 – 13.8 mm shell length). All animals were individually observed in a 1.1 L clear plastic aquarium that was initially filled with 0.45µm-filtered seawater at a salinity of 30 and temperature of ~20ºC. Before observations were made, individuals were transferred from their aquaria to the observation tank and allowed to acclimate in the observation tank for 10 minutes. They were then observed for 15 minutes

(while submerged); the times of all lifting or clamping movements were recorded. Following the 15 minutes submerged observation period, water was slowly drained from the bottom of the observation tank to simulate the outgoing tide. Once the tank was empty the lifting and clamping behaviors of individuals were observed for an additional 15 minutes while animals were exposed to the air. A total of 75 animals (25 in each treatment) were observed, after which the percentage of time spent lifted, the average number of movements (lift or clamp) and the maximum continuous time spent lifted were calculated for all groups of animals while they were both submerged and exposed to the air.

Though *C. fornicata* adults are sessile, juvenile *C. fornicata* have been described as mobile (e.g. Coe 1936), but no explicit tests of their mobility have been performed on either submerged or air-exposed individuals. Small *C. fornicata* lab-reared juveniles (2.7 – 5.7 mm shell length) were individually placed in the center of a plastic aquarium filled with 0.45µm-filtered seawater at a salinity of 30 and temperature of ~20ºC. The bottom of this tank was covered with gridlines (squares were 15 x 15 mm). Each juvenile was transferred separately from its aquarium to this observation tank, and then observed for 10 minutes. The number of gridlines that each animal crossed was counted as a means to estimate the total distance travelled. Following the 10 minute observation period, water was drained from the tank and the juveniles were observed for an additional 10 minutes while they were exposed to the air. To mimic natural conditions and ensure that the type of substrate had no effect on their movement, the experiment was repeated with juveniles on a piece of slate (with gridlines), and amount of distance travelled by the juveniles was not different when they were on the two substrates (plastic vs. slate; submerged, $t_{38} = 0.405$, $p = 0.69$; in air, $t_{38} = 0.427$, $p = 0.67$).

Aerial respiration and water loss

To determine the ability of *C. fornicata* to respire in air and how their respiration is affected by clamping behavior, lab-reared animals (10 – 18.2 mm shell length) were individually tested in chambers for CO_2 production. Each animal was separately placed on a glass slide, which was inserted into a Loligo® Systems CH10000 respirometry chamber and CO_2 was measured using differential open-flow respirometry. The Li-Cor 6262 CO_2/H_2O analyzer (Li-Cor, Lincoln, NE) was first calibrated using a gravimetric calibration mixture (Scott Specialty Gases, Plumsteadville, PA) and was always re-zeroed between measurements on different animals. A Sable Systems mass flow control system was used to maintain a flow of 200 ml min^{-1} of medical grade compressed air at 20ºC. Animals were either free to lift off of and clamp down to the glass slide, or they were artificially clamped to the substrate using an elastic band. Animals were placed in the chamber and the flow of gas was started; animals were allowed to acclimate for approximately 3 minutes during the chamber washout, after which data was recorded for 15 minutes. Animals were then removed from the chamber and either secured to the substrate with an elastic band, or their band was removed (depending on their initial treatment). Animals were then placed back into the respirometry chamber and the above procedure was repeated. When *C. fornicata* were free to move in the chamber, every time an animal lifted off of the substrate or clamped down, a mark was made on the respirometry trace.

To determine the degree to which lifting behavior affects tissue water loss, real-time water loss was measured in *C. fornicata* juveniles that were free to move or artificially clamped to the substrate. Juveniles (2.8 – 5.5 mm shell length) were placed on pre-weighed glass slides and they were either allowed to move or they were artificially clamped to the substrate with an elastic band. Each animal was then placed in a Mettler Toledo model AL104 balance at 20ºC and approximately 75% relative humidity. Weight (loss) was recorded every 30 seconds for 10

minutes, after which juveniles were removed from the balance and placed in individual aquaria filled with 0.45µm-filtered seawater. After approximately 6 h recovery time, the experiment was repeated but animals that were free to move were now artificially clamped and vice versa. Percent water loss was computed according to Sokolova and Pörtner (2001) (see above).

Statistical analysis

All statistical analyses were done in GraphPad Prism v. 4.03. To meet the assumption of the statistical tests, all percentage data were arcsine transformed before comparisons were made. To determine the effect of animal origin (intertidal, subtidal, or lab-reared) and condition (submerged or exposed) on lifting behavior, two-way, repeated measures ANOVAs were run for the average amount of time spent lifted, the average number of movements min^{-1}, and the maximum continuous time spent lifted, with animal origin and experimental condition as independent variables. Bonferroni post-tests were run to determine individual differences in behavior among animals from different origins while they were either submerged or exposed to the air. For the distance that juveniles travelled, animals that were submerged and exposed to the air were compared using a two-tailed, paired *t*-test.

The effects of exposure time and relative humidity on *C. fornicata* juvenile survival were compared using a two-way ANOVA; because no adults died following even a 10 h exposure to 75% relative humidity, desiccation tolerance of intertidal and subtidal adults could not be statistically compared. To determine if there was a potential size escape from desiccation stress, linear regressions were performed to determine the relationship between juvenile size and the percentage of water lost at all three relative humidity levels (32%, 75%, and 85%) tested.

Respirometry data were transformed in Datacan V before statistical analyses. For animals that were free to move, noting the times at which animals lifted or clamped allowed us to isolate

CO_2 production values for stretches of time that animals were willfully lifted and willfully clamped. Average rates of CO_2 production during those times were then compared to average rates of CO_2 production when the animals were artificially clamped to the substrate, using a one-way ANOVA with Bonferroni post-tests. Finally, the percentage of water lost from animals that were free to move and those that were artificially clamped to the substrate was compared using a two-tailed, paired *t*-test.

Results

When exposed to air in the laboratory at 32% RH, almost half of the juvenile *C. fornicata* died after only 3 h of desiccation stress (Fig. 1). The duration of aerial exposure (2-way ANOVA, $F_{5,72} = 67.80$, $p < 0.0001$) and the relative humidity that juvenile *C. fornicata* were exposed to (2-way ANOVA, $F_{3,72} = 79.61$, $p < 0.0001$) had a significant effect on their mortality, and the interaction between those two variables was also significant (2-way ANOVA, $F_{15,72} = 15.65$, $p < 0.0001$) (Fig. 1, Table 1). Though all juveniles exposed to 75% RH for 6 h died, none of the adults, either intertidal or subtidal, died when exposed to the same stress. In fact, all intertidal and subtidal adults exposed to air for 10 h survived (Fig. 1 inset).

When out of the water, all *C. fornicata* juveniles that lost less than 38.5 % of their tissue water survived (Fig. 2A). Conversely, all juveniles that lost more than 75.5 % of their tissue water died; the fate of *C. fornicata* juveniles losing between 38.5 and 75.5 % of their tissue water could not be predicted, as some of these juveniles died while others survived (Fig. 2A). As juveniles grew larger, they lost significantly less water over time than did smaller juveniles (Fig. 2B); though juveniles subjected to lower relative humidity levels lost more water, the trend

(larger size, less water lost) was true regardless of humidity level (85% RH, $r^2 = 0.87$, $p<0.0001$; 75% RH, $r^2 = 0.89$, $p<0.0001$; 32% RH, $r^2 = 0.86$, $p<0.0001$; Fig. 2B).

At low tide in the intertidal zone at Bissel Cove in Narragansett Bay, RI, average absolute humidity levels varied from approximately 6.3 – 12.8 g vapor mm^{-3} (corresponding to a range of relative humidity levels of 58 – 80 %) during the months of July to December of 2012. During that same time, the percentage of *Crepidula fornicata* found lifted off of the substrate in the field varied from 33 – 85 % (Fig. 3B). If observed for at least a couple of minutes, individuals periodically lifted off of and clamped themselves to the substrate.

In the laboratory, adults that had been collected from both the intertidal zone and the subtidal zone, and lab-reared juveniles that had never been exposed to air all lifted off of the substrate when exposed to air (Fig. 4). Lifting behaviors were significantly affected by both the animals' origin (intertidal, subtidal, or lab-reared) (except for time spent lifted) and whether they were submerged or exposed to air, and the interaction between the two variables was also significant (except for time spent lifted) (Table 1). During submersion, intertidal adults, subtidal adults, and lab-reared juveniles were almost always seen to be lifted off of the substrate (Figs. 4, 5A). During 15-min observation sessions in the laboratory, animals from all three groups rarely moved while submerged (Fig. 5B), allowing them to keep their tissues continuously exposed to the water for long periods of time (Fig. 5C). However, when exposed to the air, members of all three groups spent approximately only half of the time lifted off of the substrate, and the amount of time they spent lifted depended on their origin (intertidal adults < subtidal adults and lab-reared juveniles, Bonferroni post-tests, $p < 0.05$, Fig. 5A). Additionally, intertidal adults lifted off of and clamped to the substrate significantly more often than subtidal adults or lab-reared juveniles when exposed to the air (Bonferroni post-tests, $p < 0.05$, Fig. 5B), which significantly

reduced the duration of time that they remained continually lifted (Bonferroni post-tests, $p < 0.05$, Fig. 5C). Juveniles that were exposed to the air also spent significantly less time crawling around the aquarium (paired $t_{39} = 4.55$, $p < 0.0001$).

While exposed to the air and free to lift off of the substrate, *C. fornicata* were able to respire aerobically (Fig. 6). When individuals clamped themselves to the substrate, however, they effectively isolated themselves from the surrounding environment, and carbon dioxide began to build in their mantle cavities (Fig. 6). When they did lift again, they released that built up CO_2 and then continued to produce CO_2 at a relatively constant rate (Fig. 6). Indeed, when individual *C. fornicata* willfully clamped to the substrate, their ability to isolate themselves from the surrounding environment was as effective as when they were forcefully clamped to the substrate (one-way ANOVA, Bonferroni multiple comparisons test, $p > 0.05$; Fig. 7A); when clamped, individuals produced significantly less aerial CO_2 than when they were lifted off of the substrate (one-way ANOVA, $F = 59.21$, $p < 0.0001$; Bonferroni multiple comparisons test, $p < 0.05$; Fig. 7A). The ability to respire when lifted off of the substrate in the air, though, was accompanied by significantly greater water loss when lifted than when clamped to the substrate (paired $t_{20} = 13.48$, $p < 0.0001$; Fig. 7B).

Discussion

At Bissel Cove in Narragansett Bay, Rhode Island, intertidal individuals of *Crepidula fornicata* lifted off of their substrate when exposed to the air (Fig. 3). In fact, at any given time approximately half of the organisms observed over the six month sampling period were lifted off of the substrate during any observation period. They performed this behavior while exposed to a wide range of both humidity levels and temperatures. Some mollusks that are typically found

very high in the intertidal zone ('eulittoral fringe', McMahon 1990) and species that are typically found subtidally often completely isolate themselves from the environment for extended periods of time when exposed to air (e.g. Nicchitta & Ellington 1983, McMahon 1990). However, periodic exposure of tissues to the outside environment when exposed to the air (termed 'gaping', 'mushrooming', or 'lifting') is actually a behavior that is typical of many intertidal mollusks (e.g. Lent 1968, Boyden 1972, Widdows et al. 1979, Garrity 1984, Widdows & Shick 1985, Byrne & McMahon 1994, Dowd & Somero 2013); though it should exacerbate tissue water loss, it should provide other benefits (e.g. aerial oxygen uptake, evaporative cooling). The fact that *C. fornicata* performs this behavior upon aerial exposure seems to suggest that they have a long association with intertidal life.

Though some studies have casually observed lifting behavior in *C. fornicata* during aerial exposure (e.g. Newell & Kofoed 1977, Hoagland 1984, Diederich & Pechenik 2013), this study provides additional in-depth information about this behavior in this species. When submerged, individuals of *C. fornicata* were almost always lifted off of the substrate (Fig. 5), presumably for feeding and respiration. However, when exposed to air individuals of *C. fornicata* spent about half of their time clamped to the substrate, and the specific nature of their behavior (time spent lifted, maximum time lifted, number of movements) depended on their prior exposure regime (Fig. 5). Subtidal adults and lab-reared juveniles – neither of which had previously experienced aerial exposure – spent significantly more time lifted off of the substrate, lifted and clamped significantly fewer times, and stayed continuously lifted for significantly longer than intertidal adults in air (Fig. 5). The behavior of subtidal adults and lab-reared juveniles in the air seemed to match their behavior when they were submerged, while the frequent lifting and clamping movements of intertidal individuals in the air were drastically different then their behavior while

submerged. Thus, though the periodic exposure of tissues to the air is a behavior that all individuals of *C. fornicata* perform, the specific pattern of behavior depends upon past experience. The more frequent clamping behavior that results in less frequent exposure of tissues to the air in intertidal *C. fornicata* may be a learned trait that allows intertidal individuals to retain more water than subtidal individuals upon aerial exposure. Indeed, some high intertidal mollusks have been shown to be more tolerant of desiccation stress than their low intertidal or subtidal conspecifics (e.g. Morton 1957, Davies 1969, Wallace 1972, Sokolova et al. 2000). We did not find differences in desiccation tolerance between intertidal and subtidal adults of *C. fornicata* (Fig. 1), and although adults in our study lived through an aerial exposure much longer than they would experience in nature (10 hr), increased temperatures and multiple exposures could eventually reveal differences in their physiological tolerance to aerial exposure.

Since we found lifting behavior during aerial exposure to increase water loss (Fig. 7), there must be some adaptive benefit to such a behavior, or the animals would completely isolate themselves from the outside environment for the duration of the exposure. Though lifting behavior may help to cool tissue through evaporation when air temperatures are high in the intertidal zone (Williams et al. 2005, Nicastro et al. 2012), there was no threshold temperature at which individuals of *C. fornicata* lifted off of their substrate, as is true for other species (Williams et al. 2005, Dowd & Somero 2013). We suggest that individuals of *C. fornicata* perform this behavior, then, for respiratory purposes. We found that *C. fornicata* were very effective at sealing themselves off when they willfully clamped to the substrate (Fig. 6), and that CO_2 builds in their mantle cavity during this time. This ability to completely isolate from the external environment has been demonstrated in other calyptraeid gastropods as well, though in response to other stressors (low salinity, Chaparro et al. 2009; Montory et al. 2014). Because

they are able to efficiently utilize oxygen in the air (aerial respiration rate was approximately 70% of the submerged rate over the temperature range 5 – 25ºC, Newell & Kofoed 1977), lifting off of the substrate for short periods of time allowed these organisms to release built up CO_2 and replenish their aerial oxygen supply. This adds *C. fornicata* to the list of several intertidal mollusks that apparently perform this behavior for respiratory purposes (e.g. Lent 1969, Boyden 1972, Widdows et al. 1979, Dowd & Somero 2013). Their ability to maintain aerobic metabolism in air and control their water loss with periodic clamping and lifting are important factors that must in part be responsible for the broad vertical distribution of this species.

This lifting behavior had never been explored in *C. fornicata*, which is surprising because individuals of this species can be found intertidally in both their native (Diederich et al. 2011, Hoch & Cahill 2012, Diederich & Pechenik 2013) and invasive (Thieltges et al. 2003, Viard et al. 2006) ranges. Additionally, before our study, little work had been done concerning the contribution of this behavior to water loss and the tolerance of *C. fornicata* to desiccation stress. Hoagland (1984) found that large individuals of *C. fornicata* were able to survive in air for much longer than juveniles. In our study, we too found that at relatively benign temperatures and humidity levels, there was a size escape from desiccation stress in *C. fornicata* (Fig. 2). Size escape from desiccation stress has been found in other mollusks too (e.g. Davies 1969, Branch 1975, McQuaid 1982, Williams et al. 2005, Jenewein & Gosselin 2013), and is not surprising as larger animals have more water to lose and a lower surface area to volume ratio. A loss of approximately 40% of total water proved dangerous for juveniles in our study, and no juvenile was able to lose more than 75% of their total water without dying (Fig. 2). This amount of water loss corresponded to approximately 23 – 39% total weight loss, which agrees with the results of Hoagland (1984), who found that small individuals of *C. fornicata* could only tolerate a weight

loss from desiccation of approximately 30%. The amount of water loss that intertidal mollusks can tolerate is highly variable, with some species able to tolerate over 80% water loss (Wolcott 1973, Price 1980). More commonly, though, individuals tend to die after losing 10 to 70% of their tissue water (e.g. Lent 1968, Davies 1969, Branch 1975, Marshall & Mcquaid 1992, Williams & Morritt 1995, Sokolova & Pörtner 2001, Hicks & McMahon 2003), a range that encompasses the amount of water loss that caused mortality in *C. fornicata* in our study.

Some juveniles in our study died after an aerial exposure of only 2 – 3 hours, which is a realistic amount of aerial exposure time in nature (Diederich & Pechenik 2013). Thus, desiccation alone may be important for controlling the upper distribution of this species. However, the short time that individuals of *C. fornicata* are susceptible to desiccation stress (as young juveniles), is also the time when they are mobile (Fig. 5). Indeed, small juvenile *C. fornicata* were very active in our study, often constantly crawling across the substrate when submerged. Though they generally reduced crawling upon aerial emersion, they did travel short distances (Fig. 5). This mobility could allow small *C. fornicata* to position themselves in moist areas to avoid desiccation stress when exposed to the air. Most *C. fornicata* can be found living upon one another in stacks of up to ~15 members (Coe 1936). Smaller individuals often position themselves at the shell margin of larger females, which is generally thought to be for mating purposes (Coe 1936). This positioning of small individuals may have an additional benefit, though, as lifting by females when exposed to air often results in the release of a small volume of fluid that was retained in the female mantle cavity (personal observation, C. Diederich). Small juveniles at the shell margin can thus be wetted by this mantle cavity fluid, allowing them to tolerate aerial exposure until they are large enough to be highly tolerant of desiccation stress.

As individuals of *C. fornicata* grow larger, their upper distribution is more likely to be determined by factors other than resistance to desiccation stress. Most notably, both high (Diederich & Pechenik 2013) and low (Thieltges et al. 2004) temperatures have been reported as possible determinants of range limits in *C. fornicata*, and individuals of this species must spend enough time submerged to collect an adequate amount of food. As is true for many other species, though, it may be a combination of many stresses associated with intertidal life – including desiccation – that limit the upper distribution of this species (e.g. Firth & Williams 2009, Williams et al. 2011, Iacarella & Helmuth 2012). Even so, our study highlights the importance of desiccation stress and lifting behavior in the life history of this species. Individuals cannot tolerate long periods of aerial exposure when they are very small, so *C. fornicata* juveniles must be able to avoid desiccation for at least the first few days or weeks after metamorphosis. After that time, the lifting behavior and ability to maintain aerobic metabolism in the air documented here suggest that this species has long been associated with the intertidal zone, despite the fact that individuals of this species are also commonly found subtidally.

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Tables

Table 3.1: Summary of two-way ANOVA statistics for lifting behavior and desiccation tolerance of *Crepidula fornicata*.

Relevant Figure	Variable	Source	df	MS	*F*	P
1	Survival	Time	5	3.44	67.80	**<0.0001**
		% Relative Humidity	3	4.04	79.61	**<0.0001**
		Interaction	15	0.79	15.65	**<0.0001**
		Residual	72	0.051	-	-
4A	Time spent lifted	Animal Origin	2	0.16	1.56	0.22
		Condition	1	7.85	103.80	**<0.0001**
		Interaction	2	0.42	2.78	0.069
		Residual	72	0.076	-	-
4B	Movements min^{-1}	Animal Origin	2	14.71	39.19	**<0.0001**
		Condition	1	32.60	92.49	**<0.0001**
		Interaction	2	9.44	26.78	**<0.0001**
		Residual	72	0.35	-	-
4C	Max. time lifted	Animal Origin	2	153.70	8.45	**0.0005**
		Condition	1	605.80	51.97	**<0.0001**
		Interaction	2	66.21	5.68	**0.0051**
		Residual	72	11.66	-	-

Figures

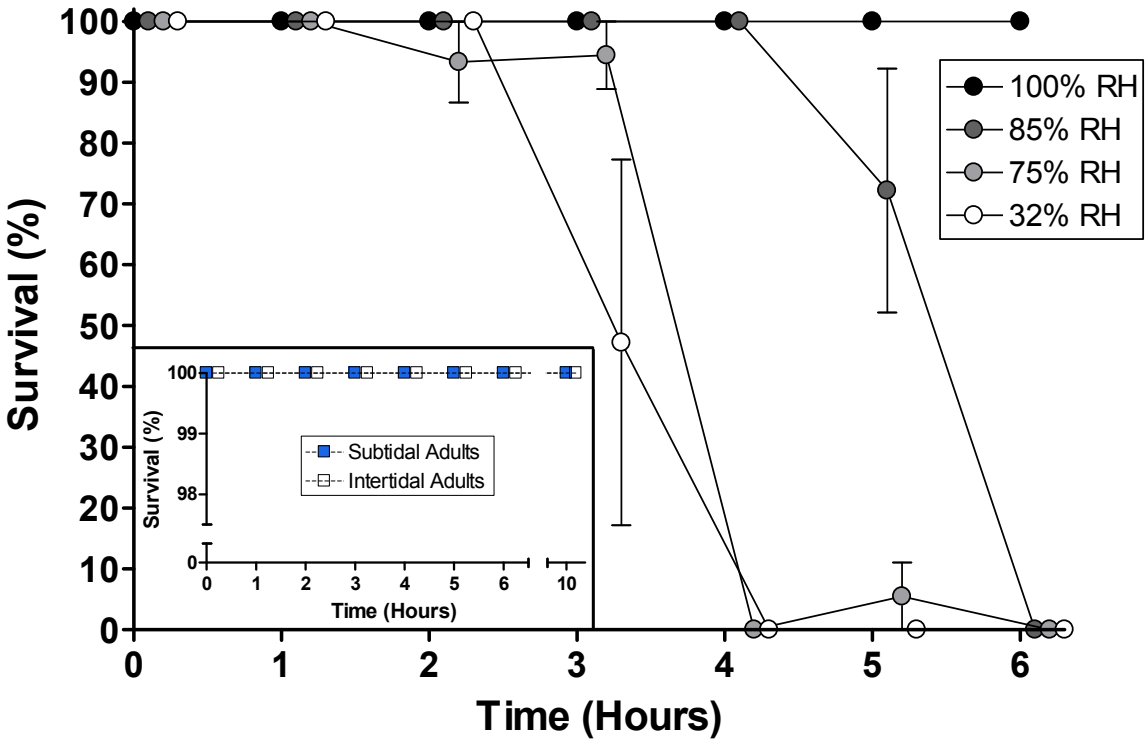

Figure 3.1: Desiccation tolerance of lab-reared juvenile *Crepidula fornicata* (large graph) and intertidal and subtidal adults (small, inset graph). Individuals were exposed to air only once, for 0 – 6 hr. For juveniles (1.3 – 4.6 mm shell length), 5 – 6 individuals per replicate (3 – 6 replicates per treatment) were exposed to the indicated relative humidity level at 20ºC; error bars are SEM. See Table 1 for detailed statistical analyses. For adults, 12 – 15 freshly-collected intertidal (white squares) or subtidal (blue squares) individuals per treatment were desiccated at 75% RH and 26ºC.

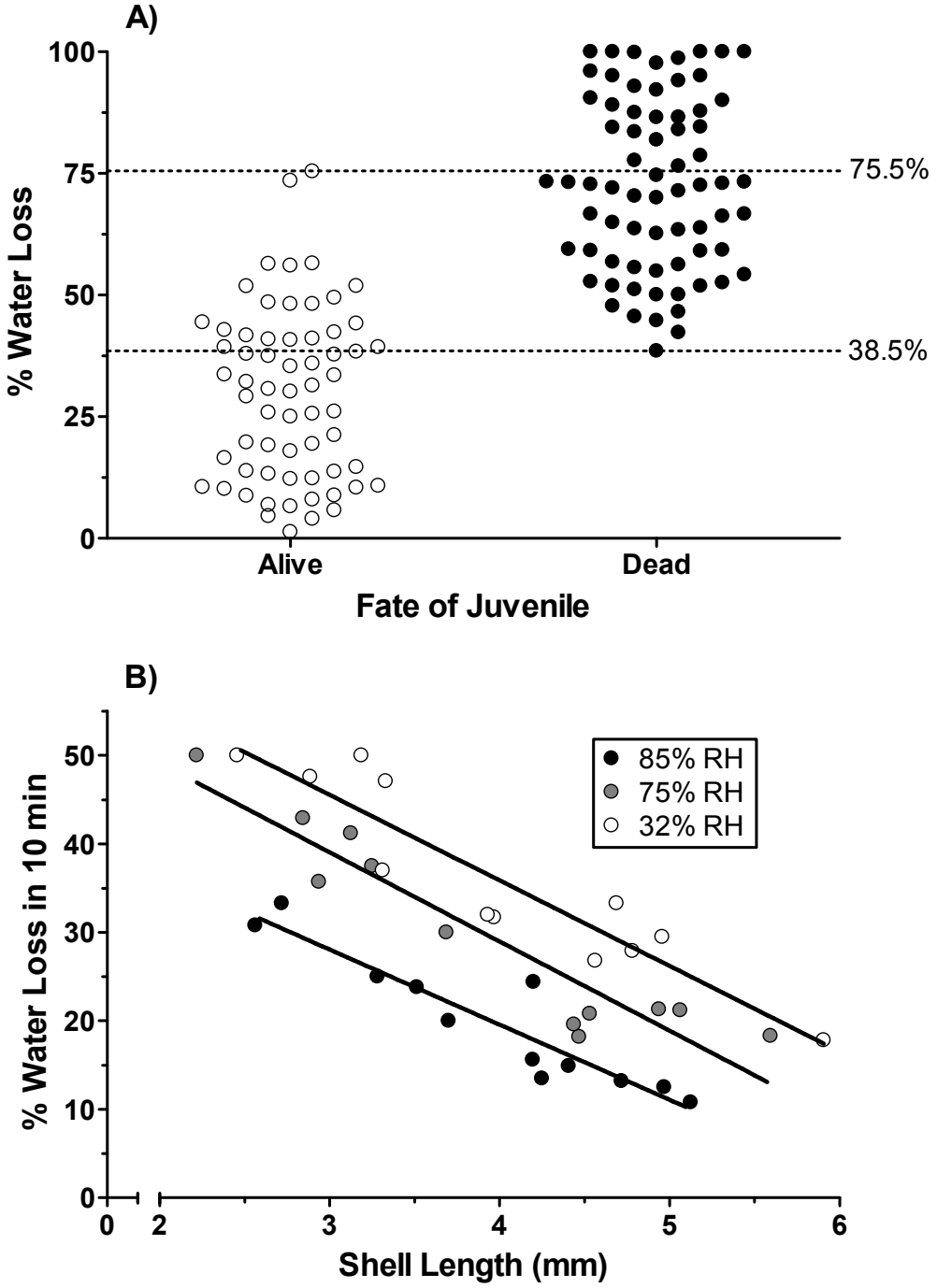

Figure 3.2: Characteristics of aerial water loss in lab-reared, juvenile *Crepidula fornicata*. (A) Amount of water loss required to kill juvenile *C. fornicata*; individuals (N = 133) were subjected to 32 – 100% relative humidity (RH) at 20ºC for up to 5 hr. (B) Size escape from desiccation stress; juveniles (N = 36) were desiccated at 85% RH (black circles, $r^2 = 0.87$, $p<0.0001$) 75% RH (gray circles, $r^2 = 0.89$, $p<0.0001$) or 32% RH (white circles, $r^2 = 0.86$, $p<0.0001$) for ten minutes at 20 ºC.

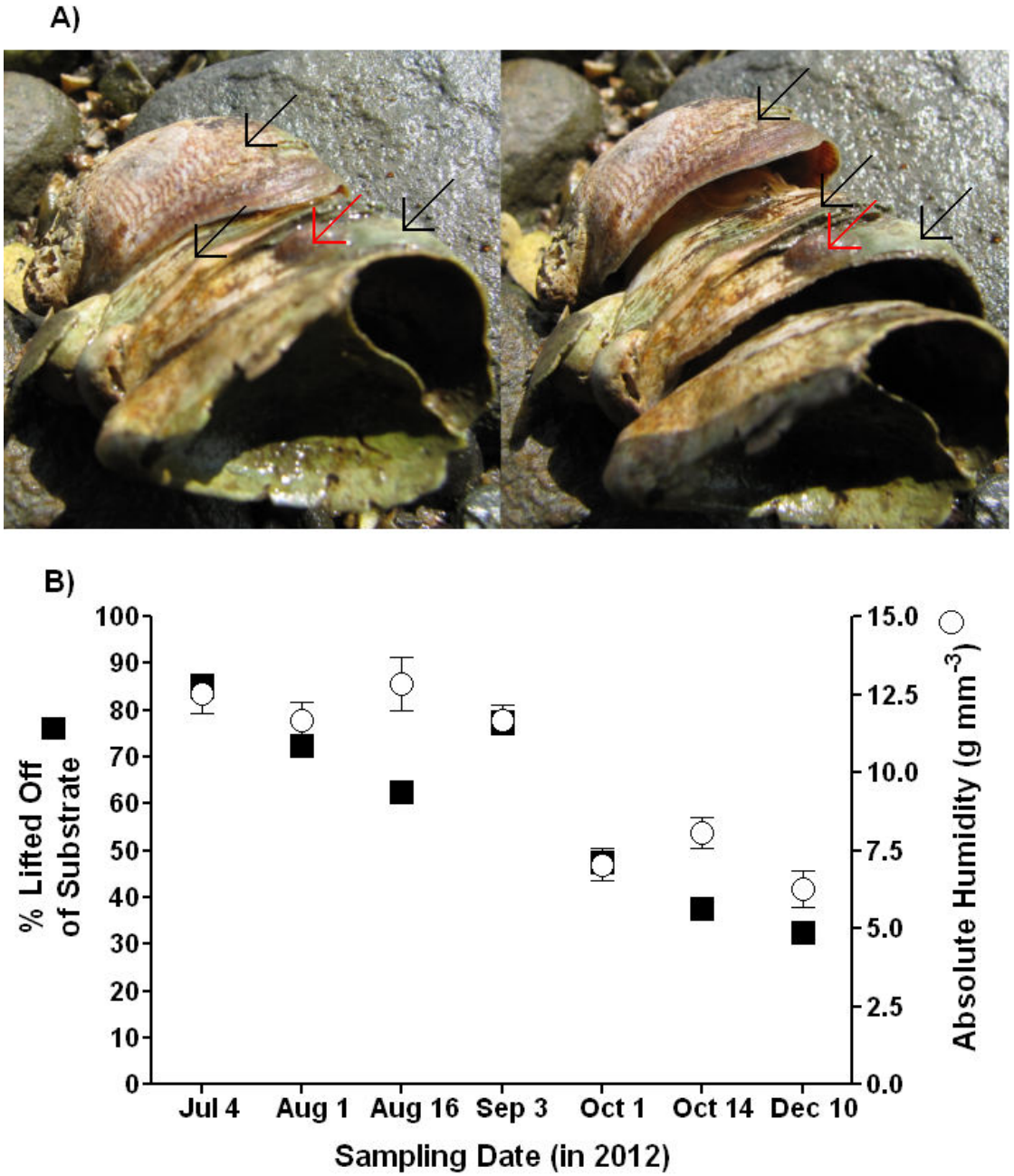

Figure 3.3: Proportion of *Crepidula fornicata* found lifted off of their substrates in the field and sample relative humidity levels at Bissel Cove in Narragansett Bay, Rhode Island. (A, left panel) A stack of 3 adults (black arrows, bottom-most adult is on an empty shell) and 1 small juvenile (red arrows) all clamped to the substrate. (A, right panel) Seconds later, the top-most and bottom-most members of the stack have lifted off of the substrate. (B) On each date, 40 individuals of *C. fornicata* were observed for their behavioral state (lifted off of the substrate or clamped to the substrate, black squares), and 20 relative humidity measurements were made in the vicinity (< 20 cm) of living *C. fornicata* (open circles, mean ± SD).

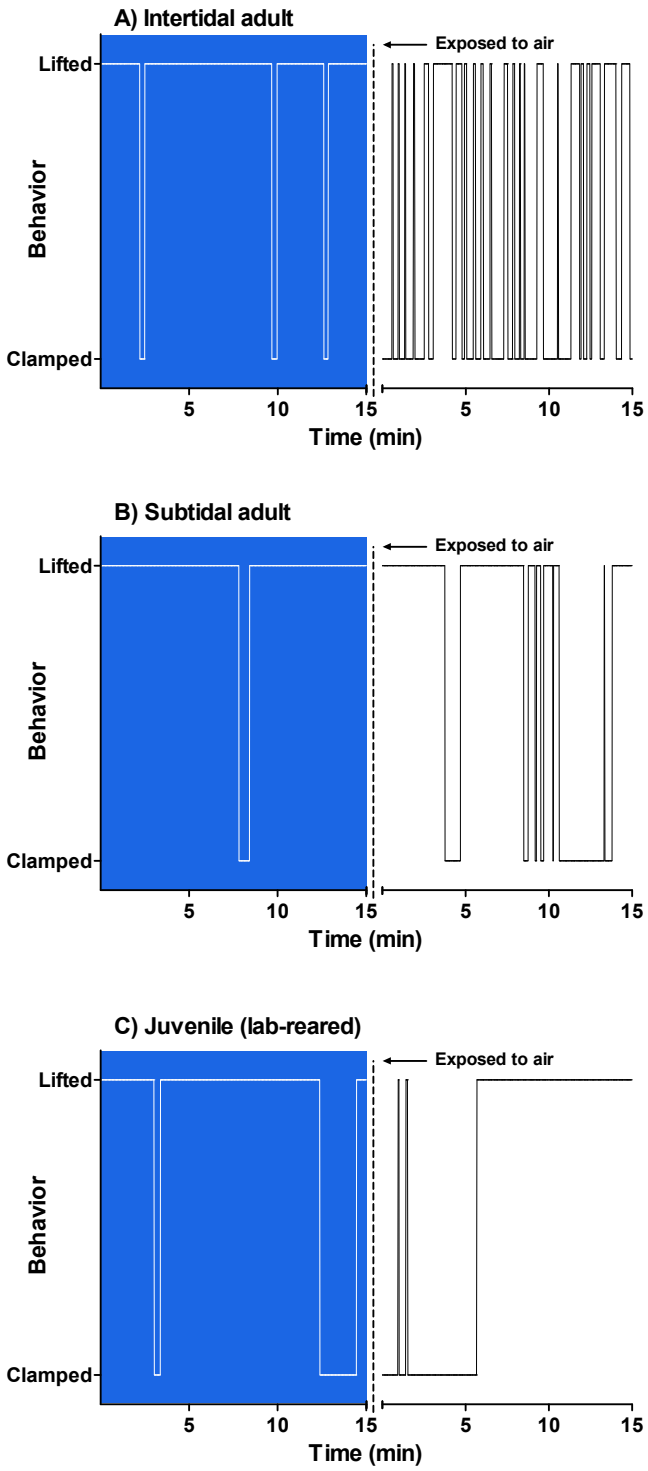

Figure 3.4: Representative lifting behavior of (A) an intertidal adult, (B) a subtidal adult, and (C) a lab-reared juvenile of *Crepidula fornicata* when submerged (blue panels) or exposed to the air (white panels). Individuals were submerged in a transparent tank for 15 minutes, after which the water was drained from the tank and they were observed for an additional 15 minutes.

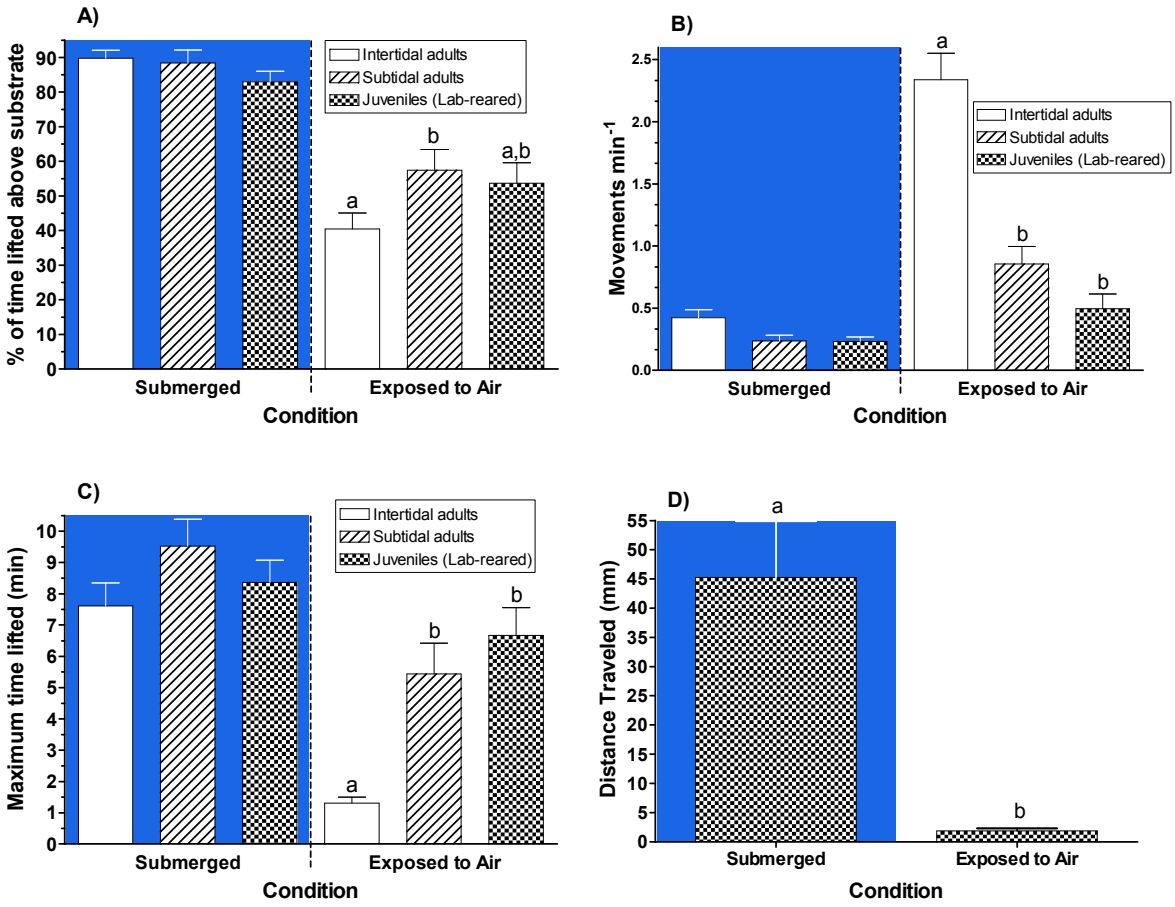

Figure 3.5: Lifting behavior of intertidal adults (white bars, N = 25), subtidal adults (striped bars, N = 25), and lab-reared juveniles (checkered bars, N = 25 for A – C, N = 40 for D) of *Crepidula fornicata* when submerged (blue panels) or exposed to the air (white panels); error bars are SEM. (A) Average percent of total time that animals spent lifted off of the substrate. (B) Average number of lifting or clamping movements during the experimental time. (C) Maximum amount of continuous time that animals spent lifted off of the substrate. (D) Distance that mobile juveniles traveled while submerged or exposed to the air. Different letters above bars indicate significant differences in behavior ($p < 0.05$, A – C, within a condition, Bonferroni testes following two-way, repeated measures ANOVA, see Table 1; D, paired *t*-test).

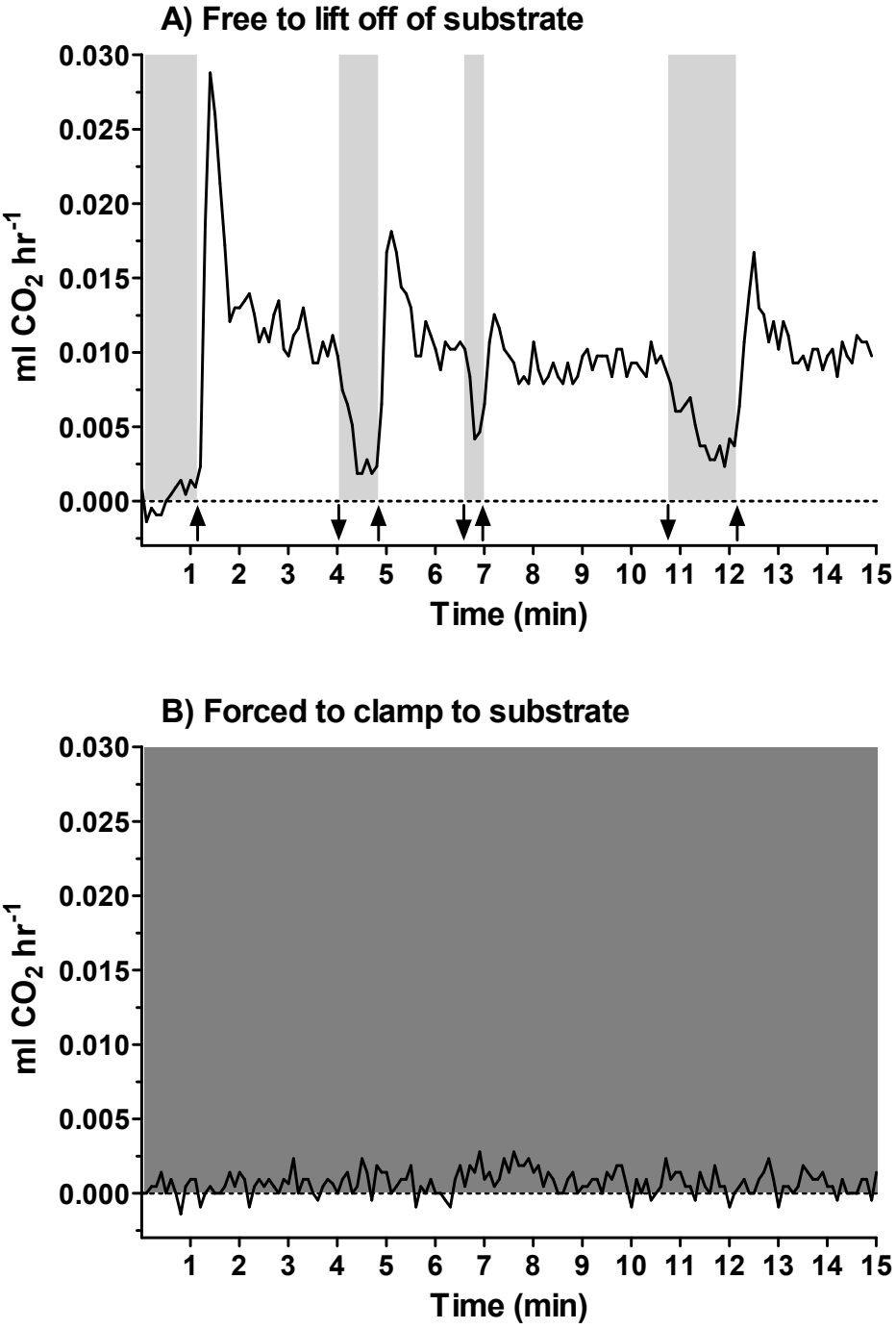

Figure 3.6: Aerial respiration of *Crepidula fornicata* juveniles measured as CO_2 output. (A) Sample respirometry trace for an individual that was free to move on the substrate. Arrows pointing up indicate the times as which the animal lifted its shell off of the substrate, arrows pointing down (and associated gray-shaded areas) indicate when the animal clamped its shell to the substrate. (B) Sample respirometry trace for an individual that was artificially clamped to the substrate (with an elastic band) so that it could not lift.

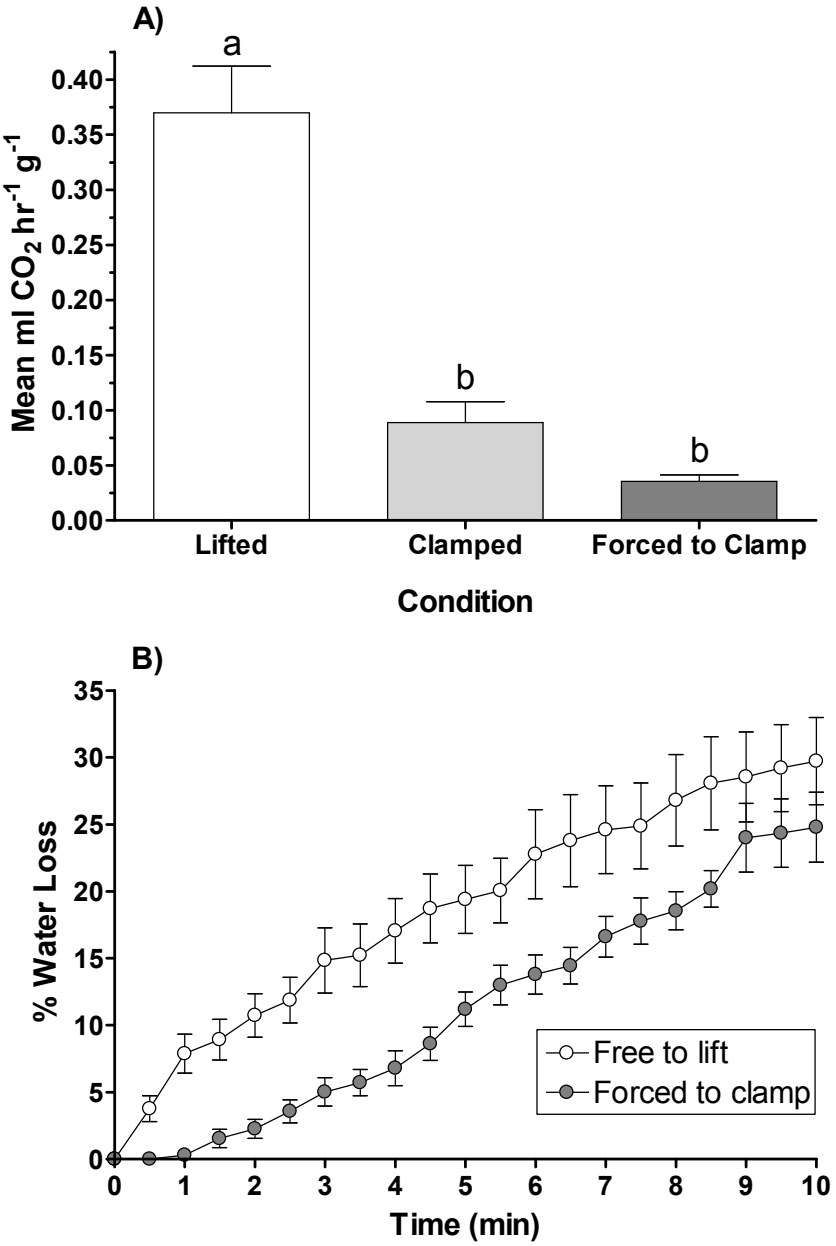

Figure 3.7: The tradeoff imposed by lifting behavior in *Crepidula fornicata* juveniles. (A) Mean (+ SEM, N = 20 per treatment) weight corrected rate of aerial respiration when individuals were free to move and lifted off of the substrate (white bar), free to move but voluntarily clamped to the substrate (gray bar), or forcibly secured to the substrate with elastic bands (dark grey bar). Different letters above bars indicate significant differences among treatments ($p<0.05$, Bonferroni multiple comparisons test); example respirometry traces can be found in Figure 6. (B) Individuals were placed on glass slides in 75% RH air either free to lift off of the substrate (white circles) or secured firmly to the substrate with elastic bands (dark grey circles); N = 12 per treatment, error bars are SEM. Banded individuals lost significantly less water over the sample period (paired *t*-test, $t=13.48$, d.f. = 20, $p < 0.0001$).

Chapter 4

Differences in feeding adaptations in intertidal and subtidal suspension-feeding gastropods: studies on *Crepidula fornicata* and *Crepipatella fecunda*

Note: Submitted to *Marine Biology*

Abstract

Suspension-feeding organisms living in the intertidal zone experience reduced feeding times associated with periodic aerial exposure. We investigated the potential morphological and physiological adaptations to this reduced feeding time for two closely-related gastropods, *Crepidula fornicata* and *Crepipatella fecunda*. Intertidal *C. fornicata* had heavier gills than subtidal conspecifics, a difference mediated by larger gill surface areas and greater numbers of gill filaments among intertidal individuals of a given size. In contrast, the gills of intertidal and subtidal *C. fecunda* were morphologically indistinguishable. Despite relatively larger food-collecting organs, individuals of *C. fornicata* from the intertidal zone had clearance rates (CR) that were statistically equivalent to those of subtidal conspecifics. In contrast, the CR of intertidal *C. fecunda* were significantly lower than those of subtidal conspecifics. The low CR of intertidal *C. fecunda* seems to be at least partially be explained by significantly lower particle transport velocities across their gills. In the context of feeding, intertidal individuals of *C. fornicata* were able to perform at least as well as subtidal conspecifics, resulting in a population whose adults are as large as those found subtidally. This suggests that *C. fornicata* has had a long association with the environmental heterogeneity associated with intertidal life. In contrast, intertidal individuals of *C. fecunda* were on average smaller than subtidal individuals, suggesting that this species is not yet well-adapted for intertidal life.

Introduction

The physical and ecological factors that control species' distributional ranges have recently received heightened attention due to anticipated climate change and an increased number of biological invasions (Sexton et al. 2009). Because species are usually distributed over large areas latitudinally, species range limits are most often studied on a broad geographic scale (e.g.Sagarin and Gaines 2002; Sorte and Hofmann 2004; Kuo and Sanford 2009). Such studies have been important for identifying potential biotic and abiotic limiting factors for a host of different species and those species' potential adaptations at range edges (e.g. Fawcett 1984; Zacherl et al. 2003; Lima et al. 2007; reviewed by Bridle and Vines 2007; Sexton et al. 2009). However, the distances separating central populations and those at range edges can make the study of limiting factors and adaptation at range edges difficult, as a variety of different conditions (e.g. climate, temperature, salinity, wave action, available substrate, species composition, species interactions, food sources) vary considerably over such large distances. Additionally, the dynamic nature of range boundaries and the subjectivity in defining them often cause difficulties in accurately identifying range boundaries (Gaston 2003).

Though underutilized in studies involving adaptation at range boundaries, the intertidal zone represents an abrupt spatial limit over very short distances for many marine organisms (Holt and Keitt 2005). Though some marine species are only found subtidally (e.g. Tomanek and Somero 1999; Collin 2000; Stillman and Somero 2000), and some are found chiefly in the intertidal zone (e.g. Connell 1972; Perez et al. 2009; Bourdeau 2011), a number of species are abundant in both areas (e.g. Dame 1972; Fletcher 1984; Saier 2002; Schaffmeister et al. 2006; Dong et al. 2011; Diederich and Pechenik 2013) and so experience a remarkable range of conditions over very short distances. Species whose populations extend from the intertidal zone

(the upper edge of their range) into the adjacent subtidal zone can be studied in the context of range boundaries, but on a much smaller spatial scale than is typical in most studies. At high tide, intertidal organisms experience many of the same conditions that subtidal organisms do, as the two groups are often only meters apart, and a well-established gradient of stressors exists as one moves from the subtidal up through the intertidal zone (Menge 1976; Newell 1979; Denny 1985; Helmuth 1998). Thus, the limiting factors associated with aerial exposure in the intertidal zone and species' adaptations to those factors can be studied by investigating the characteristics of species whose members are living both intertidally and subtidally.

The gradients of stressors most closely associated with intertidal life are usually linked to environmental heterogeneity from aerial exposure: when compared with subtidal organisms, intertidal organisms experience periodic exposure to desiccation (e.g. Garrity 1984) and to hypoxia (e.g. Brinkhoff et al. 1983), rapid changes in salinity (especially in tide pools - e.g. Morris and Taylor 1983), and greater and more rapid temperature fluctuations (e.g. Diederich and Pechenik 2013). However, sessile suspension-feeding animals, such as those investigated in the present study, are at an added disadvantage if they live intertidally, as they will be unable to feed for long periods of time while they are exposed to the air.

A number of studies have documented differences in feeding organs or feeding strategies among some populations of marine invertebrates living in different conditions (e.g. Morton et al. 1957; Payne et al. 1995; Ito et al. 2002; Chaparro et al. 2004; Dutertre et al. 2007). For example, Newell et al. (1971) found that although the periwinkle *Littorina littorea* only feeds while submerged, periwinkles living high in the intertidal zone were able to compensate for reduced feeding times with quicker radular rasping rates when compared to those of periwinkles living in the low intertidal zone. Similarly, Drent et al. (2004) found that gill-to-palp ratios (which

determine particle sorting efficiency) of the deposit feeding clam, *Macoma balthica*, were flexible and depended upon the grain size of the material that the clams were feeding on. In general, intertidal grazers typically change their feeding activities depending on the duration of aerial exposure (Newell et al. 1971; Zeldis and Boyden 1979; Little 1989; Little et al. 1991; Santini et al. 2004; but see Underwood 1984) but the situation is not as clear for suspension-feeders (Morton et al. 1957; Griffiths and Buffenstein 1981; Widdows and Shick 1985; Bayne et al. 1988; Kreeger et al. 1990; Charles and Newell 1997). At least some suspension-feeders have the ability to compensate for differences in water turbidity with flexibility in the sizes of their feeding organs (Payne et al. 1995; Honkoop et al. 2003; Drent et al. 2004; Dutertre et al. 2007).

The closely-related calyptraeid gastropods *Crepidula fornicata* (native to the east coast of USA) and *Crepipatella fecunda* (native to the Chilean coast) can be found living both intertidally and subtidally (Gallardo 1979; Collin 2003; Diederich and Pechenik 2013). These animals eat by collecting particles from the water using their modified gill (ctenidium) and transporting those particles to a food groove, where they are concentrated, directed to the mouth (via the food groove in the neck), and ingested (Chaparro et al. 2002). Because *C. fornicata* and *C. fecunda* do not move as adults, and have only limited mobility as juveniles (and as small males) (Conklin 1897; Chaparro et al. 2001a), animals like *C. fornicata* and *C. fecunda* living at the upper edge of their intertidal range have less time to collect food than their subtidal conspecifics. Unless they have a mechanism that compensates for reduced feeding time, this could potentially limit their vertical distribution, especially if slower growth delays a potential escape in size from mortality in the intertidal zone (Gosselin and Qian 1997; Hunt and Scheibling 1997).

In this study we investigated the gill morphology and feeding physiology of *C. fornicata* and *C. fecunda* that were exposed to reduced suspension feeding-time at their vertical range

edge. We compared various allometric relationships of the gill among intertidal and subtidal individuals living only meters apart at the same field sites for both *C. fornicata* and *C. fecunda*. We then used laboratory-based clearance rate (CR) experiments to assess the extent to which any differences in gill morphology actually affected the rate at which animals cleared food particles from the water. In addition, the speed of particle transport across the gill was quantified for *C. fecunda* using endoscopic analysis. Finally, size frequency distributions were used to estimate maximum growth potential for both species in the intertidal and subtidal zones.

Materials and Methods

Collection and maintenance of animals

For all experiments, *Crepidula fornicata* were collected from Bissel Cove, Rhode Island, USA, where they can be found both intertidally and subtidally in large numbers (Diederich and Pechenik 2013). Collections were made for the studies of gill morphology in April, 2011 and for clearance rates (CR) in May, 2012. Adults (14 – 40 mm shell length) were collected at low tide from approximately 0.5 meters above (+0.5 m) the mean low lower water mark (MLLW) (hereafter, "intertidal") and 1 meter below (-1.0 m) MLLW (hereafter, "subtidal"), and transferred to the laboratory in aerated seawater. In Chile, *Crepipatella fecunda* were collected from Pelluco Beach in Puerto Montt, where they can be readily found both intertidally and subtidally (personal observation C. Diederich, O. Chaparro). Collections were made for the gill morphology studies and clearance rate experiments in October, 2012 and for particle velocity experiments in January, 2013. Adult females (35 – 45 mm shell length) were collected at low tide from approximately +1 m above MLLW (hereafter, intertidal) and -1 to -2 m below MLLW (hereafter, subtidal), and transferred to the laboratory in aerated seawater.

Gill morphology

For studies of gill morphology, all animals (N = 80 for each species) were rinsed in deionized water and immediately preserved in 10% formalin (diluted with 0.5µm-filtered seawater) buffered with sodium borate. Gills were dissected and photographed under a stereomicroscope fitted with an ocular micrometer. The number of gill filaments (minimum length of 1 mm) in each gill was subsequently determined. The gill and remaining tissue were then dried separately on pre-weighed foil at 60ºC for 48 hours to determine gill and total dry tissue weight. Using photos taken under a stereomicroscope and analyzed using ImageJ (v1.43; National Institutes of Health, Bethesda, MD, USA) for *C. fornicata* and Image-Pro Plus for *C. fecunda*, the longest 5 gill filaments were measured from the base to the most distal section of the filament bulb. Finally, the gill surface area was determined using the imagine software by tracing the area of the intact, excised gill of each animal.

Clearance rates

For the clearance rate (CR) measurements, the experimental animals were cleared of all epibionts and maintained individually on their original substrate in glass aquaria with aerated, 0.5µm-filtered seawater at 22ºC (*C. fornicata*) or 12 – 14ºC (ambient temperature, *C. fecunda*) and salinity of 30. In order to avoid acclimation to laboratory conditions, all experiments were performed within 24 hours of collection (Rising and Armitage 1969; Widdows and Bayne 1971; Mcmahon and Payne 1980). Intertidal animals were exposed to air for 3 hours (*C. fornicata*) or 6 hours (*C. fecunda*) prior to experimentation to simulate low tide conditions.

Animals were placed in individual aquaria containing 3 L (*C. fornicata*, N = 95) or 10 L (*C. fecunda*, N = 79) of 0.5µm-filtered seawater at salinity of 30 and at 22ºC (*C. fornicata*) or 14ºC (*C. fecunda*). Air was continuously bubbled into each aquarium to ensure adequate mixing

and to maintain a sufficient supply of dissolved oxygen. Pure cultures of either *Duniella tertiolecta* (for *C. fornicata*) or *Isochrysis galbana* (for *C. fecunda*) were used as the food sources for the animals, and these microalgae were added to each aquarium to achieve initial concentrations of approximately 30,000 cells ml^{-1}.

One-ml water samples were taken from each aquarium at the start of the experiment and cell concentrations were measured (in triplicate) using a model ZM (*C. fornicata*) or Z2 (*C. fecunda*) electronic particle counter (Beckman Coulter Electronics). Animals were then allowed to filter the microalgae from the water for 2 – 4 hours before final concentrations were determined. Pilot studies revealed this amount of time to be appropriate in order to detect a reduction in cell concentration without allowing too many cells to be grazed from the suspension. However, clearance rates are notoriously variable from individual to individual in *C. fornicata*, and even within an individual over time (Newell and Kofoed 1977a). Because CR can be underestimated using this method if too much of the algal suspension is grazed (Coughlan 1969), any animal that grazed more than 35% of the algal suspension (Mardones et al. 2013) was excluded from the final statistical analyses (but retained in the graphic presentation with distinct symbols, see below). In addition to measuring algal concentration in all experimental aquaria, an additional 2 – 6 control tanks were set up with microalgae but without animals to determine settling or growth rates of algal cells during the experimental period. The clearance rate of each individual was estimated following the approach of Coughlan (1969):

$$CR = V[((Log_e C_0 – Log_e Ct)-a)/t]$$

where V is the volume of suspension, C_0 is the initial concentration, C_t is the final concentration, a is the rate at which particle concentration changed in the control suspension, and t is the

duration of the experiment. At the end of the CR experiments, all animals were then removed from their substrate to determine their brooding status.

Particle transport velocity on the gill

The speed at which particles moved along the gill filaments of intertidal and subtidal *Crepipatella fecunda* was measured using endoscopy (OLYMPUS model OTV-S4 endoscope, power source OLYMPUS model CLV-10). This method has previously proven successful for determining particle velocity in this and closely related species (Mardones et al. 2013; Shumway et al. 2014). Freshly collected (see above) intertidal and subtidal animals were removed from their substrates and allowed to adhere to transparent plastic plates. A small hole (diameter 3 mm) was first drilled in each plate where the endoscope would later be inserted and particle velocities were measured after animals were allowed to adhere securely to the plates for 24 hours. This procedure was performed on many more animals than were actually used in the analysis (10 intertidal, 11 subtidal) because some animals moved slightly after being placed on the substrate, and thus did not allow for accurate placement of the endoscope. To measure particle velocity, intertidal and subtidal animals were maintained individually in 1 liter aquaria with seawater at salinity of 30 and at 14ºC. Animals were fed *Isochrysis galbana* at approximately 30,000 cells ml^{-1}. The tip of the endoscope was inserted through the hole in the plastic substrate and into the pallial cavity of the animal until the gill could be visualized. Filming was initiated once animals began filtering, and then a suspension of non-toxic bright orange particles (2 - 10 µm diameter, Chaparro et al. 2001b; Chaparro et al. 2002) was added to the water near the incurrent feeding stream. These particles are easy to visualize and they move along the gill at the same speed as does *I. galbana* (Mardones et al. 2013). Videos were visualized on a Trinitron Sony monitor and recorded to VHS for analysis of particle velocity. To determine particle velocity, the

displacement of orange particles along the gill was measured between two points. In order to ensure that only particles driven by ciliary filament actions (not water flow in the mantle cavity) were measured, only particles in very close contact with the gill were selected. Then, the time elapsed was determined by stepping through the video frame by frame (NTSC format: 30 frames s^{-1}, Ward et al. 1991) and the distance travelled by the particles was determined using the width of the gill filaments as a reference (Ward et al. 1991; Beninger et al. 1992; Mardones et al. 2013). Gill filament widths were determined after the particle velocity experiments were finished by dissecting the gill out of each animal and measuring filament width with a dissecting microscope at 40X. The filaments that were measured for width were in close proximity to the location where the particle movements were recorded.

Maximum size

Maximum size (shell length) was used to estimate the growth potential of animals in the intertidal and subtidal zones. For *C. fornicata*, intertidal (N = 206) and subtidal (N = 203) animals >15 mm were collected in the spring of 2011, 2012, and 2013 and the longest shell dimension of each animal was measured to the nearest 0.1 mm with calipers. For *C. fecunda*, intertidal (N = 147) and subtidal (N = 91) animals >25 mm were collected in spring season of 2012, and the longest shell dimension of each animal was measured to the nearest 0.01 mm with calipers. Starting at 15 mm for *C. fornicata* and 25 mm for *C. fecunda*, animals were grouped into 2 mm size classes.

Statistical analyses

For the studies of gill morphology, the size of each animal needed to be accounted for before the gills of intertidal and subtidal animals could be compared. An information-theoretical approach was used to model selection based on biological hypotheses and previously used

models (Burnham and Anderson, 2002; Johnson and Omland, 2004) to determine the model that best suited each data set. Linear ($y = ax + b$) and power ($y = ax^b$) models were compared using a corrected Akaike Information Criterion score (AICc) (Akaike 1973) (Graphpad Prism V4.0). After the appropriate model was selected, regressions of each gill variable against the dry weight of the animal for intertidal and subtidal subpopulations were compared using either ANCOVA (when the best model was linear) or extra sum-of-squares F-test (when the best model was non-linear) (Graphpad Prism V4.0).

Clearance rates (CR) were standardized for animal weight as they have been shown to increase as the snails grow (Navarro and Chaparro 2002). For both species, CR was standardized to one gram of dry tissue weight using the allometric exponent of 0.56 found by Navarro and Chaparro (2002) to describe the relationship between CR and tissue weight for *C. fecunda*, which was achieved following Bayne et al. (1987):

$CR_s = (1/W)^b CR_e$

where W is the dry tissue weight of the animal, b is the allometric exponent 0.56, and CR_e is the experimentally determined clearance rate. This method of standardization has also been used successfully for *C. fornicata* (Barille et al. 2006) and the exponent used is very close to the universal scaling exponent for weight and oxygen uptake in gastropods (0.58 – 0.62, Marsden et al. 2011). Following standardization, clearance rates of intertidal and subtidal animals within each species were compared using an unpaired, two-tail t-test.

The individuals of *Crepipatella fecunda* used for the particle velocity experiments differed little in shell length (<12 mm range) and particle velocity was not correlated to animal size (linear regression of shell length vs. particle velocity: intertidal, $r^2 = 0.04$, $F_{1,8} = 0.34$, $p = 0.58$; subtidal, $r^2 = 0.00069$, $F_{1,9} = 0.0063$, $p = 0.94$). Thus, particle velocities were not

standardized to the size of each animal. The velocities of particles were averaged for each individual and the data for intertidal and subtidal subpopulations were then compared using an unpaired, two-tail t-test.

Size frequency distributions of intertidal and subtidal populations within each species were compared using contingency table analyses.

Results

Individuals of *Crepidula fornicata* and *Crepipatella fecunda* showed dramatically different patterns of gill morphology across the intertidal-subtidal boundary. In particular, all measures of gill morphology were more strongly correlated with animal size for *C. fornicata* than for *C. fecunda* (Figs 1 – 4).

Intertidal members of *C. fornicata* had gills that were as much as 50% heavier than those of subtidal members of the same species, with the differences being greatest among larger adults (Fig. 1; $F_{1,76} = 10.53$, $p = 0.001$). This difference was not mediated by differences in the length of the gill filaments (Fig. 2; $F_{2,76} = 0.95$, $p = 0.39$), but rather by their larger surface area (Fig. 3; $F_{1,76} = 4.75$, $p = 0.032$), in part because the gills of intertidal individuals had more filaments than those of subtidal individuals (Fig. 4; $F_{2,76} = 5.69$, $p = 0.0050$). The gills of the largest intertidal *C. fornicata* were about 80% greater in surface area and about 30% greater in the number of gill filaments than those of the largest subtidal members of the same species (Figs 3, 4).

Unlike *C. fornicata,* however, the gills of intertidal and subtidal *C. fecunda* showed no significant differences in weight (Fig. 1; $F_{1,76} = 0.82$, $p = 0.37$ for slope; $F_{1,77} = 0.094$, $p = 0.76$ for intercept), maximum filament length (Fig. 2; $F_{1,76} = 1.45$, $p = 0.23$ for slope, $F_{1,77} = 0.0026$, p

= 0.96 for intercept), surface area (Fig. 3; $F_{2,76} = 0.99$, $p = 0.38$), or number of filaments (Fig. 4; $F_{2,76} = 0.51$, $p = 0.61$).

Clearance rates for both intertidal and subtidal *C. fornicata* were highly variable. The range of CR for intertidal animals (0.24 – 1.84 l hr^{-1} g^{-1}) was somewhat greater than that for subtidal animals (0.03 – 1.48 l hr^{-1} g^{-1}). Due to the methodology used, more fast-clearing *C. fornicata* from the intertidal group (15) were removed from the analysis than were removed from the subtidal group (12), and animals feeding slower than 0.2 l hr^{-1} g^{-1} were only found in the subtidal group (Fig. 5). However, average CR did not differ significantly between intertidal and subtidal *C. fornicata* (Fig. 5; $t_{65} = 0.21$, $p = 0.84$). Conversely, intertidal *C. fecunda* had significantly lower clearance rates than subtidal *C. fecunda* (Fig. 5; $t_{59} = 2.85$, $p = 0.006$). Excluding fast-clearing intertidal (4) and subtidal (14) *C. fecunda*, on average subtidal animals still fed ~60% faster than intertidal animals. Because *C. fecunda* that are brooding embryos have been shown to have higher clearance rates (Mardones et al. 2013), data for intertidal and subtidal *C. fecunda* were analyzed separately for brooding and non-brooding individuals and compared. Intertidal *C. fecunda* that were brooding had lower clearance rates than subtidal brooders ($t_{36} = 1.91$, $p = 0.064$) and intertidal non-brooders had significantly lower clearance rates than subtidal non-brooders ($t_{19} = 2.96$, $p = 0.0081$), in agreement with the previous more general pattern.

In *Crepipatella fecunda*, particles were transported across the gills of intertidal animals significantly more slowly than those being transported across the gills of subtidal animals (Fig. 6; $t_{19} = 2.38$, $p = 0.028$). Though particle velocities across the gills of intertidal and subtidal animals varied widely (intertidal range = 42 – 307 μm sec^{-1}, subtidal range = 77 – 660 μm sec^{-1}), the average velocity of particles moving across gills of subtidal animals was 294.7 (±164.8 SD)

μm sec^{-1} whereas the average velocity across gills of intertidal animals was only 153.3 (±93.8 SD) μm sec^{-1} (Fig. 6), ~50% slower than that for subtidal animals.

The size structures (shell length) of intertidal and subtidal populations of *C. fornicata* differed significantly ($\chi^2 = 38.11$, d.f. = 14, $p = 0.0005$), as there were generally more larger (>27 mm) intertidal individuals. However, members of both intertidal and subtidal subpopulations reached nearly identical maximum sizes (44.6 mm subtidally, 43.3 mm intertidal). Conversely, *C. fecunda* attained a maximum shell length of 66.2 mm in the subtidal zone, a length that was ~34% larger than that of intertidal *C. fecunda* (46.4 mm). The size structures of intertidal and subtidal populations of *C. fecunda* also differed significantly ($\chi^2 = 85.13$, d.f. = 20, $p < 0.0001$), due principally to a greater number of large (>45 mm) subtidal individuals.

Discussion

Though *Crepidula fornicata* and *Crepipatella fecunda* are closely related (Collin 2003) and have similar ecological niches, our results demonstrate that members of the intertidal, range edge populations of these two species have responded very differently to the feeding disadvantages imposed by periodic aerial exposure. The gills of intertidal individuals of *C. fornicata* were considerably heavier than those of subtidal conspecifics (Fig. 1), a difference mediated both by a larger overall gill surface area (Fig. 3) and a greater number of gill filaments (Fig. 4). No such differences were found for *C. fecunda*: members of intertidal and subtidal populations of that species were morphologically indistinguishable for the gill parameters that we measured (Figs 1 – 4).

Morphological differences in the feeding organs of other conspecific suspension-feeders from different environments have previously been reported, mostly in bivalves (e.g. Franz 1993;

Payne et al. 1995; Honkoop et al. 2003; Drent et al. 2004; Dutertre et al. 2007; Dutertre et al. 2009; Yoshino et al. 2013). For example, Franz (1993) showed that mussels (*Geukensia demissa*) from Jamaica Bay, NY living high in the intertidal zone had relatively larger gills than those living in the low intertidal. Additionally, several previous studies of gastropods have demonstrated plasticity in feeding organs (radula and siphon) and feeding behavior depending on tidal height or food source (Newell et al. 1971; Zeldis and Boyden 1979; Little et al. 1991; Padilla 1998; Reid and Mak 1999; Watanabe and Young 2006), though these organisms are grazers or carnivores, not suspension feeders.

In *C. fornicata*, the larger gills of intertidal animals were not accompanied by a significant increase in clearance rate (CR) in our study; intertidal and subtidal *C. fornicata* fed at comparable rates (Fig. 5). When data from intertidal and subtidal animals were pooled, the average CR was approximately 0.85 L hr^{-1} g^{-1} for *C. fornicata*, which agrees with values of CR previously found for this species (Newell and Kofoed 1977a; Newell and Kofoed 1977b; Shumway et al. 2003; Barille et al. 2006; Harke et al. 2011). Though the gill morphology data (Figs 1 – 4) suggest a mechanism by which *C. fornicata* may compensate for reduced feeding time in the intertidal, our CR experiments were not able to detect any such compensation (Fig 5). Though some suspension-feeders show considerable flexibility in feeding behavior (Bayne 2004) and compensatory increases in CR in response to aerial exposure have been found in some cases (Morton et al. 1957; Charles and Newell 1997; Marsden and Weatherhead 1999; Galimany et al. 2013), many suspension-feeders do not show increased CR or ingestion as compensation for aerial exposure in the intertidal zone (Griffiths and Buffenstein 1981; Widdows and Shick 1985; Bayne et al. 1988; Kreeger et al. 1990). For example, Kreeger et al. (1990) found that mussels (*Geukensia demissa*) held under intertidal conditions (50% exposure time) did not compensate

for reduced feeding time with an increased ingestion rate when compared to mussels held in subtidal conditions. Charles and Newell (1997) confirmed these results, but did find increased CR in mussels held under conditions simulating a much higher tidal level (75% exposure). Though we did not detect CR compensation in intertidal *C. fornicata*, it is important to note the high variability in CR among individuals in our experiments, which may have prohibited us from detecting meaningful compensation in this species; either way, intertidal *C. fornicata* performed no worse than subtidal *C. fornicata*.

In our study, pooled data for clearance rates of *C. fecunda* also fell within the expected range determined by previous studies (Navarro and Chaparro 2002; Mardones et al. 2013). However, intertidal *C. fecunda* had a significantly lower average CR than subtidal *C. fecunda* (Fig. 5), contrary to what we found for *C. fornicata*. Since gill morphologies did not differ among intertidal and subtidal *C. fecunda* (Figs 1 – 4), it was surprising to find such a dramatic difference in CR among those two groups. Moreover, the difference was not in the direction that we had initially anticipated. Though brooding individuals of *C. fecunda* have faster clearance rates than non-brooding individuals (Mardones et al. 2013), our results were not due to an over-abundance of brooders in our subtidal sample, as the same trend (subtidal CR > intertidal CR) was observed among brooders and non-brooders. Additionally, our endoscopic results (Fig. 6) indicate that intertidal individuals started to collect phytoplankton immediately upon immersion. Thus, clearance rates of intertidal individuals were not artificially low because of a reluctance of these animals to begin feeding after they were submerged.

Assuming that decreased particle velocity across the gill surface also reflects a reduced capacity of the gill cilia to collect food, the slower CR for intertidal individuals of *C. fecunda* could be in part due to a decreased particle velocity along the gills of intertidal individuals (Fig.

6). Slower particle velocity could be the result of reduced ciliary beat frequency on the gills of intertidal *C. fecunda*, something that could be examined in a future study. In mussels, lateral gill ciliary beat frequency is mediated by several neurotransmitters and is markedly slowed by the release of dopamine (Catapane et al. 1979); increases in dopamine levels have been reported in the tissues of many mollusks in response to stress (e.g. Ottaviani and Franceschi 1996; Lacoste et al. 2001; Malham et al. 2003). However, our studies focused on particle movement along frontal gill cilia; the responses of these cilia to stressors are a potential area of future study.

Physiological stress has previously been associated with reduced performance (i.e., feeding rate) in some marine animals (Abel 1976; Menge 1978; Widdows et al. 1981; McCormick et al. 1998). The pattern of CR and particle velocity transport across the gill (subtidal > intertidal) in *C. fecunda* suggests that intertidal individuals could be stressed and in relatively poor physiological condition; this species seems to be not especially well-adapted to live intertidally. Given the stressful nature of the intertidal zone for marine organisms (e.g. Brinkhoff et al. 1983; Morris and Taylor 1983; Garrity 1984; Petes et al. 2007; Harley et al. 2009; Miller et al. 2009; Diederich and Pechenik 2013), it is possible that intertidal *C. fecunda* are members of a sink population that are in fact poorly adapted for living under such conditions. Such animals would be expected to perform worse (e.g. to exhibit a lower CR) than physiologically more robust subtidal animals. The fact that *C. fecunda* living in the intertidal zone did not reach the maximum sizes typically found among large adults of this species living subtidally (Fig. 7) also suggests that intertidal members of this species, unlike those of *C. fornicata*, are not well suited for intertidal life. Individuals of *C. fecunda* may of course have other physiological adaptations for maximizing energy gain in the intertidal zone that we did not quantify. Energetic compensation for aerial exposure time in other species has been linked to a

number of other processes not explored here, including changes in metabolic rate, gut passage time, and absorption or assimilation efficiency (Griffiths 1981; Kreeger et al. 1990; Charles and Newell 1997; Marshall and McQuaid 2011).

If larger gill size in intertidal members of *C. fornicata* is driven by adaptive plasticity, what mechanisms might control such a change? Like the compensatory feeding of the mussel *Geukensia demissa* (Charles and Newell 1997, see above), *C. fornicata* may experience a threshold shore level above which it is advantageous to have a relatively larger gill. However, intertidal individuals of *C. fornicata* are usually relegated to the mid to low intertidal zone (Diederich and Pechenik 2013), and even short bouts of aerial exposure may trigger a shift in resource allocation towards growing relatively larger gills. It is interesting that intertidal *C. fornicata* had larger gills but similar CR when compared to subtidal conspecifics, while intertidal *C. fecunda* had reduced CRs relative to those of subtidal individuals despite no difference in gill sizes. This may suggest that *C. fornicata* has a longer history of association with the environmental heterogeneity found in the intertidal zone. Indeed, *C. fornicata* is also relatively eurythermal (Diederich and Pechenik, 2013) and tolerant of long periods of aerial exposure (Hoagland 1984) (traits necessary for occupation of the intertidal zone), though intertidal individuals in some areas may be living close to their thermal maximum (Diederich and Pechenik 2013). As it relates to feeding, the intertidal *C. fornicata* in our study seem much better equipped to cope with the disadvantages associated with aerial exposure than those of *C. fecunda*. This may help to explain the ability of intertidal *C. fornicata* to attain sizes equivalent to those of subtidal animals, while intertidal *C. fecunda* did not seem to grow to be as large as subtidal *C. fecunda* (Fig. 7).

Our studies highlight the value of investigating organisms of the same species from intertidal and subtidal zones in the context of range boundaries. Though Gaston (2003) may characterize the intertidal zone as an "internal" boundary (because the intertidal zone is a specific habitat type), that does not diminish its utility in investigating limiting factors and adaptation at range boundaries. At a single field site some organisms will experience conditions associated with 'central' (subtidal) populations while conspecifics only meters away can be characterized as experiencing 'range edge' conditions (intertidal). Additionally, abiotic conditions in intertidal and subtidal areas may be nearly identical when the tide is in, and when the tide is out the suite of abiotic conditions that change are both easily measurable (time emersed, temperature, water lost, etc.) and well-characterized (Newell 1979; Denny 1985; Helmuth 1998; Helmuth and Hofmann 2001).

Finally, our results may have implications for the relative success of these species as it relates to their potential for invading new areas. In fact, *C. fornicata* is an extremely successful invasive species along European coastlines (and other areas, Blanchard 1997) while *C. fecunda* has not been found outside its native range. While the repeated importation of *C. fornicata* individuals into European waters (e.g. on oyster shipments, Blanchard 1997) caused their initial introduction, the reasons for their remarkable success as an invasive species are less clear (Dupont et al. 2006; Viard et al. 2006; Blanchard 2009; Bohn et al. 2012). When viewed in the context of habitat colonization, the ability of *C. fornicata* – and the relative inability of *C. fecunda* – to perform well in the stressful living conditions that characterize in the intertidal zone may be contributing to their relative abilities to invade new areas.

Acknowledgements

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Figures

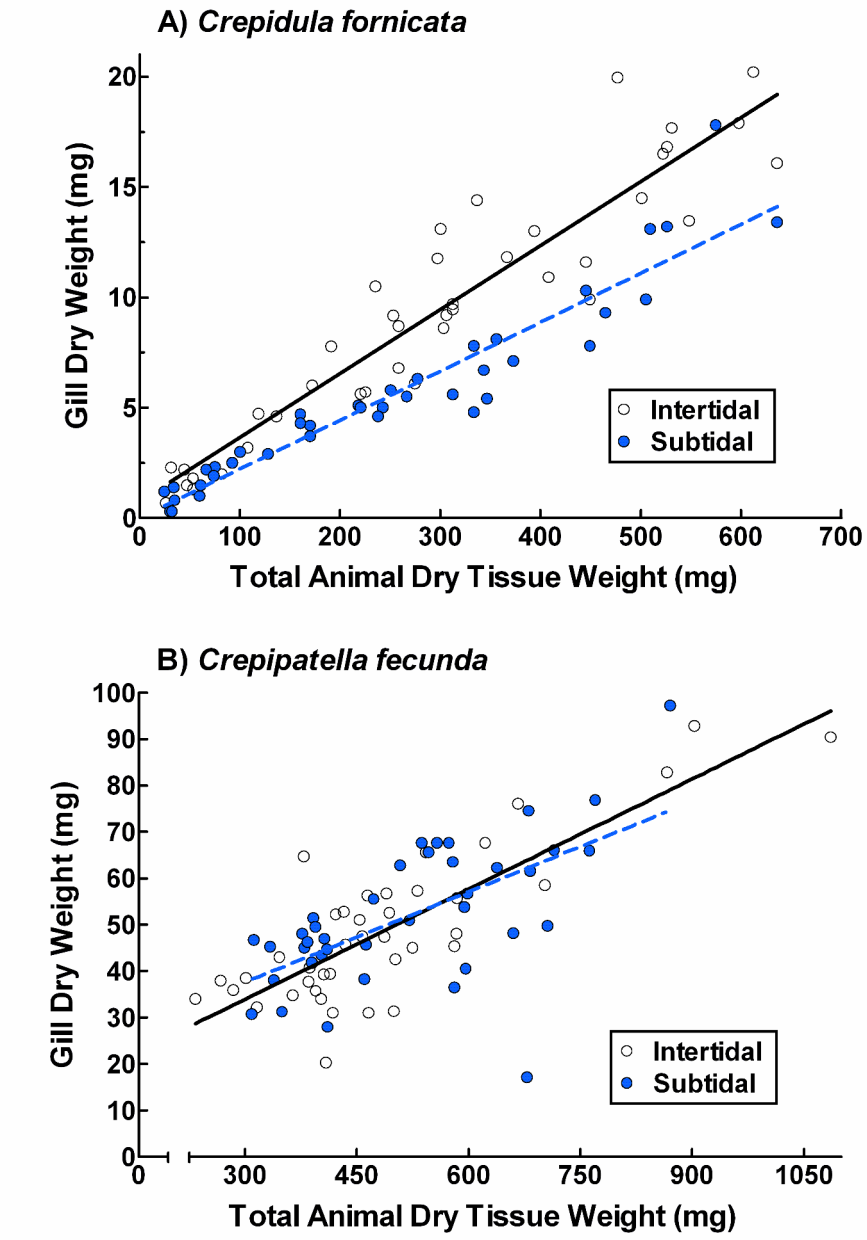

Figure 4.1: Influence of habitat on the relationship between gill weight and total animal dry tissue weight for (A) *Crepidula fornicata* from Bissel Cove in Narragansett Bay, RI, USA and (B) *Crepipatella fecunda* from Puerto Montt, Chile. Gills of 40 individuals from each habitat were excised, dried, and weighed; each point represents data from one individual. (A) Linear regressions diverge significantly for *C. fornicata*; intertidal, $r^2 = 0.88$, $y = 0.029x + 0.75$; subtidal, $r^2 = 0.91$, $y = 0.022x + 0.0099$. (B) Linear regressions do not diverge significantly for *Crepipatella fecunda*; intertidal, $r^2 = 0.68$, $y = 0.079x + 10.26$; subtidal, $r^2 = 0.39$, $y = 0.065x + 18.17$.

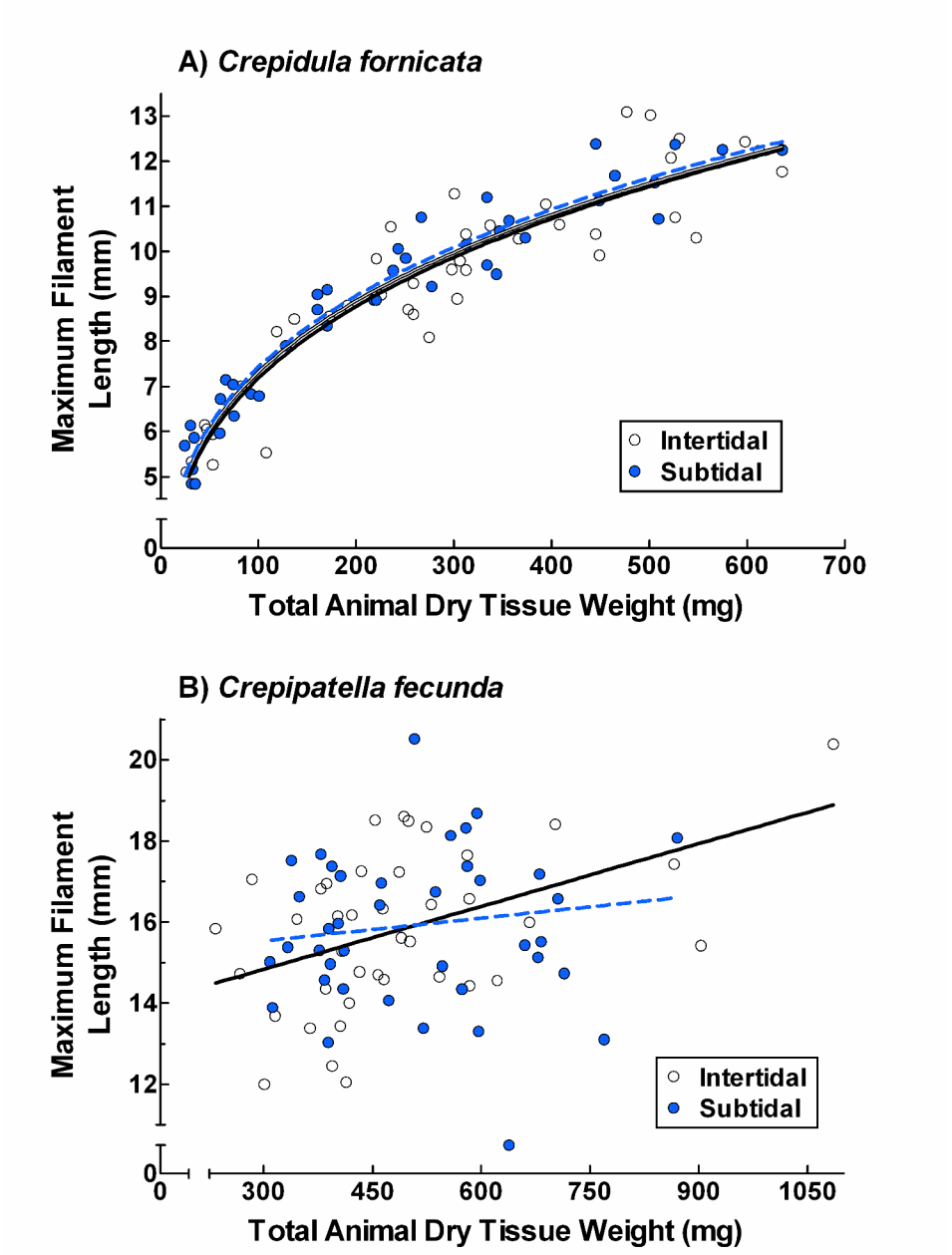

Figure 4.2: Influence of habitat on the relationship between the maximum length of gill filaments and total animal dry tissue weight for (A) *Crepidula fornicata* from Bissel Cove in Narragansett Bay, RI, USA and (B) *Crepipatella fecunda* from Puerto Montt, Chile. Gills of 40 individuals from each habitat were excised, photographed using a dissecting microscope, and the 5 longest filaments for each animal were measured and averaged; each point represents data from one individual. (A) Power models ($y=ax^b$) do not diverge significantly for *C. fornicata*; intertidal, $r^2 = 0.86$, $y = 1.90x^{0.29}$, subtidal, $r^2 = 0.94$, $y = 2.06x^{0.28}$. (B) Linear regressions do not diverge significantly for *Crepipatella fecunda*; intertidal, $r^2 = 0.21$, $y = 0.0052x + 13.29$; subtidal, $r^2 = 0.018$, $y = 0.0019x + 14.97$.

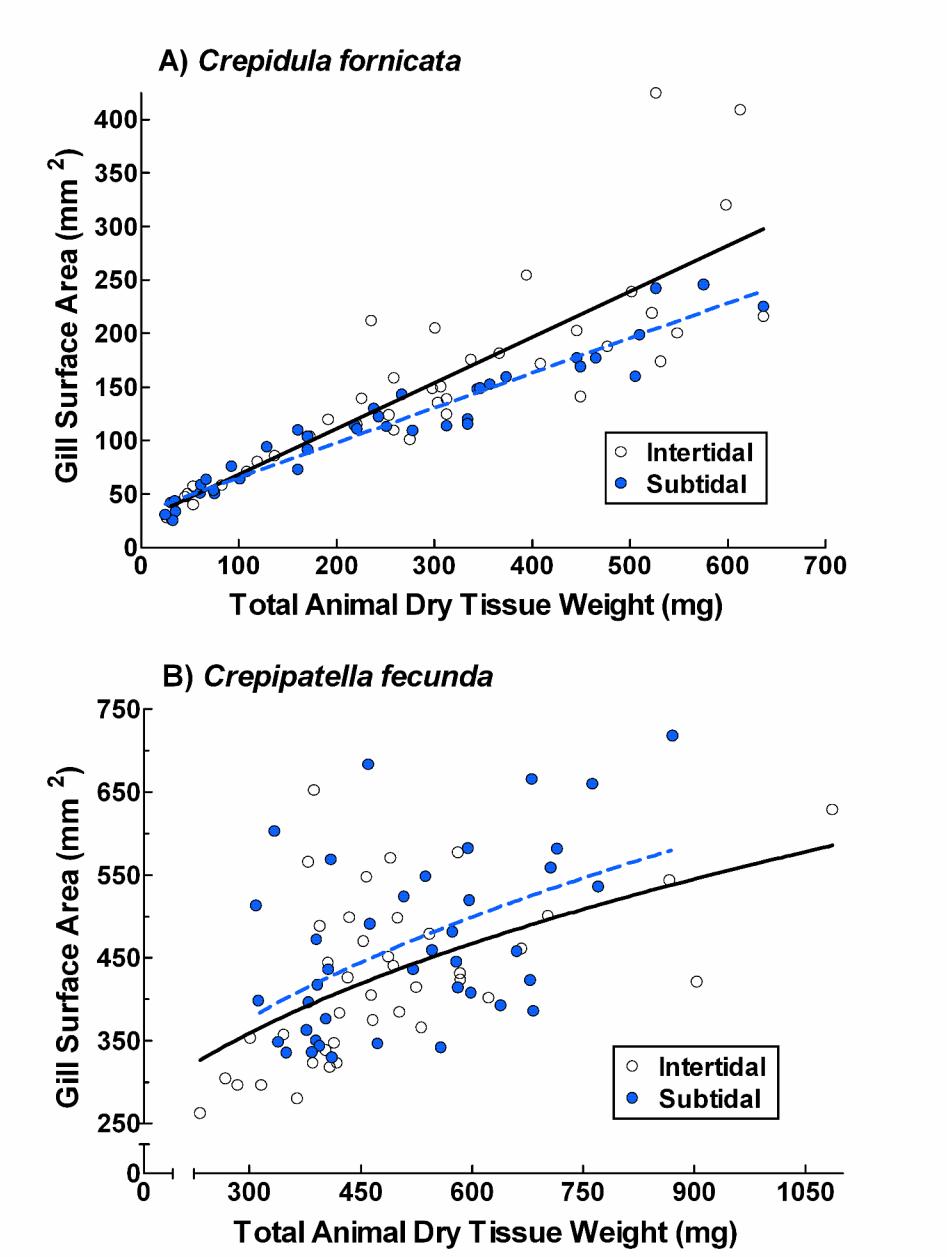

Figure 4.3: Influence of habitat on the relationship between gill surface area and total animal dry tissue weight for (A) *Crepidula fornicata* from Bissel Cove in Narragansett Bay, RI, USA and (B) *Crepipatella fecunda* from Puerto Montt, Chile. Gills of 40 individuals from each habitat were excised and then photographed using a dissecting microscope, allowing us to determine the total surface area of each gill; each point represents data from one individual. (A) Linear regressions diverge significantly for *C. fornicata*; intertidal, $r^2 = 0.71$, $y = 0.43x + 25.60$; subtidal, $r^2 = 0.93$, $y = 0.33x + 32.88$. (B) Power models ($y=ax^b$) do not diverge significantly for *Crepipatella fecunda*; intertidal, $r^2 = 0.31$, $y = 41.05x^{0.38}$, subtidal, $r^2 = 0.23$, $y = 37.51x^{0.40}$.

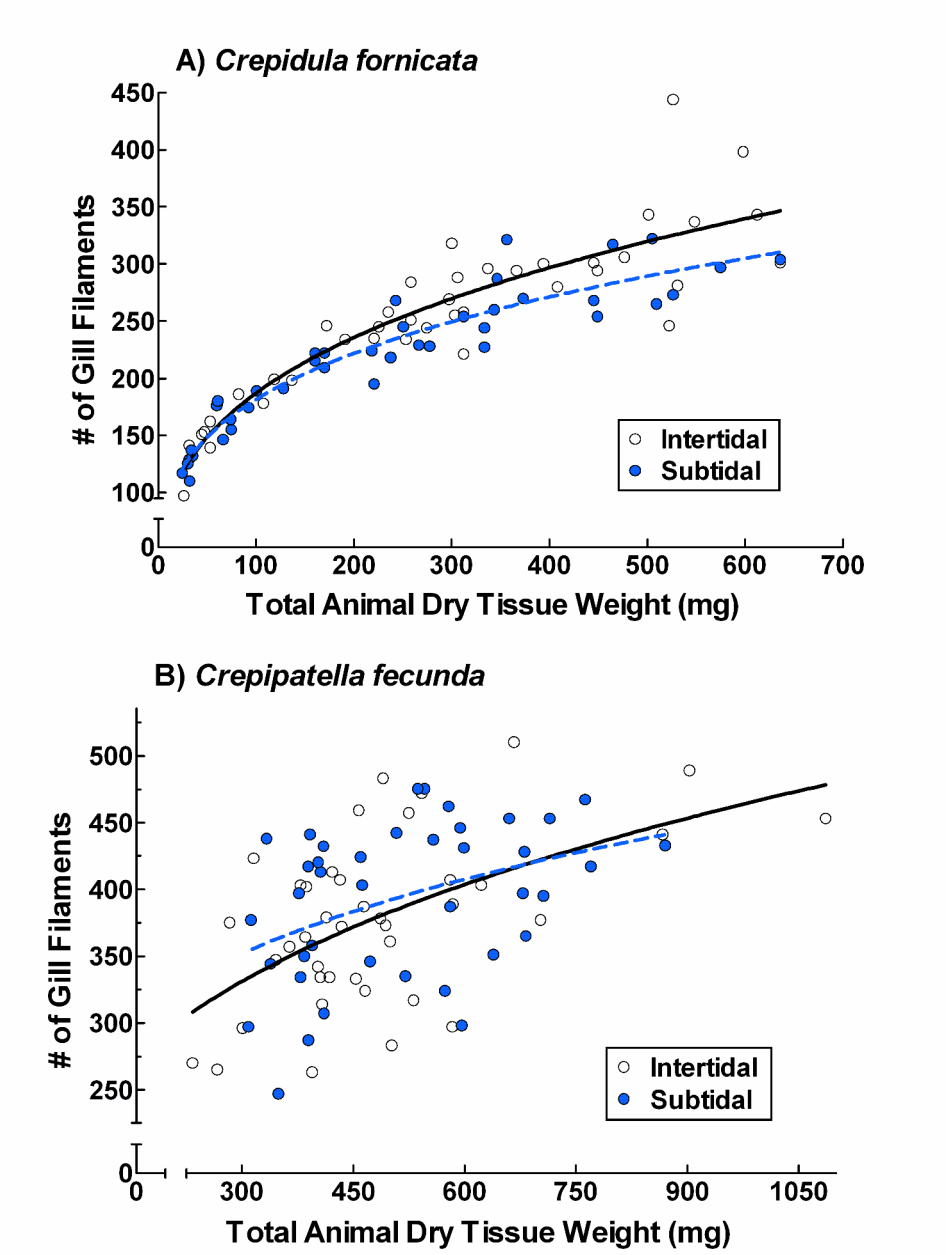

Figure 4.4: Influence of habitat on the relationship between the number of gill filaments and total animal dry weight for (A) *Crepidula fornicata* from Bissel Cove in Narragansett Bay, RI, USA and (B) *Crepipatella fecunda* from Puerto Montt, Chile. Gills of 40 individuals from each habitat were excised, and filaments (>1 mm) were counted by hand under a dissecting microscope; each point represents data from one individual. (A) Power models ($y=ax^b$) diverge significantly for *C. fornicata*; intertidal, $r^2 = 0.81$, $y = 40.30x^{0.33}$, subtidal, $r^2 = 0.90$, $y = 47.68x^{0.29}$. (B) Power models ($y=ax^b$) do not diverge significantly for *Crepipatella fecunda*; intertidal, $r^2 = 0.31$, $y = 64.80x^{0.29}$, subtidal, $r^2 = 0.16$, $y = 104.7x^{0.21}$.

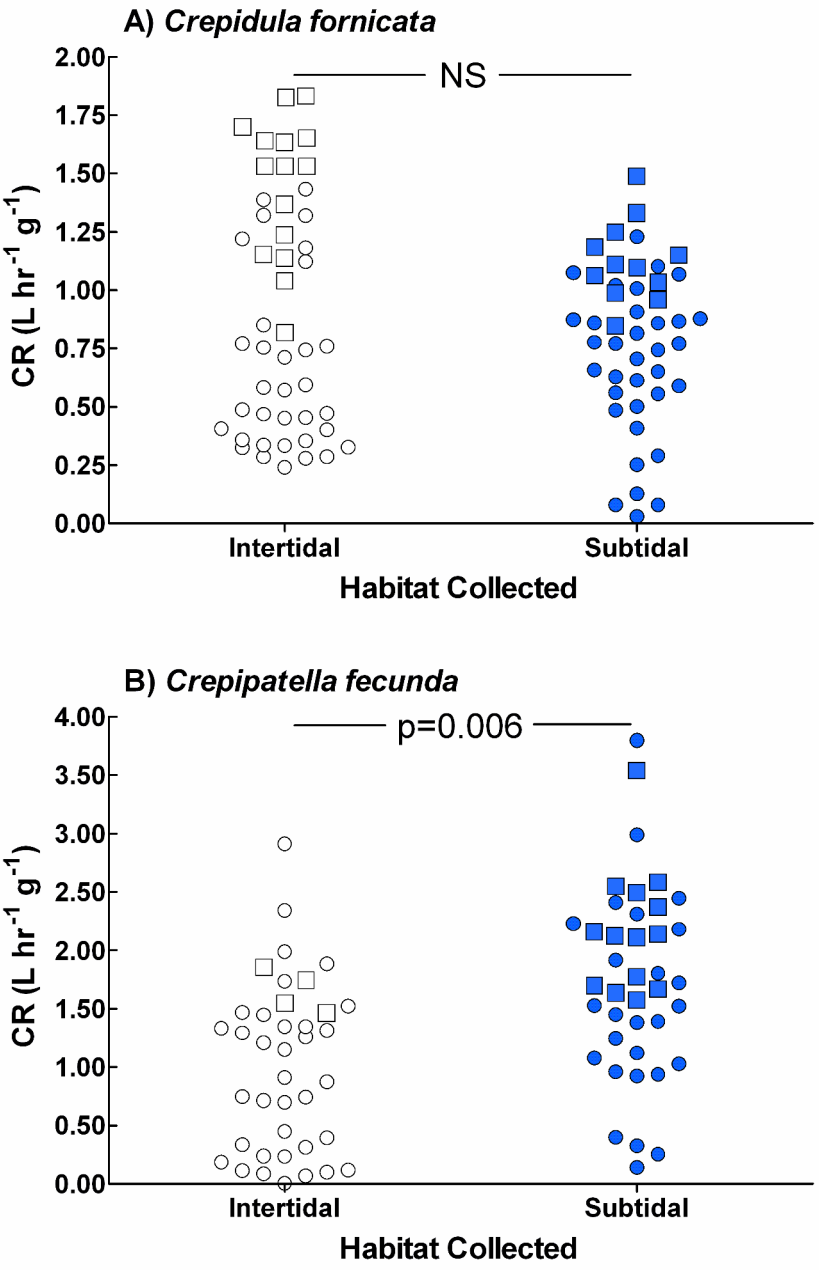

Figure 4.5: Clearance rates of intertidal and subtidal (A) *Crepidula fornicata* from Bissel Cove in Narragansett Bay, RI, USA and (B) *Crepipatella fecunda* from Puerto Montt, Chile. Each data point represents one individual, but square data points were removed from statistical analyses because those animals cleared more than 35% of the algal suspension in the experimental time; results of unpaired t-tests are indicated above the bars. Clearance rates were determined by measuring the rate at which phytoplankton disappeared from suspension over 2 – 4 hours; intertidal animals were first given a 3 hour (*C. fornicata*) or 6 hour (*C. fecunda*) emersion period. For both species, clearance rates were standardized to one gram of dry tissue weight using the allometric exponent 0.56 from Navarro and Chaparro (2002).

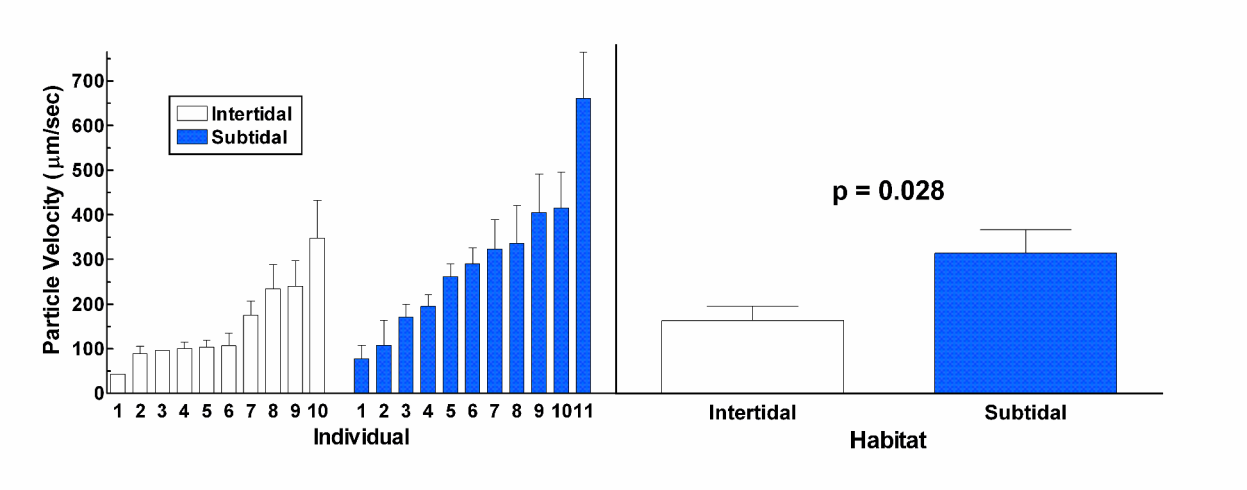

Figure 4.6: Rate (±SEM) at which intertidal and subtidal *Crepipatella fecunda* moved particles across the gill toward the food groove. Animals were collected from Puerto Montt, Chile and experiments were performed within 48 hours of collection. The movements of 1 – 12 particles per individual were monitored by endoscopy. Mean particle velocities along the gills of each individual are shown in the left panel, and mean particle velocities of all individuals grouped by habitat are shown in the right panel (result of unpaired t-test is shown above the bars).

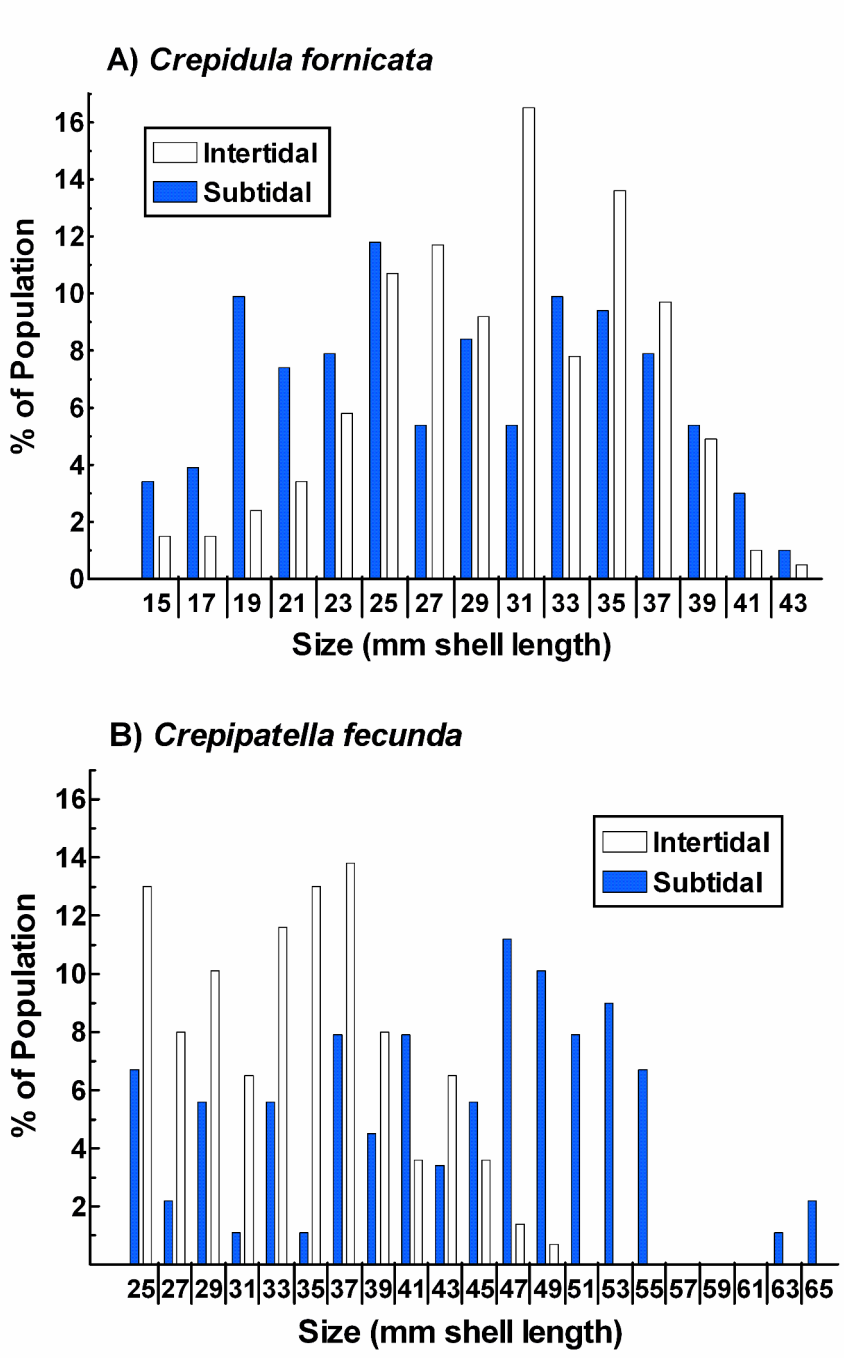

Figure 4.7: Size distributions for intertidal and subtidal individuals of (A) *Crepidula fornicata* collected from Bissel Cove in Narragansett Bay, RI, USA and (B) *Crepipatella fecunda* collected from Puerto Montt, Chile. Numbers on the x-axis represent the lower end of each size class (15 = 15 – 17 mm) (A) Frequency distributions of intertidal (N = 206) and subtidal (N = 203) subpopulations are significantly different ($\chi^2 = 38.11$, d.f. = 14, $p = 0.0005$), though the animals reach similar maximum sizes. (B) Frequency distributions of intertidal (N = 147) and subtidal (N = 91) subpopulations are significantly different ($\chi^2 = 85.13$, d.f. = 20, $p < 0.0001$), with subtidal individuals reaching a maximum size 34% greater than intertidal individuals.

Chapter 5

Reproductive dynamics across the intertidal-subtidal gradient of an important invasive species, *Crepidula fornicata* (Gastropoda), in its native range

Note: Submitted to *Marine Biology*

Abstract

Populations of *Crepidula fornicata* extend from the subtidal zone up into the intertidal zone (the upper edge of its range). Though the intertidal zone is usually thought of as an extremely stressful environment and many organisms have reduced reproductive output there, there is little information on the reproductive dynamics of *C. fornicata* in either habitat in its native range, the western Atlantic. Because *C. fornicata* is a very successful invasive species it has garnered much attention in the European research literature, but comparable studies on reproduction in its native range are rare. We studied the reproduction of *C. fornicata* in Narragansett Bay, Rhode Island, USA. The brooding period began in April for both intertidal and subtidal *C. fornicata*, and lasted into September for intertidal individuals. Additionally, on many occasions throughout the reproductive season, intertidal females had a significantly higher absolute reproductive output than did subtidal females. However, a greater percentage of smaller females were found brooding subtidally than intertidally. The number of embryos, number of egg capsules, number of embryos per egg capsule, and mean egg capsule size all increased linearly with female shell length, but there was little variation in egg size. Intertidal females achieved higher fecundity than subtidal females by crowding egg capsules with more eggs, rather than by producing more or larger egg capsules. Thus, not only were adults of *C. fornicata* not particularly stressed at their intertidal range edge in this location, they actually outperformed subtidal conspecifics in several measures of reproductive capacity. Other basic reproductive characteristics of *C. fornicata* in Narragansett Bay were very similar to those in the more northern latitudes of its invaded range.

Introduction

The intertidal zone is an extremely stressful environment for most marine organisms. When the tide recedes, organisms that cannot retreat to the subtidal are exposed to a suite of environmental stressors during aerial exposure. These stressors include, but are not limited to, tissue desiccation (e.g. Garrity 1984), reduced available feeding time (e.g. Gillmor 1982), hypoxia (due to shell closure or inability to utilize aerial oxygen) (e.g. Brinkhoff et al. 1983), and rapid and dramatic fluctuations in temperature and salinity (e.g. Morris and Taylor 1983; Diederich and Pechenik 2013). Not surprisingly, many marine organisms never venture into the intertidal zone (e.g. Collin 2000; Stillman and Somero 2000). Often, though, the species that do live intertidally are found predominantly there (e.g. Perez et al. 2009; Bourdeau 2011), probably due to intense competition or predation subtidally. However, some species are abundant both intertidally and subtidally (e.g. Saier 2002; Schaffmeister et al. 2006; Dong et al. 2011; Diederich and Pechenik 2013), exposing subpopulations of those species to drastically different conditions, even though the animals in these subpopulations may be living only meters apart.

For those species that live in both habitats, the intertidal zone represents a range boundary (Holt and Keitt 2005). Studies undertaken on a large latitudinal scale have found that reproductive output is often impeded at the range edge (see Gaston 2009), though this is certainly not a universal finding (e.g. Angert 2006; Lester et al. 2007, Ling et al. 2008). Regardless, reproductive output is an important trait that is commonly studied to assess species performance at range boundaries (e.g. Lewis 1986; Herbert et al. 2003; Ling et al. 2008; Pecorino et al. 2013). Though the study of reproductive dynamics at range boundaries has been fruitful on a latitudinal scale, the effects of aerial exposure on reproduction at intertidal range boundaries has been less well studied.

Measuring absolute reproductive output at the intertidal range edge can give insight into the degree to which organisms living in the intertidal zone are stressed and negatively impacted by the conditions associated with aerial exposure (e.g. temperature fluctuations, desiccation). Results to date have generally shown reproduction to be hindered in the intertidal zone. Due to a shift in resource allocation to somatic maintenance and stress resistance, most organisms (e.g. barnacles, urchins, several species of mussel) that experience longer periods of aerial exposure have been found to have reduced reproductive output compared to conspecifics living lower in the intertidal zone or subtidally (e.g. Palmer 1980; Bayne et al. 1983; Page 1984; Borrero 1987; Byrne 1990; Petes et al. 2007; Petes et al. 2008). For example, Borrero (1987) found that not only did mussels living high in the intertidal zone have a delayed reproductive season compared to low-intertidal mussels, their gametic output was also reduced, and by as much as ~60%. However, presumably to ensure some measure of reproductive success in the face of stressful conditions, other organisms have shown increased allocation to reproduction (or earlier spawning) in response to aerial exposure or food limitation (e.g. McKillup and Butler 1979; Thompson 1983; Byrne 1990; Franz 1997). In those cases where allocation to reproduction was higher in stressful environments, however, absolute reproductive output (e.g. total gonadal production, total fecundity) was often still lower than that of conspecifics living in less physically stressful environments (Franz 1997). Though the common gastropod *Crepidula fornicata* has a broad vertical range, such studies have never been reported for this species.

Native to the east coast of the United States, the gastropod *C. fornicata* (Calyptraeidae) is an important member of both intertidal and subtidal communities (Diederich and Pechenik 2013). Members of this species are sessile suspension feeders and protandrous hermaphrodites; adults usually live on one another in ‘stacks’, ensuring reproductive success (Dupont et al. 2006)

with larger, older females occupying the lower portion of each stack and smaller males the upper portion (Coe 1936). Females brood embryos in gelatinous egg masses beneath the shell for several weeks until releasing feeding veliger larvae into the plankton, where they grow to approximately 0.7 – 1 mm in shell length before becoming competent to metamorphose (Pechenik 1980). Metamorphosis is eventually achieved after contact with conspecific cues, so that this species is often found patchily distributed and in high numbers. In the 1870's, *C. fornicata* was introduced to European waters with oyster shipments, and has since become an extremely successful invader (Blanchard 1997; Thieltges et al. 2003; Streftaris and Zenetos 2006; McNeill et al. 2010; Doğan et al. 2014), often to the detriment of some native marine organisms (e.g. Orton 1926; Le Pape et al. 2004; Thieltges 2005; Leloup et al. 2008; Kostecki et al. 2011).

Due to its importance as an invasive species, some information has already been published on the reproductive dynamics of *C. fornicata* in its invaded range (Chipperfield 1951; Deslous-Paoli 1985; Richard et al. 2006; Valdizan et al. 2009; Beninger et al. 2010; Valdizan et al. 2011; Bohn et al. 2012). However, surprisingly little is known about the reproduction of this species in its native range, particularly about variation in fecundity or even the duration of the reproductive season. In three studies focusing on aspects of *C. fornicata* biology other than reproduction (embryology, size at sex change, and male reproductive success) there have been limited estimates of fecundity and only one mention of the duration of the reproductive season (Conklin 1897; Collin 1995; Proestou et al. 2008). The lack of information on the reproductive dynamics of such an important species in its native range is surprising, and in no study (native or invasive range) have *C. fornicata* in the intertidal and subtidal zones been explicitly compared.

In this study we investigated the reproductive dynamics of *C. fornicata* across the intertidal-subtidal boundary in one of its native range populations. Our main goal was to determine if the reproductive characteristics of *C. fornicata* were affected by the marked changes in physical conditions at the edge of their vertical range in the intertidal zone. Specifically, we compared the length of the brooding season and the fecundity of intertidal and subtidal *C. fornicata* at a field site in Narragansett Bay, Rhode Island. Additionally, we estimated the relative size structures of reproductively active subpopulations. Finally, in order to investigate the mechanism controlling any fecundity differences among members of intertidal and subtidal subpopulations, we measured a variety of egg mass characteristics from *C. fornicata* females collected from both habitats: total number of embryos, number of egg capsules, number of embryos/egg capsule, egg capsule size, and embryo size.

Materials and Methods

Study Site

All studies were performed at Bissel Cove in Narragansett Bay, Rhode Island; collections were not made in the cove itself but on the portion of the coastline adjacent to the cove that faces the bay, which is subject to a diurnal tidal regime typical of much of the northeast coast of North America. At this site, *Crepidula fornicata* is abundant both intertidally and subtidally (Diederich and Pechenik 2013). All intertidal specimens were collected from approximately 0.5 meters above (+0.5 m) the mean low lower water mark (MLLW) (hereafter, intertidal) and all subtidal specimens were collected from approximately 1 meter below (-1.0 m) MLLW, a depth at which the substrate is never exposed to the air (hereafter, subtidal). Intertidal and subtidal sampling sites were approximately 50 meters apart. At this site, temperatures may differ substantially

between the two collecting areas when the tide is out: *C. fornicata* biomonitors reached temperatures above 40°C in the intertidal zone in the summer of 2011, while subtidal biomonitors reached only 27°C during the same time period (Diederich and Pechenik 2013). Additionally, while they are exposed to the air intertidal individuals may experience temperatures much lower than subtidal individuals for short periods of time (Diederich and Pechenik 2013). Freshwater input at Bissel Cove is typically low, and salinity levels remain high, above 27 psu (Welsh 1975; McKinney et al. 2001), especially on the bay side of the cove where we sampled (personal observation, C. Diederich).

Reproductive Season

Females of *C. fornicata* deposit their fertilized eggs in a cluster of thin-walled egg capsules whose stalks join at a central peduncle, which is attached to the substrate (Conklin 1897). This entire egg mass is brooded under the shell between the foot and neck of the mother for approximately 20-30 days until planktonic veliger larvae hatch out (Conklin 1897; Brante et al. 2008). To estimate the duration of the reproductive season for intertidal and subtidal *C. fornicata*, we measured the percentage of animals that were brooding egg masses on many dates (see below) over the course of one year. On each sampling date, 20 intertidal and 20 subtidal females were collected (except for May 21st, 2013 when 53 females were collected from each subpopulation). After measuring the female shell length (longest dimension, anterior to posterior), she was gently pried off of her substrate and the presence or absence of an egg mass was noted. The sampling dates for this study were May 4th, 2012; June 1st, 2012; July 5th, 2012; August 1st, 2012; September 3rd, 2012; October 1st, 2012; October 14th, 2012; December 12th, 2012; February 11th, 2013; March 10th, 2013; April 13th, 2013; April 25th, 2013; and May 21st, 2013.

Fecundity, Developmental Stage, and Egg Capsule Measurements

In order to determine the effect of habitat (intertidal or subtidal) on reproductive dynamics, *C. fornicata* egg masses were collected intact from brooding females on each of the collection dates listed above. While checking for brooding females (see above), all egg masses were collected from sampled females in both subpopulations unless >50% of females were brooding, in which case the first 10 egg masses were collected from each subpopulation. In addition to the dates listed above, egg masses were also collected specifically for determinations of fecundity on two earlier dates: June 22nd, 2010 and August 30th, 2011. On each sampling date, females were very gently removed from their substrates so as not to damage the egg masses which remained attached to the substrate. Egg masses were removed from the substrate at the peduncle-substrate attachment point with a fine pair of forceps, and immediately preserved in 5% formalin buffered with sodium borate.

Egg masses were examined in the laboratory using a dissecting microscope fitted with an ocular micrometer. Egg masses were separated into their constituent egg capsules. The capsules were counted and the lengths and widths of 5 haphazardly selected egg capsules from each egg mass were measured. Each egg capsule is essentially triangular, and though we did not measure the thickness of the egg capsule, we could estimate the size of egg capsules by computing the triangular area (Fig. 1).

All egg capsules were then dissected and the developmental stage of the embryos was determined; we were able to assign a single developmental stage to each egg mass, as all embryos within an egg mass were at a similar stage of development (Richard et al. 2006; Bohn et al. 2012). Developmental stages were determined using a scale adapted from Chipperfield (1951) (see Richard et al. 2006; Bohn et al. 2012) in which embryos were scored either as morulas,

trocophores, or veligers. These stages can be seen more plainly in Figure 2 of Henry et al. (2010): morula (Henry et al., 2010 Figure 2L-2S), trocophore (Henry et al., 2010 Figure 2T-2V), and veliger (Henry et al., 2010 Figure 2W-2Y). Morula and trocophore stages together comprise approximately the first half of the 20 – 30 day brooded development period (Maeda-Martinez 2008).

After dissecting all of the capsules in an egg mass, the embryos were mixed and the longest dimension ("length") was measured for 10 haphazardly chosen embryos. Because the morphology of embryos changes markedly throughout development (see Henry et al. 2010), any analyses that we performed on embryo size were restricted to embryos that were found to be in the earliest developmental stage (morula). Finally, we determined the total number of embryos in each egg mass by counting all of them individually.

Data Analysis

The main goal of these studies was to investigate the reproductive dynamics of *C. fornicata* at its intertidal range edge (i.e., to compare the intertidal individuals with the subtidal individuals). Fisher's exact tests were performed to compare the ratio of brooding to non-brooding females on any given sampling date.

Because habitat may have an effect not only on broods per season, embryos per brood and other short-term aspects of reproductive dynamics but also on long-term aspects (e.g. lifetime number of broods produced per female), we wanted to estimate the size at which females became reproductively active in the intertidal zone and subtidal zones. To do this, multiple logistic regression was run with female shell length and habitat (intertidal or subtidal) as independent variables and brooding status (yes or no) as the response variable; estimates for the

probability of brooding across a wide size range (16.0-44.6 mm shell length) of females were generated for members of both habitats.

To compare the measurement variables associated with reproductive dynamics (number of egg capsules, embryos per egg capsule, capsule area, and embryo size) between intertidal and subtidal subpopulations, we regressed each variable against the mother's shell length. We first used an information-theoretical approach to model selection (Burnham and Anderson 2002; Johnson and Omland 2004) and a corrected Akaike's Information Criterion (AICc) score (Akaike 1973) to compare linear ($y = ax + b$) and power ($y = ax^b$) models, and found that linear models best described the relationship between the mother's shell length and the number of egg capsules, the embryos per egg capsule, and the capsule area. There was no statistically significant relationship between the mother's shell length and embryo size (linear regression, $r^2 = 0.0033$, $F_{1,31} = 0.10$, $P = 0.75$). An ANCOVA was run for each variable with habitat (intertidal vs. subtidal) as a factor and female size as the covariate. After homogeneity of slopes was confirmed, intercepts were compared to determine potential differences in each variable between intertidal and subtidal organisms. Statistical significance was set at the level of $\alpha < 0.1$.

Results

In Narragansett Bay, the reproductive season for *C. fornicata* began in April of 2013 (Figs. 2, 3). Though females were not checked for brooding in April 2012, they were checked on May 4th, 2012 and nearly all females were found brooding; the presence of some embryos in the trocophore and early veliger developmental stage indicates that those egg masses had probably been laid in April of that year. Significantly more intertidal than subtidal females were brooding egg masses in June 2012 (Fisher's Exact Test, $P = 0.014$), August 2012 (Fisher's Exact Test, $P =$

0.020), and September 2012 (Fisher's Exact Test, $P = 0.0033$), and the proportion of brooding females did not differ between intertidal and subtidal subpopulations on any other date (Fig. 2). In 2012, the brooding season for intertidal *C. fornicata* extended into September, while we found no subtidal individuals brooding embryos after July (Fig. 2). On any given sampling date, the developmental stage of the embryos from both the intertidal zone and subtidal zone was not uniform (i.e. morula, trocophore, and veliger embryos were all found simultaneously, Fig. 3). The presence of morula-stage embryos in September of 2012 in the egg masses of intertidal females indicates that at least some intertidal *C. fornicata* had deposited egg masses that late in the season.

After standardizing for female size, intertidal *C. fornicata* produced significantly more embryos per egg mass than subtidal animals in June 2010 (*t* test, $t_{36} = 4.13$, $P = 0.0002$), August 2011 (*t* test, $t_9 = 2.06$, $P = 0.069$), and July 2012 (*t* test, $t_{17} = 3.17$, $P = 0.0056$) (Fig. 4). Statistical tests were not conducted for August and September of 2012 because there were no brooding females in the subtidal zone at that time. In May and June 2012, there were no significant differences in average fecundity between intertidal and subtidal females (Fig. 4, *t* test, $t_{18} = 0.27$, $P = 0.79$; $t_{18} = 1.52$, $P = 0.15$, respectively). Both intertidal and subtidal fecundity varied significantly over time (Fig. 4, subtidal ANOVA, $F_{4,46} = 12.23$, $P < 0.0001$; intertidal ANOVA, $F_{6,64} = 7.29$, $P < 0.0001$). For animals in both subpopulations, the egg mass collected early in the season (May, 2012) contained significantly more embryos than all other times (except for August, 2011; Fig. 4, Bonferroni multiple comparisons tests, $P < 0.05$).

Female size was an important indicator of whether or not *C. fornicata* were found brooding (Fig. 5, multiple logistic regression, Wald $\chi^2 = 43.26$, d.f. = 1, $p < 0.0001$). To a lesser degree, location (intertidal or subtidal) was also important for predicting brooding status (Fig. 5,

multiple logistic regression, Wald $\chi^2 = 43.26$, d.f. = 1, p = 0.081), as more of the smaller females were found brooding egg masses subtidally than intertidally (Fig. 5).

The number of embryos produced per female increased significantly with female size (number of embryos = –14,080 + 674.5 X shell length, $r^2 = 0.25$, $F_{1,120} = 40.75$, $P < 0.0001$). Only the regressions for density of embryos in each capsule (vs. female size) were significantly different between intertidal and subtidal females (with embryo densities higher in intertidal egg capsules, Fig. 6b, $F_{1,119} = 4.53$, $P = 0.035$). Regressions for the number of egg capsules (Fig. 6a, $F_{1,119} = 0.38$, $P = 0.54$) and the average size of egg capsules (Fig. 6c, $F_{1,119} = 0.13$, $P = 0.72$) were not different between intertidal and subtidal individuals; the size of a *C. fornicata* female had no effect on the size of the embryos that she produced (Fig. 6d; linear regression, subtidal, $r^2 = 0.024$, $F_{1,10} = 0.25$, $P = 0.63$; intertidal, , $r^2 = 0.013$, $F_{1,15} = 0.19$, $P = 0.67$).

Discussion

Though *Crepidula fornicata* is an important member of both intertidal and subtidal communities worldwide, surprisingly little is known about the reproductive dynamics of this species in its native range, the western Atlantic. In 2013 at Bissel Cove in Narragansett Bay, Rhode Island, brooded eggs were first observed for both intertidal and subtidal *C. fornicata* in April (Fig. 2). Additionally, brooded eggs were found in early May of 2012; since the brooding period for this species lasts several weeks (Brante et al. 2008), the brooding season that year probably also started in April. In 2012, the brooding period extended into September for intertidal individuals (Fig. 2, 3). Though only sampled for one year, our results on the brooding period of this species (April – late summer) differ from the only other mention of the reproductive season for *C. fornicata* in its native range (Conklin 1897), which states that the

breeding season lasts from "early summer until about August 15" on the New England coast. If the onset of the breeding season is controlled by temperature (brooding being initiated at 10ºC in some areas, Chipperfield 1951; Richard et al. 2006 but as low as 6°C is the Wadden Sea and Wales, Thieltges et al. 2004; Bohn et al. 2012), then in the relatively warmer waters of Narragansett Bay it is not surprising that females could be brooding embryos earlier than in the colder waters along the New England open coast. For example, in April 2013 the average water temperature near our field site was 1.4°C warmer than in the coastal waters near Boston, MA (8.7°C and 7.3°C, respectively; data from NOAA National Data Buoy Center). Additionally, Conklin's (1897) remark was made nearly 120 years ago; since then coastal waters have warmed from climate change, which may have altered the time when brooded embryos can be found for this species.

Compared to *C. fornicata* in its invaded range, the length of the breeding season that we found is consistent with that of *C. fornicata* in South Wales, U.K., South England, U.K., and the North Wadden Sea, Denmark (March to September, Bohn et al. 2012; April to September, Chipperfield 1951; Thieltges et al. 2004, respectively), but quite different from that of populations of *C. fornicata* in the Bay of Brest, France (February to October, Richard et al. 2006), Marennes-Oleron, France (March to October, Deslous-Paoli 1985) and Bourgneuf Bay, France (February/March to October, Valdizan et al. 2009; Beninger et al. 2010). The report by Valdizan et al. (2011) that the brooding period in Bourgneuf Bay has lengthened over time due to increased water temperatures is consistent with the finding that *C. fornicata* in colder Rhode Island, U.K., and Wadden Sea waters have truncated brooding seasons compared with *C. fornicata* in French waters. For example, average monthly water temperatures near our field site in Narragansett Bay ranged from 2.5°C to 22.8°C from 2004 – 2012 (NOAA National Data Buoy

Center), similar – especially on the low end of the range – to the ranges reported for south Wales, U.K. (2.7 – 18.1°C, Bohn et al. 2012) and the Wadden Sea (3.4 – 18.1°C, Thieltges et al. 2004) but lower than those reported for different areas of the coast of France (8.6 – 18.7°C in the Bay of Brest, Richard et al. 2006; approximately 9 – 24°C in Bourgneuf Bay, Valdizan et al. 2011).

As it relates to fecundity, the first sampled brood of the season for both intertidal and subtidal females was approximately double the size of subsequent broods. This trend has also been found in *C. fornicata* in its invaded range (Richard et al. 2006). If the size of the brood is determined by the amount of energy reserves stored by the female, a large input of phytoplankton in the spring coupled with relatively low temperature (and low energetic costs) may contribute to such high spring fecundity in both zones.

. Fecundity in *C. fornicata* increased with female size, as is true for many other species (reviewed in Shine 1988), and has previously been found for *C. fornicata* (Collin 1995; Richard et al. 2006; Proestou et al. 2008). Not only did larger *C. fornicata* produce more embryos per brood, they also produced larger and more egg capsules, and each capsule contained more embryos (Fig. 6).The relationship between female shell length and fecundity that we found is nearly identical to that found by Proestou et al. (2008) at least five years earlier at a site several miles away to the south of our study site. Additionally, the regression line that we found is very similar to that found by Richard et al. (2006) in the Bay of Brest, France, though their regression estimated only maximum fecundity. Thus, the average reproductive output of *C. fornicata* at Bissel Cove in Rhode Island was similar to the maximum reproductive output in the Bay of Brest, France. The remarkable success of *C. fornicata* along the French coastline, then, may be more dependant on the fact that females reach larger sizes there (Bay of Brest, > 55 mm, Richard

et al. 2006; Narragansett Bay, ~44 mm) and probably have a longer reproductive season (see above).

The reproductive dynamics of *C. fornicata* individuals living in the intertidal zone were different from those of subtidal individuals but, surprisingly, not in the direction that we had anticipated. The brooding season started at similar times (April) for intertidal and subtidal individuals, but we did not find any subtidal females brooding egg masses after July in 2012, while the egg-laying season for intertidal females extended into September (Fig. 2). In addition to potentially having a relatively longer reproductive season, fecundity (average number of embryos per brood) was higher for intertidal females than for subtidal females on many occasions throughout the reproductive season, and fecundity of subtidal females at any time point never exceeded that of intertidal females (Fig. 4). Though the number of embryos in each brood varied widely (Fig. 4), the absolute reproductive capacity of intertidal *C. fornicata* was never lower than subtidal *C. fornicata*. This is surprising, as many other species are at a distinct energetic disadvantage in the intertidal zone (e.g. Palmer 1980; Bayne et al. 1983; Page 1984; Borrero 1987; Byrne 1990; Franz 1997; Petes et al. 2007; Petes et al. 2008).

After the first large brood of the reproductive season, subsequent summer broods were smaller, and intertidal fecundity stayed relatively constant, while subtidal broods were significantly smaller or nonexistent later in the reproductive season (Fig. 4). The mechanistic cause for this pattern is not clear, though it may be influenced by many factors related to rates of energy input and expenditure. Since intertidal and subtidal females were only meters apart, it is unlikely that food quality or concentration differs among the habitats; only food access time would have differed, as intertidal animals will have had less time each day to capture food particles from the water. It is possible that intertidal *C. fornicata* overcome this feeding-time

disadvantage with larger food-collecting surfaces (Diederich et al. In Review). Though conditions in the intertidal zone have been shown to be stressful for members of this species in south Wales, U.K. where post-settlement mortality can be high (Bohn et al. 2013a, b) this species is relatively eurythermal (Diederich and Pechenik 2013), and intertidal temperatures may not induce a costly stress response for adults. Increased temperature in the intertidal may positively affect food collection rates, gut clearance times, and absorption and/or assimilation efficiencies, all of which may contribute to the relatively high fecundity that we observed, issues that should be fruitful areas of study in the future (Bayne and Scullard 1978; Griffiths 1981; Dam and Peterson 1998; Sorbal and Widdows, 1997; Sanford, 2002; Yamane and Gilman 2009).

Although average fecundity was higher for intertidal *C. fornicata* in a single reproductive season, a greater percentage of small females were found brooding subtidally than intertidally (Fig. 5). If subtidal females do start reproducing at a younger age than intertidal females, this could ultimately result in a greater (or equal) number of lifetime broods for subtidal females. However, growth rates of *C. fornicata* living in the two different zones were not quantified, and though reports of determining age in this species using shell characteristics have been attempted (Le Gall 1980), this practice has not been used in natural populations. Thus, though more subtidal females may have been found brooding, we cannot know if those females were younger than the smallest brooding intertidal females. A longer-term study is necessary to truly determine if differences in lifetime fecundity exist between intertidal and subtidal *C. fornicata*.

Interestingly, intertidal females produced more embryos per female than did subtidal females, but this was mediated by neither an increase in the number or size of egg capsules, nor a decrease in the size of embryos (Fig. 6). Instead, in order to achieve a higher fecundity, intertidal females crowded each egg capsule with a greater density of embryos (Fig. 6). These results

imply that the egg capsule material is costly to make, and females with greater energy reserves should invest energy into increasing the density of embryos in each egg capsule before making more capsules. How the deposition of embryos within egg capsules is controlled in *C. fornicata* is not known. In related species, energy invested in egg capsules was found to be relatively low (<8% in *Crepipatella dilatata* and *C. fecunda*, Chaparro et al. 1999; Chaparro and Flores 2002), but the cost of production of extra-embryonic structures (e.g. capsules, stalks) in mollusks varies and can be very high, even more than 50% of total investment to reproduction (e.g. Stickle 1973; Perron 1981, Lee and Strathmann 1998). At some density of embryos within a capsule, oxygen constraints on over-crowded embryos should shift the selective advantage toward production of more egg capsules rather than increasing the number of embryos per capsule (Perron 1982). Indeed, many species with encapsulated embryos, including *C. fornicata*, experience hypoxic or anoxic conditions within egg capsules (e.g. Cancino et al. 2000; Lardies and Fernández 2002; Moran and Woods 2007; Brante et al. 2008; Chaparro et al. 2009). Though *C. fornicata* larvae may be sensitive to hypoxia after hatching (Brante et al. 2008), encapsulated embryos are particularly tolerant of very low oxygen conditions (Brante et al. 2008; Brante et al. 2009). Thus, it follows that *C. fornicata* females would crowd costly egg capsules with hypoxia-tolerant embryos to maximize fitness and the efficiency of reproductive energetic expenditure (Fig. 6).

Taken together, our results suggest that *C. fornicata* living in the intertidal zone at their upper range edge perform as well as conspecifics that live continuously submerged, despite what would seem to be a physiologically more stressful existence. In fact, contrary to the many other species whose reproduction is hindered due to high stress in the intertidal zone, reproductive season and absolute reproductive output were slightly enhanced in the intertidal zone for *C. fornicata* in our study. Physical stressors (e.g. temperature, desiccation) may ultimately control

the upper distribution of this species (Bohn et al. 2013a, b), but they do not appear to affect the reproductive output of *C. fornicata* in the intertidal zone. Their resilience in the face of such stressors is a likely factor in their demonstrated ability to colonize new environments and to become such a successful invasive species outside of the western Atlantic. This is the first instance of such an extensive data set on the native reproduction of *C. fornicata*; we hope it will be useful in informing future studies on the success of this species as it spreads further, and predicting its fate in native habitats at the margins of its geographic boundaries in the face of climate change.

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Figures

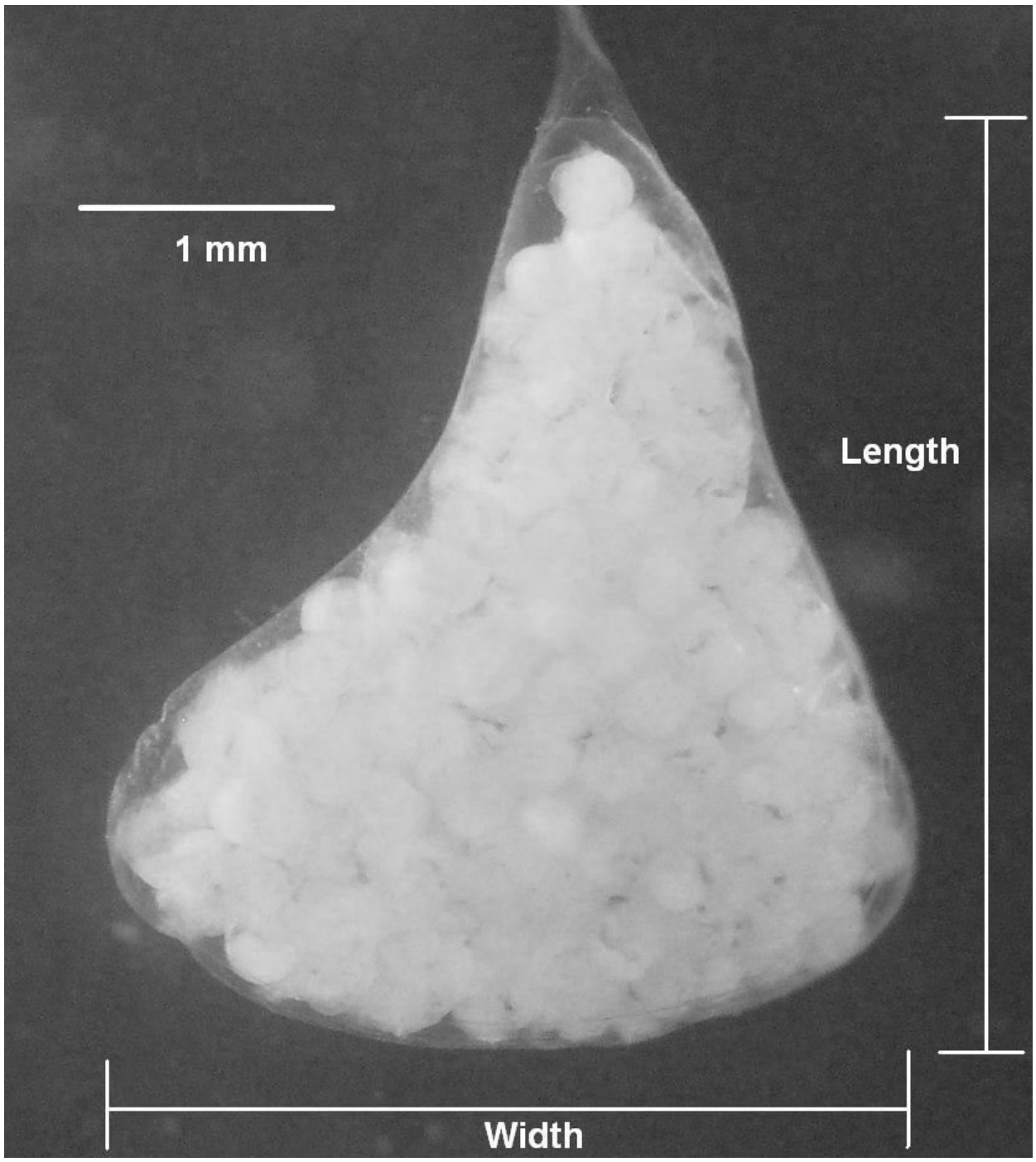

Figure 5.1: A single egg capsule from an egg mass of *Crepidula fornicata*. Capsule size was computed by measuring the length and width of each capsule and calculating the triangular area.

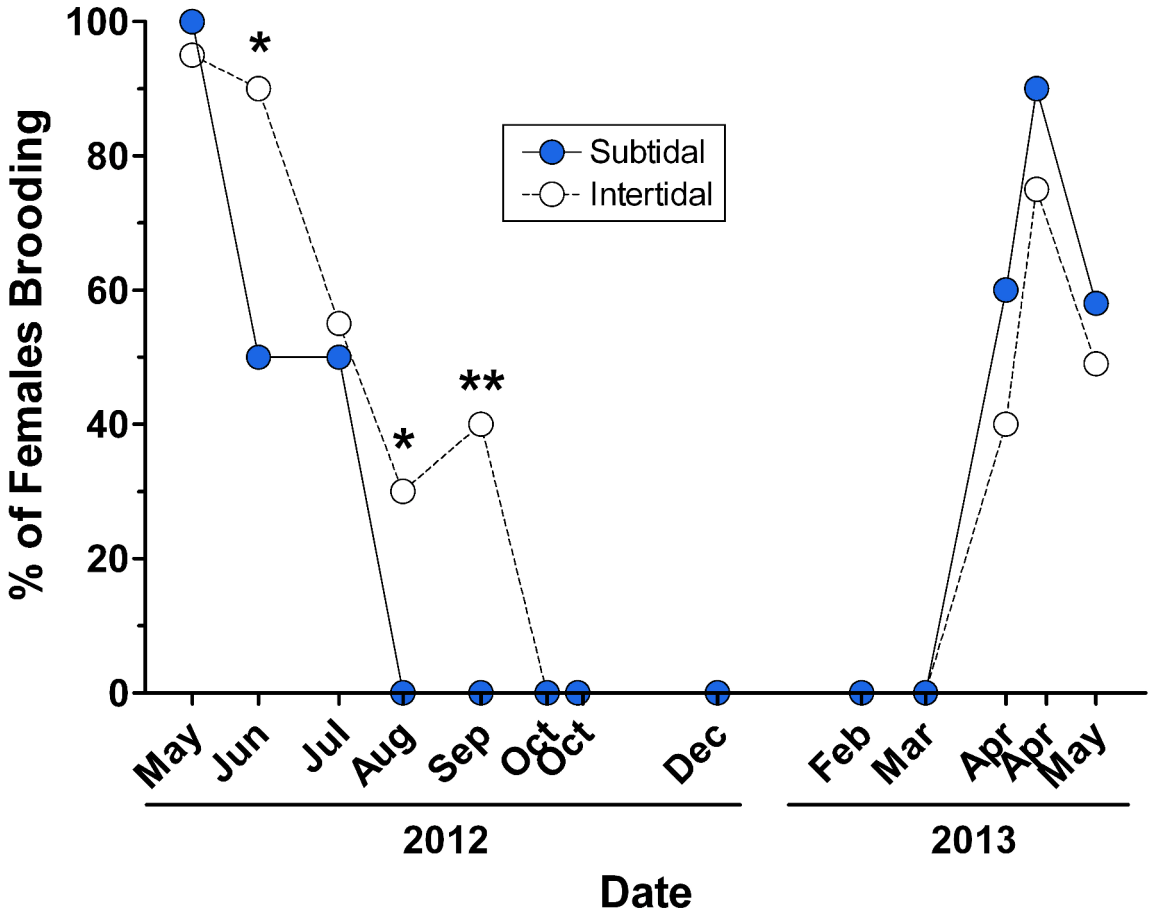

Figure 5.2: Reproductive season of subtidal and intertidal *Crepidula fornicata* at Bissel Cove in Narragansett Bay, Rhode Island. At each time point 20 intertidal and 20 subtidal females (except for May, 2013 where N=53 for each habitat) were removed from their substrate and scored for presence/absence of egg masses. Asterisks indicate significant differences in percent of brooding females at a particular time point (*, $p<0.05$; **, $p<0.01$; Fisher's exact test).

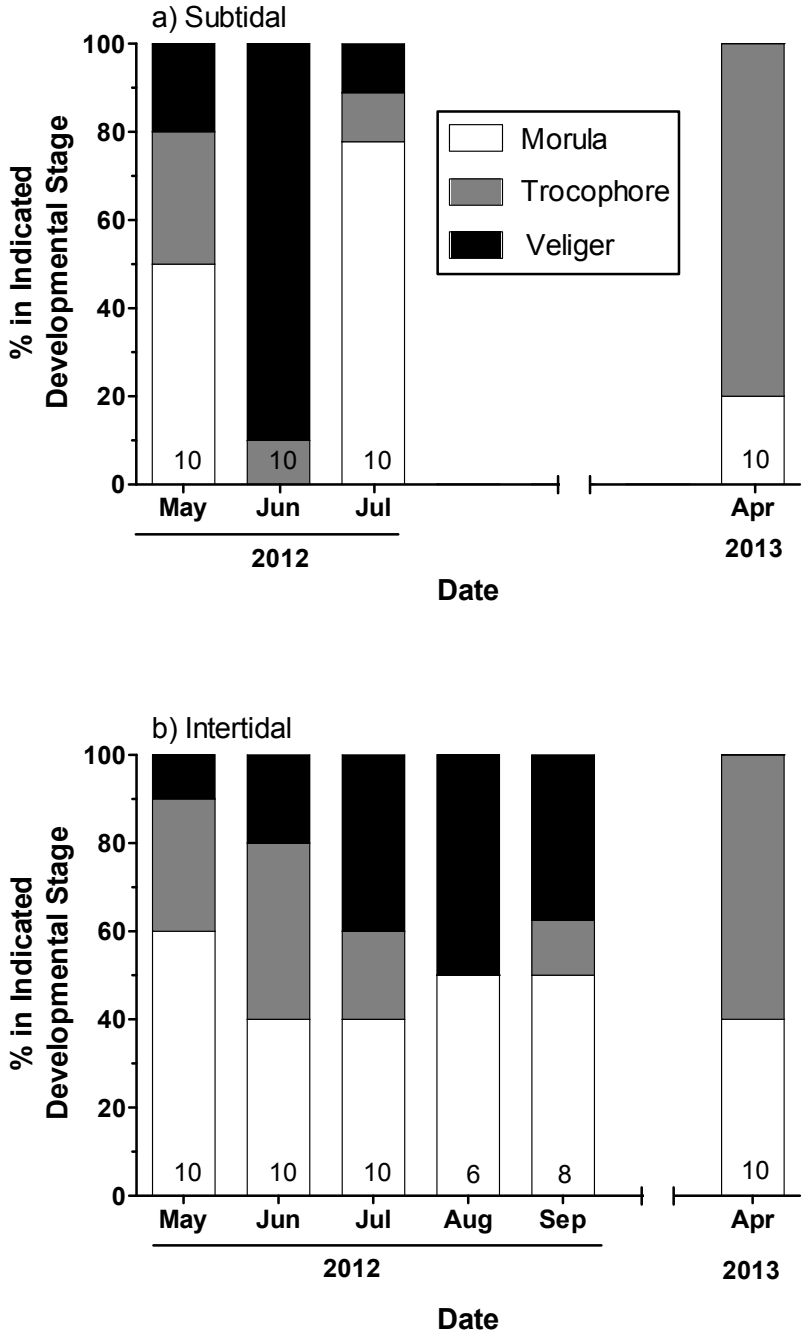

Figure 5.3: Developmental stage of brooded embryos in egg masses of (a) subtidal and (b) intertidal *Crepidula fornicata* at Bissel Cove in Narragansett Bay, Rhode Island. The developmental stage of embryos in each egg mass were determined to be morula (white), trocophore (grey), or veliger (black) using a scale adapted from Chipperfield (1951) and Richard et al. (2006). Developmental stage of all embryos within an egg mass was similar, and thus all embryos in an egg mass were assigned the same developmental stage. Sample size is displayed in each bar.

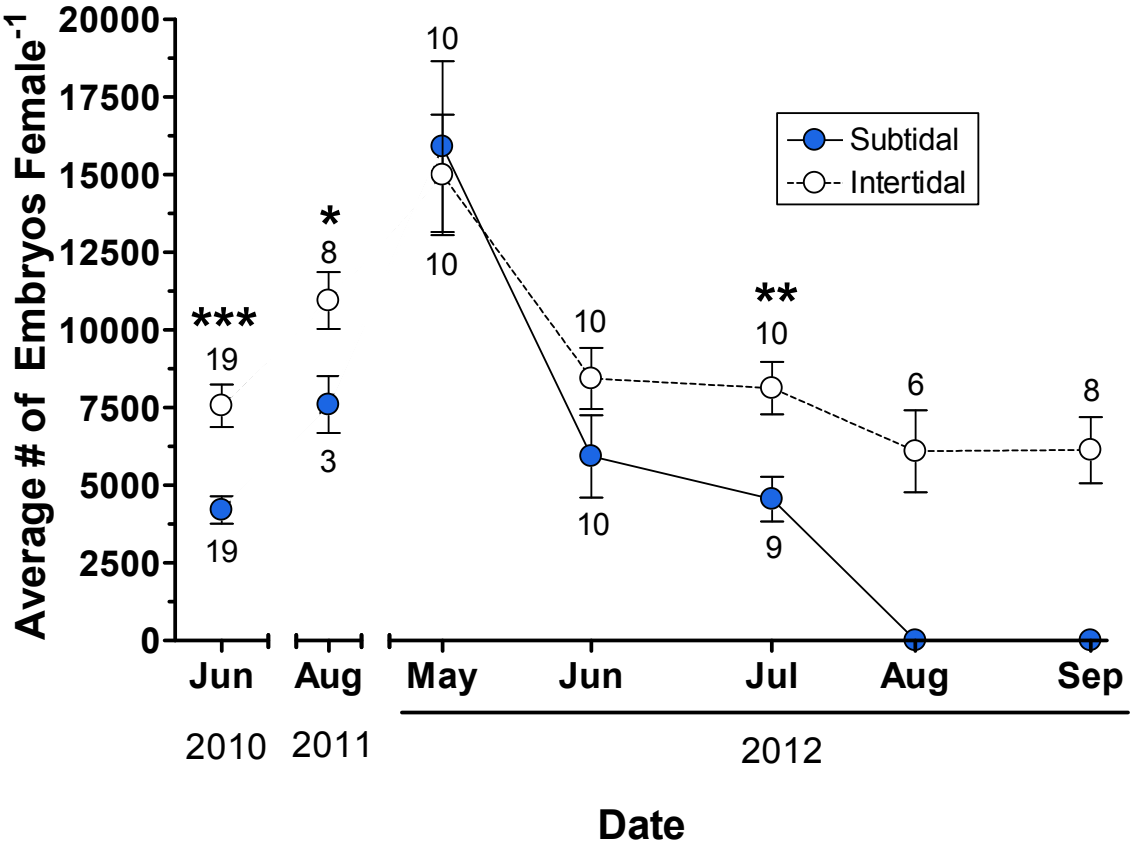

Figure 5.4: Average fecundity (±SEM) of subtidal and intertidal *Crepidula fornicata* from Bissel Cove in Narragansett Bay, Rhode Island. The number of embryos per individual were standardized by first dividing the number of embryos by the shell length of the female and then multiplying by the average female shell length (33.4mm). Sample sizes at each time point for each habitat are indicated above or underneath the symbols. Asterisks indicate significant differences in fecundity at a particular time point (*, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; t-test). Statistical comparisons were not performed for August and September of 2012 because no subtidal females were found brooding on those dates.

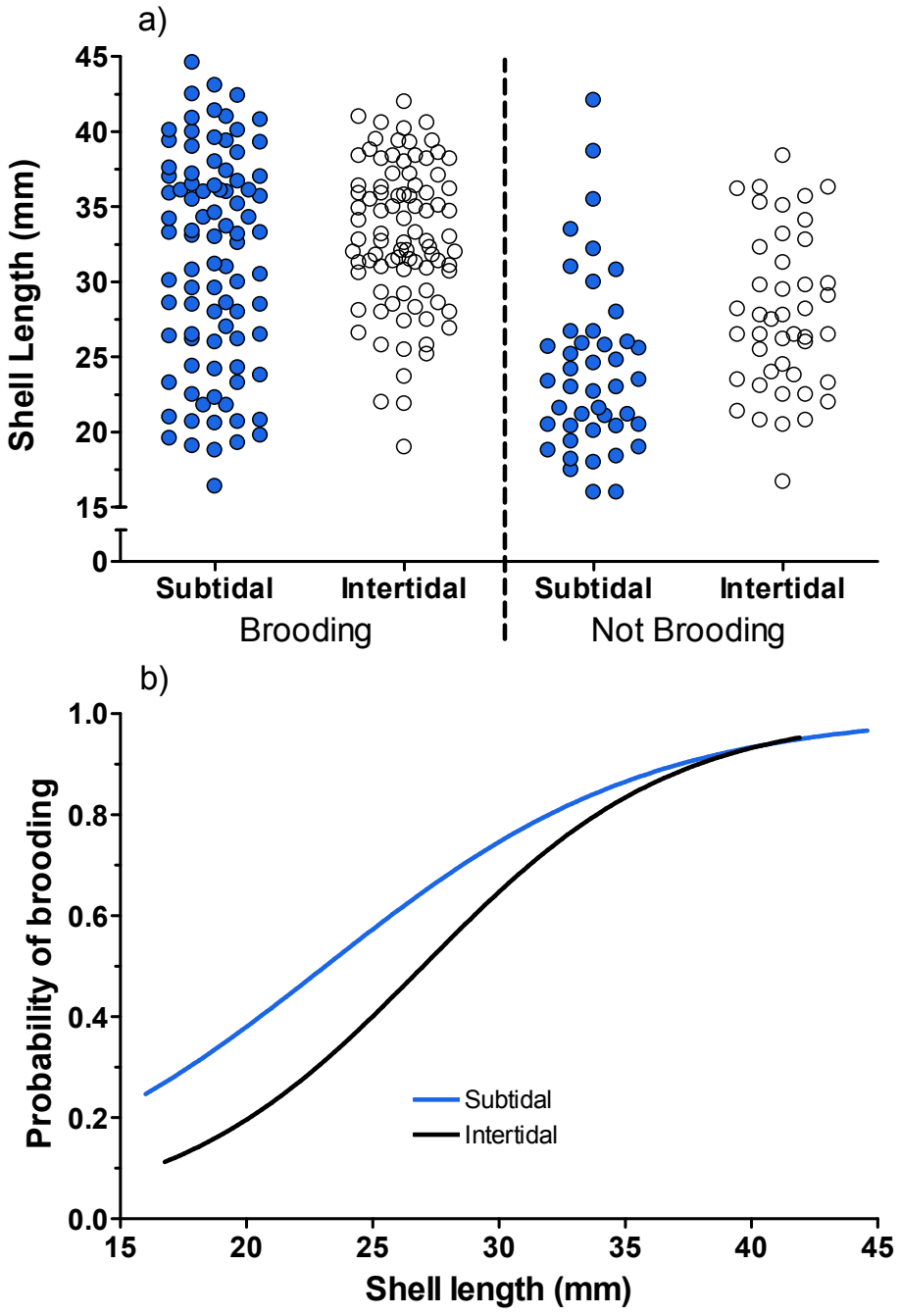

Figure 5.5: Size at reproductive maturity of subtidal and intertidal *Crepidula fornicata* from Bissel Cove in Narragansett Bay, Rhode Island. (a) Size of individuals from the subtidal and intertidal found to be brooding (N=89 for each habitat) or not brooding (N=44 for each habitat) during the reproductive season. (b) Probability of brooding for intertidal and subtidal females, generated from multiple logistic regression; effect of location (intertidal vs. subtidal), Wald χ^2 = 3.04, p = 0.0811.

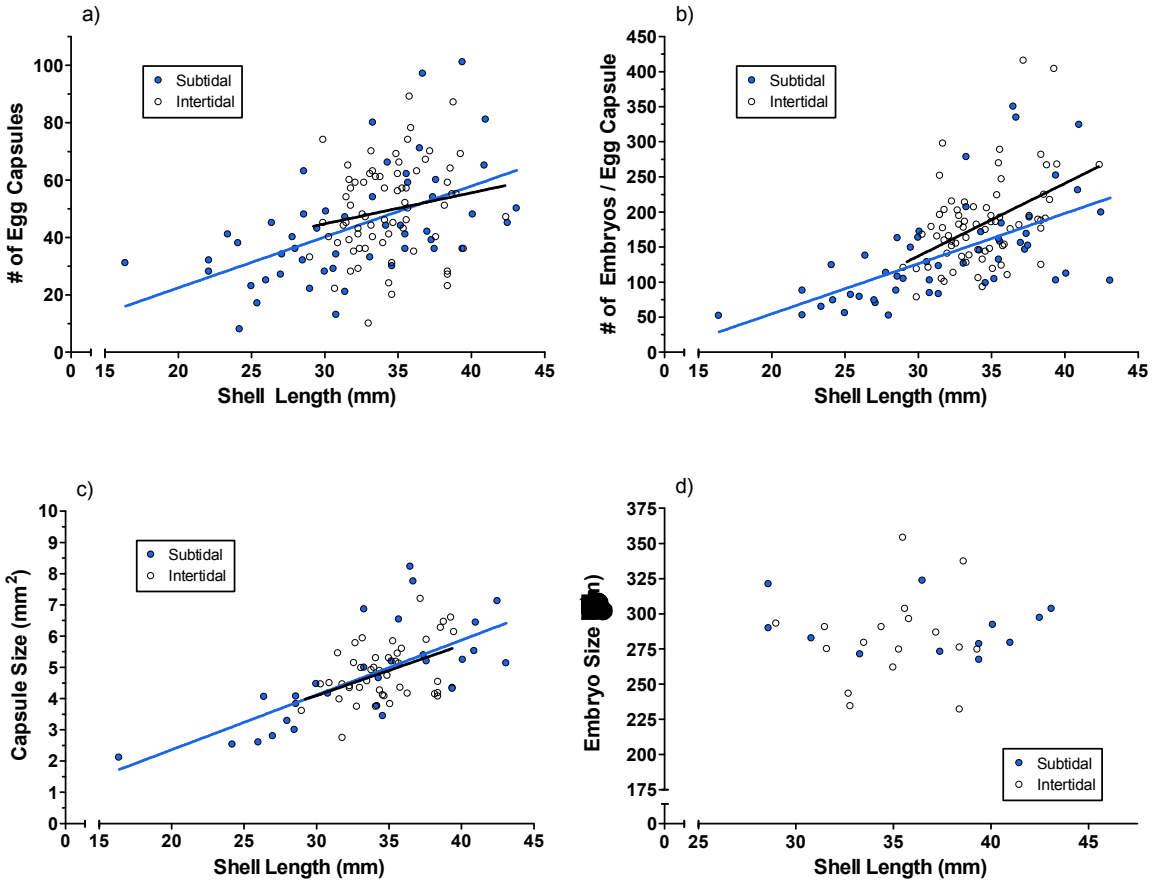

Figure 5.6: Relationships between various measures of reproduction and size of reproductively active females of *Crepidula fornicata* from intertidal and subtidal habitats at Bissel Cove in Narragansett Bay, Rhode Island. After preservation in buffered formalin, individual egg masses were dissected and (a) the total number of egg capsules were counted for each egg mass. (b) For each egg mass the total number of embryos was divided by the total number of egg capsules and (c) the area of 5 haphazardly chosen egg capsules was measured (see Methods). (d) For each egg mass containing embryos in the morula stage, 10 haphazardly selected embryos were measured (longest dimension) and averaged. Intercepts of linear regressions for intertidal and subtidal animals are significantly different only in (b), ANCOVA, $F_{1,119} = 4.53$, $p = 0.035$.

Chapter 6

Conclusions

The main goal of this dissertation was to study *Crepidula fornicata* at its internal range boundary, the intertidal zone, in order to gain a better understanding of some of the basic features of its biology and ecology. In particular, how do certain intertidal stressors – high temperature, desiccation, and food limitation – affect this species, and are there reproductive consequences from living intertidally for *C. fornicata*? Our results suggest that temperature and desiccation stress may be particularly important for limiting the distribution of *C. fornicata* in the intertidal zone, but that the role of food limitation is less clear. Though the distribution of *C. fornicata* ends abruptly in the intertidal zone, our results on the reproductive dynamics of intertidal and subtidal conspecifics suggest that intertidal members of this species are not at any noticeable disadvantage when compared to subtidal conspecifics. Taken together, these results may help investigators predict the future native and invasive distribution of this species, especially in the face of continued climate change.

How do temperature, desiccation, and food limitation affect C. fornicata *in the intertidal zone?*

In our study (Chapter 2), *Crepidula fornicata* that were living in the intertidal zone at Bissel Cove experienced temperatures close to their upper thermal limit. On several occasions, temperatures rose above those that were shown to kill *C. fornicata* in the laboratory. Though our study only assessed the susceptibility of *C. fornicata* adults and embryos to a single three hour thermal stress, the temperatures at which individuals died (approximately >35°C) indicates that high summer temperatures may be particularly important for controlling the upper distribution of this species. Water temperatures at our field site during bouts of high air temperature were at or below approximately 25°C; based on data from artificial "robosnails," heating to lethal temperatures upon aerial exposure may take up to several hours (but could be quicker) depending

on climatic conditions. Because *C. fornicata* at their upper edge in the intertidal zone spend approximately 4 – 6 hours continuously exposed to the air, their tissues may reach temperatures above 35°C and remain there for several hours. Thus, our laboratory tolerance experiments suggest that *C. fornicata* in the intertidal zone may be living very close to their thermal maximum, but that subtidal *C. fornicata* are in no danger of dying from high temperature stress. Interestingly, embryos were no less tolerant of high thermal stress than adults or juveniles, suggesting that there is no "weak link" in the life history of this species as it relates to thermal tolerance, as has been found for many other species (e.g. Crisp and Ritz, 1967; Kinne, 1970; Freitas et al., 2010).

Desiccation stress is also likely to be an important limiting factor for *C. fornicata* in the intertidal zone (Chapter 3). However, unlike for temperature stress, the life-history stage of an individual when it is exposed to the air matters for its resistance to desiccation stress. Embryos are unlikely to lose much water because they are brooded in a manner that limits their exposure to air and larvae spend their time adrift and thus submerged (Conklin, 1897); juveniles and adults, however, can lose tissue water in the air and it is juveniles that were found to be quite susceptible to desiccation stress. Only an exposure of 2 – 3 hours at relatively benign temperatures (~22°C) killed juveniles, but they lost less water as they grew larger. Juveniles are mobile, though, and could potentially seek refuge from desiccation stress while exposed to the air at low tide. Their ability to grow large enough to be able to withstand longer periods of aerial exposure, then, should be contingent on their ability to stay moist when they are very small. Staying moist during low tide while living on a stack of *C. fornicata* is feasible: juveniles could travel to the under (shaded) side of a stack or to moist crevices created by the meeting points of adult *C. fornicata* shells. Additionally, they may position themselves at the shell-margins of large

adults where they may benefit from the small amounts of water that adults expel when they clamp to the substrate (personal observation, C. Diederich). It would be interesting, in future studies, to learn if and exactly how small juveniles cope with desiccation stress in the intertidal zone. Though they may have more behavioral options to avoid desiccation stress than they do thermal stress, our results on the relatively low tolerance of small juveniles to short periods of aerial exposure nevertheless suggest that desiccation is major limiting factor for this species in the intertidal zone.

Since *C. fornicata* are sessile suspension-feeders, living intertidally means a reduction in the overall time available to collect food particles from the water, a situation that could potentially limit how high these organisms can live in the intertidal zone (Chapter 4). It is still unclear, though, if this food limitation is more or less important than temperature and desiccation stress for limiting the upper distribution of this species. We did find that, when compared to subtidal *C. fornicata,* there were major morphological differences in the gills of intertidal *C. fornicata*. The gills of intertidal organisms were relatively heavier, with a greater number of gill filaments and a greater overall surface area than subtidal conspecifics, suggesting that the gill size of *C. fornicata* is under intense selective pressure to be larger or that gill size is a phenotypically plastic trait in this species. Either way, conditions in the intertidal zone appear to be taxing enough to cause changes in the morphology of the gill, an organ used for both food collection and respiration in this species. However, we did not find a significant difference in the food clearance rates of individuals from the two subpopulations. In fact, there was very large individual-to-individual variability in the rate at which individuals of this species collected phytoplankton from the water. Because the gill is a multi-use organ, the gills of intertidal *C. fornicata* may actually be larger for respiratory, not feeding, purposes and the differences that we

found in surface area may not be large enough to cause a meaningful change in clearance rates among members of the two subpopulations. Since clearance rates among individuals collected from intertidal and subtidal habitats were not significantly different, suspended food is abundant in near shore habitats (e.g. Mandelli et al., 1970; Durbin et al., 1975; Longhurst et al., 1995), and maximum attainable size (and reproductive capacity) were not hindered in intertidal individuals, I propose that food limitation is not as important as other factors for limiting the distribution of *C. fornicata* in the intertidal zone.

In the intertidal zone, *C. fornicata* may be limited by particular environmental factors, but intertidal individuals of this species do not seem to be at any noticeable disadvantage when compared to subtidal individuals. For example, intertidal animals had food clearance rates that were comparable to those of subtidal animals, and they also grew to be as large as subtidal individuals; this was not true for intertidal members of a related species, *Crepipatella fecunda*, which appeared to be stressed in the intertidal zone (lower clearance rates and food transport rates along the gill) and did not reach the sizes that were attainable by individuals living in the subtidal zone (Chapter 4). Furthermore, there were no noticeable reductions in many measures of reproductive output (e.g. season length, number of embryos) for intertidal *C. fornicata*, though they might start reproduction at a later age (Chapter 5). These results add *C. fornicata* to the growing list of species that don't appear to suffer any fitness consequences when living at their range edge (see Gaston, 2009; Sexton et al., 2009).

Other potentially limiting factors

These limiting factors may not be the only causes for the spatial restriction of *C. fornicata* in the intertidal zone. However, I chose to study high temperature, desiccation, and

food limitation because many of the other factors that are typically associated with limiting species distributions are unlikely to be important for limiting the distribution of *C. fornicata* in the intertidal zone. We actually know very little about the major predators of *C. fornicata*, but sea stars (*Asterias* spp.) seem to be the most important consumer of this species (Hoagland, 1974; personal observation, C. Diederich). These sea stars, though, are rarely found in the intertidal zone in New England (Gaymer et al., 2001; Perez et al., 2009); though they may be important for controlling populations of *C. fornicata* subtidally, *Asterias* spp. should not be a major limiting factor for *C. fornicata* in the intertidal zone. I have also observed the oyster drill, *Urosalpinx cinerea*, attached to – and in the process of drilling holes into – *C. fornicata* on many occasions, and these species do co-exist in the intertidal zone. Additionally, though *U. cinerea* prefer to prey on mussels, they will eat small juvenile *C. fornicata* in the laboratory (Pechenik et al., 2010). However, I have almost never found holes drilled all the way through large empty *C. fornicata* shells, and I have found many *C. fornicata* shells with only partial drill holes (and often several partial drill holes on each shell). Though only an anecdotal observation, this suggests that *U. cinerea* could be food-limited in portions of the intertidal zone, and though they may attempt to eat *C. fornicata*, they do not often succeed in drilling a complete hole through the shells of *C. fornicata* adults. This interaction, though, should be explored more thoroughly in the future. Furthermore, it would be interesting to know if crabs, which have been shown to eat only small juvenile *C. fornicata* in the laboratory, (Pechenik et al., 2010) and birds, which have been observed dropping mussels (but not *C. fornicata*) from several meters high in the air to crack open their shells could be substantial intertidal predators of this species.

Crepidula fornicata is also not likely to be limited in the intertidal zone by substrate availability or competition for space. This is because of their unique tendency to "stack" on top

of one another and remain sessile as adults (Coe, 1936). Their ability to use each other as a place to live makes available substrate plentiful (see Thieltges et al., 2004). Though the lowest member of a stack of *C. fornicata* may be attached to a rock – potentially limiting substrate availability – they may also be attached to empty *C. fornicata* shells or other hard substrates, which should reduce the impact of free rock space as a limiting factor. Furthermore, since *C. fornicata* can live attached to other sessile mollusks but their own shells are almost always free of other large fouling organisms, competition with other organisms for space in the intertidal zone should not be problematic for members of this species. This is probably best demonstrated by the fact that in their invasive range they can exist in remarkably dense assemblages – thousands per square meter (e.g. Blanchard, 1995) – which suggests that, given the right conditions, substrate availability and competition for space are almost never limiting factors for this species.

Competition with other organisms for food also should not limit the distribution of *C. fornicata* in the intertidal zone because they are not usually found in dense assemblages with other suspension-feeders. In the intertidal zone, individuals of *C. fornicata* may be found on or near the occasional oyster, mussel, clam, or other suspension-feeder, but individuals are rarely found living in the dense beds of intertidal filter-feeders (e.g. *Geukensia demissa*) that are more likely to rapidly deplete the water of most of its suspended matter. Even if some *C. fornicata* are found among dense assemblages of other filter-feeders, recent studies have found no evidence of trophic competition between *C. fornicata* and other filter-feeding mollusks (the mussel, *Mytilus edulis* and the oyster, *Crassostrea gigas*; Lesser et al., 1992; de Montaudouin et al., 1999; Thieltges, 2005; Thieltges et al., 2006). Furthermore, near-shore environments are notoriously rich in algae and other suspended food particles (e.g. Mandelli et al., 1970; Durbin et al., 1975; Longhurst et al., 1995), so there should be plenty of food to go around (when the tide is in). One

might think that there would be heavy intraspecific competition for food between members of a stack, but living in a stack has actually been proposed to enhance the food-collecting abilities of *C. fornicata* (see Barille et al., 2006).

There have been very few studies on the parasites of *C. fornicata*, but those that have been performed suggest that parasitism is also unlikely to be a factor in controlling the upper distribution of this species. Trematodes are a major parasite of gastropods, but they are curiously absent altogether in *C. fornicata*, though they infect a host of other species that co-localize with *C. fornicata* (Pechenik et al., 2001). In terms of external parasites, shell-boring sponges (*Cliona celata*) can often be found on the shells of *C. fornicata*, but these sponges have been shown to have almost no effect on individuals of this species (Le Cam and Viard, 2011), and *Cliona celata* are found subtidally (Hartman, 1957) at least as much as they are found intertidally. Finally, I have observed the parasitic snail *Boonea seminuda* at the shell margins of adult *C. fornicata* in both the intertidal and subtidal zones, and a preference of *B. seminuda* for *C. fornicata* over some oysters (*Crassostrea virginica*), clams (*Mercenaria mercenaria*), mussels (*Modiolus modiolus*), scallops (*Aequipecten irradians*), and other gastropods (*C. plana*, *Littorina littorea*) has been documented (Boss and Merrill, 1965; Robertson and Mau-Lastovicka, 1979). Though they are by no means omnipresent on *C. fornicata*, at times they can be common; the physiological effects of *B. seminuda* and potential as an ecological limiter should be further studied in the future.

Finally, it is possible that other abiotic factors could pose a threat to *C. fornicata* in the intertidal zone. Specifically, changes in salinity and/or oxygen availability have been cited as harmful for intertidal organisms (e.g. Brinkhoff et al., 1983; Przeslawski, 2004; Altieri, 2006). During periods of heavy rain, organisms in the intertidal zone (in tide pools) or in shallow water

can experience rapid reductions in salinity to almost fresh water conditions (e.g. Pechenik, 1982; Chaparro et al., 2008). However, *C. fornicata* can clamp to the substrate so that their tissues do not contact waters that are at such low salinity levels, as other calyptraeid gastropods do (Chaparro et al., 2009). Even if their tissues were exposed to water of low salinity, *C. fornicata* appears to be a relatively euryhaline species (Diederich et al., 2011; unpublished data, Samuel Bashevkin) that is already found in estuaries in some areas (e.g. Cole and Baird, 1953; Walne, 1956), so low-salinity stress is unlikely to be a major factor that excludes this species from some intertidal areas. Some organisms cannot effectively utilize aerial oxygen in the intertidal zone, and are thus only found subtidally or must rely on anaerobic metabolism to survive short bouts aerial exposure. *Crepidula fornicata*, though, is not one of these species (Chapter 3; Newell and Kofoed, 1977b); individuals of this species have the ability to aerobically respire in the air, though not at the same rate that they do while submerged. However, our studies on the gill morphology of intertidal and subtidal *C. fornicata* (Chapter 4) could implicate oxygen limitation as a potential factor for limiting the upper distribution of *C. fornicata* in the intertidal zone (see above). Still, in studies of desiccation tolerance (Chapter 3), *C. fornicata* adults were able to survive aerial exposures of at least 10 hours (several hours longer than they would experience at the very upper edge of their intertidal range). The metabolic dynamics (aerobic and anaerobic) of intertidal and subtidal members of this species should be a topic of future research.

Basic biology of Crepidula fornicata: *what else have we learned?*

In addition to investigating the limiting factors and reproductive dynamics of *C. fornicata* in the intertidal zone, this dissertation provides novel information on many basic characteristics of *C. fornicata* in its native range. This is the first (and only) comprehensive study on the thermal

tolerance of this species. In some other experiments designed to test various aspects of larval development and metamorphosis, investigators have found that *C. fornicata* larvae from mothers collected in their native range die after exposure to temperatures above approximately 35ºC, depending on exposure time (Lucas and Costlow, 1979; Pechenik and Eyster, 1989; Gaudette et al., 2001). Since the free-swimming larvae of *C. fornicata* are not likely to be exposed to such high temperatures, we did not assess the thermal tolerance of larvae in our study. However, field-collected embryos and adults from Bissel Cove in Narragansett Bay, RI, and lab-reared juveniles had upper lethal limits that were similar to those previously reported for larvae (Chapter 2). Field-collected embryos were found to be no more susceptible to (in fact, they were slightly more tolerant of) high thermal stress. Finally, there was variability in high thermal tolerance limits both within and among hatches of laboratory-reared juveniles, suggesting that high tolerance limits could be the target of selection in the intertidal zone in future global warming situations.

Additionally, this is the first time that shell lifting behavior has been characterized in this species. The fact that these organisms lift their shells off of the substrate at all (as opposed to just clamping tightly to the substrate for the duration of the aerial exposure period to prevent desiccation) is reason to suggest that *C. fornicata* has had a long evolutionary relationship with the intertidal zone. Most often it is the species that are intimately associated with the intertidal zone (e.g. *Cerastoderma* cockles, *Mytilus* mussels, *Siphonaria* limpets, *Perna* mussels, *Modiolus* mussels, *Cellana* limpets, *Cardium* mussels, *Chiton* chitons) that perform behaviors similar to the lifting behavior of *C. fornicata* ("gaping" in mussels and "mushrooming" in limpets) (e.g. Lent, 1968; Boyden, 1972; Widdows et al., 1979; Garrity, 1984; Widdows and Shick, 1985; McMahon et al., 1991; Hicks and McMahon, 2003; Williams et al., 2005; Dowd and Somero,

2013). Species normally found subtidally or at the very upper fringe of the intertidal zone are more likely to completely isolate their tissues from the outside environment during aerial exposure (McMahon, 1990). The shell lifting behavior that *C. fornicata* exhibits, though, appears to depend upon past experience. Intertidal adults lifted their shells off of the substrate and clamped tightly against the substrate much more frequently than both subtidal adults and lab-reared juveniles. This difference in behavior effectively reduced the amount of time that intertidal adults had their tissues exposed to the air. We also demonstrated the tradeoff between water loss and aerial respiration imposed by shell lifting behavior, and since their behavior in the air replenishes aerial oxygen to the tissues very quickly, the quick lifting and clamping movements of intertidal adults is a good way to conserve tissue water while still oxygenating the mantle cavity. Finally, our results on both the size escape from desiccation and the amount of exposure time that kills individuals of *C. fornicata* (< 3 hours for small individuals, > 10 hours for large individuals) generally agrees with the results of Hoagland (1984), the only other study that has included any investigation of desiccation tolerance in this species.

Of the four data chapters in this dissertation, the chapter concerning the gill morphology and feeding biology of *C. fornicata* is the most previously well-studied. We know quite a bit about how *C. fornicata* collects food, though debate on whether or not these animals use a "mucus net" to feed has only been settled recently (they do not: Shumway et al., 2014). However, the variability in different trait aspects of the gill (size, surface area, number of filaments, length of filaments) that may be a result of plasticity or that – with intense enough selection – may lead to adaptation had not been studied until now. Our results on the clearance rates of *C. fornicata* and *C. fecunda* demonstrate that members of these species have a remarkably high individual-to-individual variability in the rate at which they collect food. This

result, though, has been corroborated by others (Personal communication, S. Shumway, O. Chaparro) and the average clearance rates that we found for both species is similar to those found in other studies of *C. fornicata* in both its native (Shumway et al., 2003; Harke et al., 2011) and invasive range (Newell and Kofoed, 1977a; Newell and Kofoed, 1977b; Barille et al., 2006) and for *C. fecunda* (Navarro and Chaparro, 2002; Mardones et al., 2013) as well.

Our study on the reproductive dynamics of *C. fornicata* is novel for this species in its native range, and provides valuable information that might be compared with studies of the reproduction of *C. fornicata* in portions of its invasive range. Prior mentions of the reproductive dynamics of *C. fornicata* in its native range have either been anecdotal or incomplete (Conklin, 1897; Collin, 1995; Proestou et al., 2008), but here we provide a wealth of information on the length of the brooding season of this species in part of its native range, in addition to the average fecundity per brood, the number of broods per season, the size of adults at reproductive maturity, the number of embryo capsules per egg mass, the number of embryos per capsule, the size of capsules, the size of morula-stage embryos, and the relationship of many of these variables to female size in both intertidal and subtidal individuals, albeit at a single field site. When compared with populations of *C. fornicata* along European coastlines, the reproductive dynamics of *C. fornicata* at Bissel Cove in Narragansett Bay, Rhode Island were similar to those found for more northern populations in the U.K. and Denmark (Chipperfield, 1951; Thieltges et al., 2004; Bohn et al., 2012), but were markedly different (especially in the length of the brooding season) from those found for populations found along the coast of France (Deslous-Paoli, 1985; Richard et al., 2006; Valdizan et al., 2009; Beninger et al., 2010). Though the reproduction of *C. fornicata* is also apparently unhindered at its northernmost range boundary in the U.K. (Bohn et al., 2012), it would be interesting to see if the same is true at the southern range boundaries of

this species, along the coasts of Spain and Italy (Blanchard, 1997) and in the Gulf of Mexico (Collin, 2001).

Future distributional patterns

From a latitudinal standpoint, *C. fornicata* already occupies a wide thermal niche, so increasing global temperatures are not likely to affect this species the way they have others (Parmesan and Yohe, 2003; Bellard et al., 2012). That is, based on temperature alone, since our experiments showed a relatively high thermal tolerance for *C. fornicata* (>35°C) even at more northern latitudes, potentially more thermally-tolerant populations of this species in the south are unlikely to be displaced by water temperatures that rarely reach only ~32°C. However, the future amelioration of low winter temperatures at northern latitudes may allow for an extension of the range of *C. fornicata*, particularly in its invasive range (Thieltges et al., 2004; Bohn et al., 2012). Additionally, warmer temperatures are most-likely the cause for extended brooding periods and the large adult sizes of *C. fornicata* along French coastlines (Valdizan et al., 2011), so more northern populations of this species could see large population booms in the future. From an internal range boundary standpoint, however, individuals of *C. fornicata* appear to be rapidly killed by high summer temperatures in the intertidal zone. Though the populations that I have observed seemed relatively healthy and showed enhanced reproduction at the range edge, those summer air temperatures should only get hotter in the years to come, which may force this species out of the intertidal zone altogether. Their relatively high thermal tolerance (in cooler ocean waters) of individuals of this species, though, coupled with their resistance to desiccation beyond the young juvenile stage, their ability to collect a vast amount of food from the water in short periods of time, their ability to respire in the air, and their high reproductive output will

ensure that this species will not only continue to thrive subtidally, but will probably expand its range at northern latitudes, either from niche expansion (e.g. Chapter 4), or colonization of new locations following unintended introductions.

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