Genomic and physiological adaptation to temperature in the invasive golden star tunicate (*Botryllus schlosseri*)

by

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ABSTRACT

Because non-indigenous species (NIS) often encounter novel environments during colonization and expansion, species invasions present useful opportunities to investigate the mode and pace of adaptive change in natural populations. In this dissertation, I use the range expansion of the invasive golden star tunicate, *Botryllus schlosseri*, as a natural experiment to study how a pernicious NIS adapts its thermal physiology on contemporary time scales. In Chapter 2, I applied low-coverage whole genome sequencing (lcWGS) to investigate patterns of population genetic structure and signatures of local adaptation to temperature. In addition to illustrating the potential for rapid adaptation of thermal tolerance at the genomic level, this chapter demonstrated that the molecular basis of thermal adaptation on either coast is distinct, providing valuable evidence for parallel adaptation being driven by divergent molecular means. In Chapter 3, I performed a physiological study to investigate differentiation of post-larval heat tolerance across five populations across a major biogeographic break on the east coast of North America. I found that northern populations are more susceptible to heat stress than their southern, warm-exposed counterparts, providing evidence for adaptive shifts of thermal tolerance. Further, by taking advantage of natural temporal variability in temperature, I demonstrated that temperature during development positively affects heat tolerance at later life stages, establishing developmental plasticity of thermal tolerance. In Chapter 4, I extended my physiological investigation to the west coast of North America, comparing post-larval heat tolerance across three populations spanning a 24.3° latitudinal gradient while expanding to include differentiation of cold tolerance in adults. Similar to the east coast, I observed that the two northern populations were more susceptible to heat stress than their southern counterpart. For cold tolerance, I observed a pattern of countergradient variation whereby northern populations were better able to maintain cardiac function in the cold than southern populations. This suggests compensatory genetic adaptation to the colder water temperatures at higher latitudes. Overall, my work furthers our understanding of how NIS are able to rapidly shift their thermal physiology in response to novel environments, shedding light on the potential of species more generally to adapt to environmental change on contemporary timescales.

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TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF TABLES

LIST OF SUPPLEMENTAL MATERIAL

I

INTRODUCTION

To the passing eye, the seemingly endless expanse of blue-green ocean conveys monotony, a uniformity of conditions, a great homogenous soup. But this illusion betrays a much more exciting, dynamic reality. The ocean is replete with heterogeneity. From rocky, wave-exposed coastlines to silty and placid back bays, from the cold, nutrient-rich waters of upwelling zones to the warm, oligotrophic waters of the tropics, the ocean is a realm full of rich variation. Both across space and through time, conditions in the sea are constantly in flux. And it is this diversity of environments that has shaped the great diversity of lifeforms and lifestyles we observe in the ocean.

How species respond to environmental heterogeneity is a question central to such diverse scientific disciplines as ecology, evolutionary biology, and physiology. Many species exist over broad spatiotemporal gradients of environmental conditions, gradients that span scales from microns to thousands of kilometers, from seconds to millennia. For example, in the rocky intertidal a sessile organism such as a barnacle or mussel may be submerged in cold waters at high tide and only six hours later face the prospect of desiccation in the dry heat of low tide. This tidal gradient, spanning mere meters in the spatial dimension and hours in the temporal and encompassing variation in temperature, moisture, and the presence of predators, has profound consequences not only for the physiology of individual species (Somero 2002; Tomanek and Helmuth 2002), but also for the ecological structure of the rocky intertidal community, having been the focus of seminal work in the field of marine biology (Connell 1961; Paine 1974). From this example and countless others, it is clear that species face myriad environmental conditions to which they must adapt. Understanding how species meet these challenges is not only fundamental to our understanding of their basic biology, but it is critical to predicting, and potentially shaping, their responses to the profound changes they are facing during an age of great environmental upheaval.

This dissertation is largely concerned with the mechanisms by which organisms are able to adapt to the breadth of environmental conditions they face across geography and through time, and in particular on contemporary timescales. Encompassing both the evolutionary and the physiological, species are equipped with tools to cope with abiotic stressors in their environment. In describing spatiotemporal variation in both genomic and phenotypic

characteristics of a focal species, this dissertation examines these evolutionary and physiological mechanisms in detail, providing insights into how species meet environmental challenges and how they may fare in a rapidly changing ocean.

Local adaptation and phenotypic plasticity

In the context of global change, species are often said to possess three "choices": move, adapt, or die. It is the second of these three to which this dissertation is dedicated. Adaptation, in the broadest sense, refers to "the process of changing to suit different conditions" (Cambridge Dictionary). Naturalists have long been fascinated by intraspecific variation in key phenotypes and how these might constitute adaptations to the local environment. By and large, these adaptations can arise through two modalities, often working in tandem. First, natural selection can drive the evolution of distinct traits that confer advantages upon populations within their immediate environment. Local adaptation, as it is known, implies a genetic, heritable basis to adaptive variation and arises across generations of selection (Kawecki and Ebert 2004). Contrastingly, adaptations can arise through phenotypic plasticity. Phenotypic plasticity refers to the ability of a particular genotype to express multiple phenotypes depending on environment (West-Eberhard 2003). Notably, phenotypic plasticity can have a range of effects upon fitness, being either maladaptive, neutral, or adaptive. Generally, phenotypic plasticity operates within generations (though transgenerational plasticity has indeed been confirmed for numerous taxa and traits) and may be reversible, where, by contrast, phenotypes shaped by local adaptation are constitutive. It should be noted that phenotypic plasticity itself can be considered a phenotype, subject to the process of local adaptation, leading to the expectation that phenotypic plasticity should evolve to become greater in highly variable environments (Bradshaw 1965; Levins 1968).

Understanding the contributions of genetics, through local adaptation, and environment, through phenotypic plasticity, to intraspecific variation in fitness-related phenotypes is a major theme in contemporary evolutionary biology (Merilä and Hendry 2014). Further, there are outstanding questions as to the complex interplay between local adaptation and phenotypic plasticity and how it may shape the evolutionary trajectory of species

(Ghalambor et al. 2007; Crispo 2008; Oostra et al. 2018; Fox et al. 2019). In a more applied vein, disentangling the contributions of genetics and the environment also has major implications for such endeavors such as plant and animal breeding (De Jong and Bijma 2002), evolutionary medicine (Stearns et al. 2010), and biological conservation (Frankham 1999).

Local adaptation and phenotypic plasticity are two critical processes that may mediate species persistence in an era of global change (Merilä and Hendry 2014). Where geographic variation in critical phenotypes such as heat tolerance has a genetic basis, natural dispersal or programs of assisted gene flow may redistribute this variation, potentially buttressing at-risk populations against the threat of a more extreme future (Aitken and Whitlock 2013). For example, one assisted gene flow experiment in the forests of California demonstrated that tree seedlings transplanted from lower elevations, typically characterized by a dry and hot climate, outperformed local seedlings at higher elevations during an anomalously hot regime resembling future climate scenarios (Young et al. 2020). This indicates that human interventions toward repartitioning adaptive variation may be a fruitful approach. Similarly, identifying or breeding the most phenotypically plastic genotypes is often a target of conservation programs, perhaps most notably in corals (Rinkevich 2021; Quigley 2024), with the goal of producing individuals capable of coping with pronounced environmental heterogeneity. Beyond understanding their respective contributions, it is important to consider the interactions between evolutionary adaptation and phenotypic plasticity in order to predict species' responses to global change (Donelson et al. 2019).

In the oceans, local adaptation has classically been assumed to be rare (Sanford and Kelly 2011). The presumption of well-mixed populations due to the prevalence of long-lived, highly dispersive larvae implied high levels of gene flow that was believed to overpower natural selection at local scales. The incessant inflow of maladapted individuals/alleles from outside of a particular selective regime can lead to gene swamping (Lenormand 2002), inhibiting local adaptation. However, over the past several decades advancements in genetics, chemical tracing methods, and physical modeling have improved our understanding of larval dispersal (Levin 2006), contradicting the long-held assumption of widespread connectivity and panmixia. Many examples demonstrate potentially high levels of local retention of larvae (reviewed by Swearer

et al. 2002), shaped by physical processes (Teske et al. 2016) and through biological phenomena such as larval behavior (Paris and Cowen 2004; Morgan et al. 2009). Additionally, recent research has shown that local adaptation is possible even in the face of strong gene flow, often mediated by specific chromosomal architectures that repress recombination among causal genetic loci (Tigano and Friesen 2016). It should be no surprise, then, that several decades of study have revealed numerous examples of local adaptation in the sea (reviewed by Sanford and Kelly 2011). Also, despite the trend toward highly dispersive larvae in marine systems, this is hardly the rule. Many species brood their young internally, oftentimes releasing them as crawling juveniles rather than as gametes or swimming larvae. Others, like many ascidians, possess larvae that are extremely short lived, sometimes on the order of hours, thus having limited dispersal potential (Svane and Young 1989). The subsequent closed nature of the populations of such species renders them prime candidates for developing local adaptation in response to spatial environmental heterogeneity. Provided sufficient standing genetic variation and selection pressure, local adaptation has the potential to evolve rapidly, especially in cases of large effective population sizes (Barrett and Schluter 2008).

The aforementioned example of the sessile marine invertebrate in the rocky intertidal speaks to the pronounced environmental challenges any individual may face in their lifetime. As such, many marine species have evolved phenotypic plasticity as a strategy to cope with variation in abiotic and biotic components of their environment (reviewed by Padilla and Savedo 2013). For example, in the bryozoan *Membranipora membrancea*, the presence of a predator cue induces the development of defensive spines (Harvell 1984). Inducible defenses are a classic type of phenotypic plasticity that is well described in aquatic organisms such as cladocerans (Harvell 1990). More recently, there has been growing attention on phenotypic plasticity's ability to buffer marine species from axes of global change such increasing temperature or decreasing pH (Somero 2010; Reusch 2013). Phenotypic plasticity may be an especially important component of adaptation to not only a hotter or more acidic future ocean, but also one that exhibits more extreme variability (Oliver et al. 2018; Burger et al. 2020).

I should note that while disentangling local adaptation and phenotypic plasticity is a common goal of many evolutionary studies, it is not strictly the goal of this dissertation. Doing

so experimentally often requires a reciprocal transplant approach, which is inadvisable for non-indigenous species (NIS), as will be seen, is the focus of this work, or rearing animals in a common garden for multiple generations, which is not tractable for the species at hand. Instead, this dissertation weaves multiple lines of evidence from both genomic and physiological investigations to describe how local adaptation and phenotypic plasticity *may* rapidly shape intraspecific variation of a critical trait – thermal tolerance.

Thermal biology

> *"Unlike many other variables that concern biologists, temperature is not just a property of life; it is a property of matter. Nothing escapes its control."* (Angilletta, 2009, p. 1)

Of all of the abiotic stressors that species face, perhaps none have effects so pervasive and fundamental as temperature. The effects of temperature upon biological systems are wide-ranging, influencing processes from the rate of enzymatic reactions to the structuring of species' geographic distributions (Hochachka and Somero 2002). And, of course, temperature is among the principal variables that is shifting as part of global climate change. Understanding how species cope with variation in temperature, then, is key to predicting their responses to global change (Chown et al. 2010; Somero 2010, 2012; Sinclair et al. 2016).

Marine ectotherms, which are mostly unable to regulate their body temperature, are especially vulnerable to changes in temperature. However, many of these species have evolved in the presence of pronounced thermal variability, whether across the tidal cycle or throughout their geographic distribution, and thus possess adaptations to cope with heterogeneity in their thermal environment. In relation to their thermal niche breadth, species are often characterized as eurythermal or stenothermal, having a broad breadth or a narrow one, respectively. Eurythermal species are often encountered in highly temporally variable environments, such as in the temperate zone and the intertidal. Stenothermal species, on the other hand, are most often found in stable environments, such as the poles or deep sea. In their ability to maintain homeostasis across a broad range of temperatures, eurytherms may at first glance appear to exhibit limited phenotypic plasticity, i.e., traits tightly correlated to fitness (growth, survival, etc.) show little change with changing temperature. However, this represents

a type of phenotypic plasticity called *phenotypic buffering* (Bradshaw 1965; Reusch 2013), whereby the processes underlying such traits (e.g. gene expression, metabolic rate, etc.) often exhibit pronounced plasticity in service of maintaining said homeostasis. Many marine ectotherms fit this pattern of phenotypic buffering, relying on plasticity in traits at smaller scales of biological organization to cope with thermal variability. For example, in the Pacific oyster, *Crassostrea gigas*, shifts in thermal tolerance coincident with tidal height are accompanied by plastic changes in gene expression of heat-shock proteins (Hamdoun et al. 2003). Eurythermal species, then, are often demonstrated to be highly plastic when investigating processes at lower levels of biological organization (e.g. Buckley and Hofmann 2002; Sokolova and Pörtner 2003; Jayasundara and Somero 2013; Tepolt and Somero 2014).

Thermal plasticity is a critical trait for species persistence in a warming world (Merilä and Hendry 2014; Seebacher et al. 2015). There is a breadth of plastic responses to temperature, or any environmental factor, that can be categorized according to the lag in life history between an environmental cue and the emergence of an altered phenotype. At the broadest scale, transgenerational plasticity refers to a change in phenotype of a descendant, typically F_1 or F_2, caused by the environment experienced by a parent or grandparent. As it relates to thermal tolerance, this has been demonstrated in a variety of systems (e.g. Massamba-N'Siala et al. 2014; Cavieres et al. 2019; Diaz et al. 2021). For example, Rivera et al. (2021) demonstrated in the anemone *Nematostella vectensis* that greater maternal temperatures prior to spawning resulted in significantly higher heat tolerance of larvae. Thermal plasticity also operates extensively within generations. When the environment at an earlier ontogenetic stage affects the thermal physiology of a later life stage, it can be considered developmental plasticity. For example, in the zebrafish (*Danio rerio*), adult fish that were reared at higher temperatures as larvae had a significantly higher critical thermal maximum (CT_{max}) than fish reared at control temperatures (Schaefer and Ryan 2006). Another type of phenotypic plasticity, acclimatory plasticity, often refers to a change in phenotype in response to environmental conditions within a particular life stage, typically adults. Many species gradually become more tolerant of temperature conditions to which they have become accustomed. This response can encompass a breadth of timescales, from seasonal to incredibly

rapid. For example, in insects, rapid cold hardening is a commonly-studied form of plasticity in which individuals become more cold-tolerant after a brief (minutes to hours) pulse of low temperature (Teets et al. 2020). The prevalence of rapid cold hardening and other fast-acting forms of acclimatory plasticity imply that much of acclimatory plasticity can occur over short time scales. Importantly, given that all of these plastic responses differ with respect to the lag between the onset of the environmental cue and the change in phenotype, those with greater lags (i.e. transgenerational plasticity) are more likely to result in mismatches between cue and response environments (Donelan et al. 2020). In an era in which not only environmental temperature means are increasing, but also variability (Salinger 2005), the lack of cue reliability could render some of these plasticity types less adaptive (Bonamour et al. 2019).

While thermal plasticity is clearly an important component of how animals cope with thermal heterogeneity in their environment, phenotypic plasticity alone is unlikely to meet the challenge of global temperature changes (Gunderson and Stillman 2015). In addition to plastic responses, evolution can drive differences in thermal physiology among populations through local adaptation. Because many marine ectotherms are sessile and thus lack the capacity to seek out thermal refugia, they may be especially prone to natural selection on thermal tolerance phenotypes compared to their terrestrial counterparts. Indeed, a meta-analysis by Sasaki et al. (2022) revealed that local adaptation to temperature is more common in the ocean than on land, perhaps due in part to the Bogert effect, which posits that the increased capacity for behavioral thermoregulation on land shields terrestrial species from selection (Bogert 1949). It is now clear from decades of study that local adaptation to temperature is common in marine systems (Sanford and Kelly 2011). Much of our knowledge of local thermal adaptation in the ocean comes from coastal and intertidal species. For example, in the intertidal gastropod *Nucella canaliculata*, a multi-generational common garden experiment revealed a genetic basis for differentiation of thermal tolerance between populations in Oregon and northern California (Kuo and Sanford 2009). Interestingly, rather than following a latitudinal cline, this coincides with a mosaic of temperature variation whereby the Oregon sites are more exposed to heat due to the timing of low tides during mid-day as opposed to at night to the south.

Such manipulative laboratory experiments, ideally across multiple generations, are the gold standard for demonstrating local adaptation to temperature (Kawecki and Ebert 2004). However, these approaches are not tractable for all species, such as those that are long-lived or a difficult to rear in the laboratory. In these circumstances, many empiricists instead acclimate wild-caught individuals to common conditions in an attempt to minimize environmental effects (acclimatory plasticity). This approach makes study of intraspecific differentiation of thermal physiology accessible to a wider range of organisms, not just those that can be reared in culture, better reflecting the diversity of evolutionary trajectories in nature. Molecular methods also enable the study of local adaptation to temperature in a broader swath of organisms. Population genomics approaches that describe variation across the genomes of many individuals from many populations have the power to detect genetic patterns that are indicative of local adaptation (Savolainen et al. 2013; Hoban et al. 2016). Beyond their ability to demonstrate putative local adaptation to temperature, or any environmental variable, they can also reveal its genetic basis. For example, Benestan et al. (2016) used restriction site-associated DNA sequencing (RAD-seq) in the American lobster (*Homarus americanus*), revealing footprints of local adaptation to temperature among dozens of loci. Some of these loci were within genes putatively involved in adaptation to temperature in other systems, bolstering their role in local adaption in *H. americanus*. This example and others demonstrate how the application of genomic and transcriptomic approaches has the potential to further our understanding of local adaptation to temperature in the oceans, especially when paired directly with laboratory experimentation.

Understanding the pace of adaptive change

As we have seen, phenotypic plasticity and local adaptation can both contribute to shifts in thermal physiology that increase fitness. However, there are open questions as to whether these two adaptive modalities will operate fast enough to meet the pace of ongoing environmental change (Duputié et al. 2015; Martin et al. 2023). While phenotypic plasticity has the potential to be fast-acting, questions about the magnitude of the adaptive response (Gunderson and Stillman 2015) and concerns about cue reliability persist (Bonamour et al.

2019). As for local adaptation, conventional wisdom posits that evolutionary change is glacially slow, inadequate to face contemporary environmental challenges. However, many recent (Reznick et al. 1990; Stuart et al. 2014; Reid et al. 2016; Campbell-Staton et al. 2017), and, indeed, some not so recent (e.g. Bumpus 1899), investigations have revealed the rapid pace at which natural selection can operate in natural populations, driving local adaptation. Still, questions remain as to whether adaptive evolution will "keep pace" with the rate of environmental change.

Understanding the magnitude and pace of adaptive evolution in response to global change is a growing theme within evolutionary biology. While evolutionary adaptation can proceed rapidly, it is often difficult to observe "in action." As such, the field has come to rely on the concept of space-for-time substitution, whereby the potential for temporal evolutionary shifts is inferred by examining putatively adaptive differentiation of phenotypes across spatial climatic gradients (Lovell et al. 2023). While an elegant and potentially useful method, there have been some questions as to whether spatial differentiation can be predictive of future temporal shifts (Damgaard 2019; Perret et al. 2024). As an alternative, experimental evolution approaches that directly apply a selective pressure and measure how traits evolve can be an especially powerful approach. Experimental evolution experiments have contributed to our understanding of the pace of evolutionary change and the conditions under which rapid adaptation is possible (Kawecki et al. 2012). However, it is only feasible for species with short generation times and that are readily kept in a laboratory or mesocosm, limiting the taxonomic breadth to which it can be applied.

Range expansions and species invasions present useful "natural experiments" to investigate the pace of adaptive evolution in wild populations (Huey et al. 2005; Sax et al. 2007; Westley 2011). At the leading edge of a range expansion or during the introduction of a NIS, colonizing individuals are often presented with novel environmental conditions to which they must adapt (Prentis et al. 2008). Provided sufficient records documenting the spread of these species, investigators can put temporal bounds around evolutionary changes they observe among populations, informing us of the pace of adaptation.

Biological invasions

Species invasions are a major facet of global environmental change (Vitousek et al. 1996, IPCC 2022). The introduction of NIS can have a profoundly destabilizing effect on native ecosystems and is among the leading causes of species extinction (Clavero and García-Berthou 2005). In addition to their often-severe ecological consequences, NIS can exert serious negative societal impacts, including to economies and human health (Pimentel et al. 2005; Pyšek and Richardson 2010). Invasion biology as a field has long focused on the ecological causes and consequences of species introductions (Elton 1958; but see Baker and Stebbins 1965), but increasingly the importance of evolutionary processes in meditating invasions has become clearer (Prentis et al. 2008). The microevolutionary forces of migration, genetic drift, and natural selection all play important roles in the establishment and spread of NIS (Lee 2002). Further, the application of evolutionary genetic approaches to the study of NIS can help to elucidate sources and vectors of introduction, an important consideration for the management of NIS (Cristescu 2015). An evolutionary lens on species invasion can not only inform us of the importance of evolutionary processes in biological invasions, but also of the pace at which evolution can proceed more generally (Westley 2011).

Research in recent decades have demonstrated the importance of adaptive evolution in the success of NIS (Maron et al. 2004; Lavergne and Molofsky 2007; Sultan et al. 2013; Turner et al. 2014). By encountering novel conditions during introduction and expansion, NIS often experience strong selective gradients. While these encounters are also likely to be mediated via phenotypic plasticity (see below), local adaptation can be critical to invasion success in these new environments (Oduor et al. 2016). For example, Sultan et al. (2013) used a resurrection approach to document rapid adaptive shifts in the invasive tufted knotweed (*Polygonum cespitosum*), with the evolution of increased reproductive output in its invasive range relative to its native range in Asia in just eleven years. This example and others like it demonstrate the rapid pace at which adaptation can proceed, serving as potent indicator for the likelihood of species more generally to adapt to novel environmental conditions under global change. Rapid evolutionary adaptation often relies on the presence of standing genetic variation upon which natural selection can act (Barrett and Schluter 2008). Characterizing the sources and extent of

genetic variation, then, is critical to informing our understanding of how rapid adaptation can proceed. Using species invasions as natural experiments can inform us of the pace of adaptive evolutionary change and the conditions under which it may be possible.

The role of phenotypic plasticity in species invasions has been a topic of consideration for invasions biologists almost as old as the field itself (Baker 1965). Broad environmental tolerances (potentially driven through phenotypic plasticity *via* phenotypic buffering) have long been hypothesized to be one of the primary characteristics shaping the invasiveness of successful NIS (Baker 1965). Since these early ideas of how phenotypic plasticity might shape invasion success, the role of phenotypic plasticity in species invasions has grown into a mature subfield in its own right, especially with regard to the study of invasive plants (Richards et al. 2006; Chown et al. 2007; Hulme 2008; Dyer et al. 2010). A meta-analysis by Davidson et al. (2011) demonstrated that invasive plants possess greater levels of phenotypic plasticity than closely related native counterparts, bolstering the role for plasticity in species invasions (but see Palacio-López and Gianoli 2011 for a countervailing view). In addition to its importance for the establishment of NIS in new environments, because phenotypic plasticity is itself a trait subject to natural selection, the potential for evolution of phenotypic plasticity in species introduced ranges has piqued the interest of evolutionary biologists and invasion biologists alike (Matesanz et al. 2010; Lande 2015). Local adaptation of phenotypic plasticity may thus be an important factor shaping the evolution and success of NIS, but one with only minimal empirical support.

Temperature is a major environmental axis along which NIS must adapt, and therefore thermal physiology can play an important role in species invasions (Kelley 2014). There are some indications that, like for other abiotic tolerance traits, invasive species exhibit broader thermal tolerance than native counterparts, particularly at warm temperatures (Slabber et al. 2007; Godoy et al. 2011; Zerebecki and Sorte 2011; Bates et al. 2013). Climate warming, then, may make ecosystems predisposed to invasion by NIS (Stachowicz et al. 2002; Rahel and Olden 2008; Walther et al. 2009; Sorte et al. 2010). In addition to potentially possessing high intrinsic thermal tolerances, thermal tolerance can evolve during species invasions. For example, in the cane toad (*Rhinella marina*), northern populations within its invasive range in Florida have

evolved greater cold tolerance than warmer populations further south. This has occurred since its introduction to Florida in 1955 (Easteal 1981), speaking to the rapid pace at which evolutionary adaptation can shape thermal physiology in an NIS.

Like any other realm, the oceans and coasts are susceptible to species invasions. The marine environment is replete with examples of NIS. Notable examples include the lionfish (*Pterois volitans*), a fish of Indo-Pacific origin that has invaded much of the Caribbean (Morris Jr et al. 2009), and *Caulerpa taxifolia*, an invasive alga that has established in the Mediterranean where it competes with native flora (Klein and Verlaque 2008). While noted in early work on NIS (Elton 1958), research on NIS in marine systems has, until recently, mostly lagged behind investigations in terrestrial and aquatic realms (Carlton 1989; Ruiz et al. 1997). Given that shipping accounts for the majority of species introductions in the oceans (Ruiz et al. 2000), humans have been redistributing marine biota for as long as they have taken to the oceans. Further, because we historically lack reliable baseline information on marine communities, it is often difficult to discern which species are native and which are invasive at any given locale (Darling and Carlton 2018). When a species' invasive status is unresolved, either globally or in a particular region, it is referred to as cryptogenic. Cryptogenesis is especially common in the oceans (Darling and Carlton 2018), which can complicate the study of adaptation in marine NIS. While many studies compare phenotypes between the native and invasive ranges of NIS to make inferences about the role of adaptation in invasion success (Prentis et al. 2008), focusing on how traits vary within the known invasive range can also inform us of evolutionary adaptation's role in invasion and range expansion, bypassing the complications stemming from cryptogenesis.

Notwithstanding the issues related to resolving whether a species is invasive or not, species invasions in the oceans, like on land and in aquatic systems, present excellent case studies for investigating how adaptive evolution and phenotypic plasticity can response to environmental changes on contemporary time scales. Marine NIS often exist over broad gradients in temperature, across which they must adapt their physiology either evolutionarily or through phenotypic plasticity. Further, given that many marine NIS are sessile, especially those within the fouling community, they have limited capacity for thermoregulation and are

thus subject to severe environmental selection for thermal tolerance, potentiating the emergence of local adaptation.

Ascidians – biofouling tunicates

Ascidians are a class of sessile tunicates that are commonly encountered in nearshore benthic environments but are especially prolific in fouling communities on marine infrastructure such as docks, bulkheads, and ship hulls (Lambert 2005). Many are invasive and have broad global distributions, spanning ocean basins and continents (Lins et al. 2018). Ascidians, like all tunicates, are chordates and are the closest living relatives to all extant vertebrates. The typical adult ascidian body plan consists of an oral siphon, through which it takes in seawater, and an atrial or exhalant siphon, where water is expelled. Ascidians are filter-feeders, taking in plankton and other suspended particles (detritus) from the water column. Like many other marine invertebrates, ascidians have a biphasic life cycle. As adults, they exist on the benthos. Their larvae, however, are planktonic. These larvae, called tadpoles, are non-feeding and typically have short larval durations, on the order of minutes to a few days, after which they settle. This means that ascidians generally have very limited natural dispersal potential (Svane and Young 1989). Most long-distance dispersal can be attributed to translocation of adults by humans or by rafting on drifting material (Worcester 1994; Lins et al. 2018). Ascidians can be solitary (e.g. *Ciona intestinalis*) or colonial (e.g. *Didemnum vexillum*), and both growth forms are highly represented in the ranks of marine NIS.

Invasive ascidians are often a dominant component of fouling communities and are well-represented in the literature on marine NIS (Zhan et al. 2015). They have a variety of ecological and economic impacts (Lambert 2001; Aldred and Clare 2014) and are especially well-known as nuisance species in mariculture (Carman et al. 2010). Adams et al. (2011) found that control of biofouling, to which ascidians make an outsized contribution, can comprise upwards of 20% of operating costs of marine molluscan aquaculture. Beyond impacts to local economies, ascidians can have devastating impact on native ecosystems (Lambert 2001). For example, the colonial tunicate *Didemnum vexillum* is an especially problematic species that

outcompetes native species for space on the benthos and can overgrow other organisms (McKenzie et al. 2017).

Like all marine NIS, management of invasive ascidians often relies on early detection. Several jurisdictions have launched programs to monitor the establishment and spread of marine NIS. Proactive solutions to mitigate further introduction of ascidians, however, have lagged behind. Existing programs to stymie marine NIS have focused on ballast water management, which, given that ascidians are primarily translocated via hull fouling and aquaculture transfers, may have limited impact. Some nations have established programs of hull husbandry to limit the introduction of fouling NIS (Zabin et al. 2018), though these are few and far between.

Because it is difficult or near impossible to rid out NIS once they are established, an important component of marine NIS management is predicting when and where a potential invader may show up next (Keller et al. 2008; Gallien et al. 2010). For this, it is vital to understand the sources and vectors of introduction (Hulme 2009). Evolutionary genetics can be an important tool for untangling the often-complex invasion histories of marine NIS (Cristescu 2015). Physiological studies are also necessary to describe the abiotic niches of NIS in order to infer the suitability of new habitats to invasion (Lennox et al. 2015). While useful, species distribution modeling of NIS tends to overlook intraspecific variation of environmental tolerances and often ignores the potential for niches to shift through evolutionary adaptation (but see Compton et al. 2010; Fraimout and Monnet 2018). Given that NIS are often capable of evolving during the course of invasion, it is important to consider how evolutionary shifts may mediate expansion of NIS into previously uninhabitable climes.

Ascidians as a whole are an excellent taxon in which to study the ecological and evolutionary dynamics mediating invasion success (Zhan et al. 2015). Several species (e.g. *C. intestinalis* and *Botryllus schlosseri*) are used as model systems in evolutionary developmental biology and thus have well-characterized organismal biology, widely available culturing techniques, and fully sequenced reference genomes. Further, given their broad geographic distribution, they exist over broad spatial gradients in temperature, enabling study

of intraspecific variation of thermal tolerance through local adaptation and/or phenotypic plasticity.

Botryllus schlosseri

Botryllus schlosseri Pallas 1766 is a colonial ascidian that has a cosmopolitan distribution, being found in most of the temperate global ocean (Fofonoff et al. 2024). Originally described in Europe, *B. schlosseri* can be found in diverse locales including East Asia, the Mediterranean, the Americas, the Indian subcontinent, Africa, and Oceania (Fofonoff et al. 2024). *Botryllus schlosseri* has a complex global invasion history. Historically, *B. schlosseri* was assumed to be European in origin and invasive throughout the rest of its global distribution. Recent molecular study revealed that *B. schlosseri* is likely a cryptic species complex, with at least five distinct clades, only one of which, clade A, has become globally invasive (Bock et al. 2012). While its status as a cryptic species complex is still under debate (see Reem et al. 2021), here I use *B. schlosseri* to refer solely to clade A, following Brunetti et al. (2020). Despite its presumed origin in Europe, Yund et al. (2015) provided evidence using mitochondrial *COI* data that *B. schlosseri* is also native to the Northwest Atlantic. Others consider *B. schlosseri* to be native to the Northwest Pacific (Lee and Shin 2021). Currently, its invasive status in various parts of the world is far from being resolved, and in many regions it should be considered cryptogenic, following Fofonoff et al. (2024).

In North America, *B. schlosseri* is present on both coasts. Its invasion history on the east coast unclear. Considered cryptogenic in the Atlantic United States and into the Bay of Fundy, in Canada it has recently expanded northward into Nova Scotia (Carver et al. 2006), Prince Edward Island (Ramsay et al. 2008), and Newfoundland (Callahan et al. 2010). Its southern limit along the Atlantic seaboard is somewhat variable, but it can be reliably collected in the environs of Virginia Beach, VA (pers. obs.), and not having been detected in a recent survey to the south in North Carolina (Villalobos et al. 2017). On the west coast, *B. schlosseri*'s invasion history better documented. First noted in San Francisco Bay in 1947 (Carlton 1979), *B. schlosseri* has since spread north and south. To the south, it was common in the waters of San Diego by the 1960's (Lambert and Lambert 1998) and has been reported as far south as La Paz, Baja California Sur,

México (Tovar-Hernández et al. 2014). To the north, *B. schlosseri* spread throughout the Pacific Northwest in the decades after its original detection in San Francisco Bay. More recently, *B. schlosseri* has spread further north into Southeast Alaska, where it was detected in Sitka, its current northern range limit in the Northeast Pacific, in 2001 (Ruiz et al. 2006) and Ketchikan in 2010 (Simkanin et al. 2016; Jurgens et al. 2018). *B. schlosseri* is also present in northern British Columbia, where it was first detected on the island of Haida Gwaii in 2007 (Gartner et al. 2016) and on the mainland in Lax Kw'alaams, near Prince Rupert, in 2018 (T. Therriault, pers. comm.).

Owing to its use as a model system, *B. schlosseri*'s organismal biology is well-characterized (Ben-Hamo and Rinkevich 2021). Like all ascidians, *B. schlosseri* begins its life as a tadpole larva. These swimming larvae settle on the benthos and quickly metamorphose into oozooids, losing their tails. Oozooids soon open their siphons and begin to feed. Being colonial, individuals (zooids) reproduce asexually, adding zooids to the colony through budding (blastogenesis). Blastogenesis occurs synchronously in *B. schlosseri*, whereby all zooids within a colony simultaneously grow new buds. These buds eventually replace their parent zooids through a process called "takeover", whereby adult zooids die back and are resorbed by the colony. The cyclical nature of blastogenesis in this species means there are discrete asexual generations. The blastogenetic cycle is also closely linked to a cycle of sexual reproduction. *Botryllus schlosseri* zooids are protogynous hermaphrodites, whereby the ova develop in advance of sperm, ostensibly to prevent selfing. Fertilization by broadcast sperm occurs when the new asexual generation of oozooid open their siphons. Embryos are then brooded internally, and development is complete shortly before takeover, at which point the embryos are released from the zooid as swimming larvae. Each zooid can produce ~4-8 larvae (Grosberg 1988); multiplied by the hundreds to thousands of zooids within a colony, this can produce many hundreds or even thousands of larvae from a single colony (Milkman 1967).

Botryllus schlosseri has a long history of study, which has been reviewed elsewhere (Manni et al. 2018; Ben-Hamo and Rinkevich 2021). Though far from the first to study *B. schlosseri*, early work by Sabbadin in Venice Lagoon established basic biological characteristics such as its asexual and sexual reproductive cycles, growth phenology, and temperature preferences, which also comprised the first successful attempt at

intergenerational laboratory culture (Sabbadin 1955a, b). He and researchers from his group went on to study the genetics of pigmentation, dynamics of colony fusion and allorecognition, blastogenesis, and a variety of other topics, mostly dealing with its organismal biology. Since these earlier studies, *B. schlosseri* has emerged as a model for understanding the genetics and evolution of allorecognition (Scofield et al. 1982; Voskoboynik et al. 2013b). Importantly, this line of research resulted in a reference genome for the species being released in 2013 (Voskoboynik et al. 2013a). Ecological and evolutionary study of *B. schlosseri* initially lagged behind. Working with a population in Eel Pond in Woods Hole, Grosberg was among the first to spearhead *B. schlosseri*'s use as a model for ecological and evolutionary questions. He demonstrated two different life history morphs, one semelparous and another iteroparous, in this population, and that this variation appears to be at least partly under genetic control (Grosberg 1988). He also established that *B. schlosseri* has limited dispersal potential by demonstrating proximity dependent mating success (Grosberg 1987), hinting at the potential for microgeographic genetic differentiation that was confirmed by others using genetically determined pigmentation morphs and allozymes (Yund and O'Neil 2000). Later study demonstrated phenotypic plasticity for reproductive output among populations of *B. schlosseri* in the Gulf of Maine (Newlon et al. 2003). Notably, there have been many population genetic studies of *B. schlosseri* in various corners of the globe using allozymes, microsatellites, and *cytochrome oxidase c subunit 1* (*COI*) sequences (e.g. Paz et al. 2003; Ben-Shlomo et al. 2006; López-Legentil et al. 2006; Lejeusne et al. 2011; Bock et al. 2012; Yund et al. 2015). These have mostly demonstrated high levels of genetic diversity and pronounced levels of genetic differentiation. More recently, RAD-seq was applied to nine global populations of *B. schlosseri* (Gao et al. 2018, 2022), an important foray into population genomics that suggested local adaptation to a variety of environmental variables.

Interestingly, aside from a handful of studies (Sabbadin 1955a; Brunetti et al. 1980, 1984; McCarthy et al. 2007; Epelbaum et al. 2009; Zerebecki and Sorte 2011), there has been little examination of *B. schlosseri*'s thermal biology. There has been still less work that examines the potential for intraspecific variation of thermal tolerance. Sorte et al. (2011) demonstrated that adult *B. schlosseri* from the east coast of North America (Lynn, MA) exhibited significantly

greater heat tolerance than individuals from the west coast (Bodega Bay, CA), coincident with higher summer water temperatures in Massachusetts compared to California and suggesting a role for local adaptation and/or adaptive phenotypic plasticity. Given *B. schlosseri*'s vast global latitudinal distribution, stretching from the Indian subcontinent (Ali et al. 2009) to Iceland (Ramos-Esplá et al. 2020), it is subject to a broad gradient in water temperature. Aside from the one bicoastal comparison (Sorte et al. 2011), little is known about *B. schlosseri*'s potential for population-level differentiation of thermal tolerance and the potential contributions of local adaptation and phenotypic plasticity.

Thesis Aims

By combining population genomic and ecophysiological methods of inquiry, this thesis aims to evaluate intraspecific variation of thermal tolerance and its genetic and physiological basis. By using a NIS with a well-characterized recent history of northward spread in North America, I will assess the potential for rapid shifts in thermal physiology, possibly mediated by evolutionary adaptation, that may underlie its success at these higher latitudes. Importantly, in querying genomic variation of many hundreds of individuals from across *B. schlosseri*'s North American range, this thesis also contributes to resolving its complicated invasion history in this region. By weaving multiple lines of evidence from genomes to physiology, this thesis comprises a truly integrative body of work that contributes to our understanding of how intraspecific variation of thermal biology can arise on compressed timescales and how it may mediate invasion success.

Thesis Overview

By querying the genomes of many individuals from many populations, population genomic methods can help resolve the demographic history of NIS and uncover signatures of local adaptation, providing evidence for the role of evolutionary adaptation in biological invasions. In Chapter 2, I use a population genomic approach to investigate patterns of genetic differentiation and signatures of local adaptation to temperature across *B. schlosseri*'s North America Distribution. Using low-coverage whole genome sequencing (lcWGS), I describe

genomic variation of hundreds of individuals across twenty-four populations, twelve from each coast of North America. Analyzing millions of single-nucleotide polymorphisms (SNPs), I describe the population structure of this species, uncovering the existence of three main evolutionary lineages within North American waters. Principal component analysis (PCA) revealed hierarchical levels of population structure within each lineage, which allowed me to assess the relationships among populations. A genomic approach facilitated the implementation of genotype-environment association (GEA) analyses to detect signatures of local adaptation to environmental temperature. Working independently in two of the three evolutionary lineages, one from each coast, I detected thousands of SNPs whose allele frequencies were significantly correlated with environmental temperature and may thus be implicated in thermal adaptation. Comparing the SNPs and genes potentially implicated in selection across either coast allowed me to evaluate the potential for parallel adaptation of thermal tolerance. While a large component of genetic diversity was shared between both coasts, the SNPs and genes exhibited signatures of selection were mostly independent, suggesting that the genomic mechanisms underlying adaptation of thermal tolerance on each coast may be independent.

Describing spatial and temporal phenotypic variation is often a useful means for investigating local adaptation and phenotypic plasticity in natural populations. In Chapter 3, I present an ecophysiological investigation of heat tolerance in five populations of *B. schlosseri* spanning 4.4° of latitude along the east coast of North America. I develop a novel approach to measure the lethal temperature of 50% survival (LT_{50}) of *B. schlosseri* post-larvae (oozooids). I found that populations with the Gulf of Maine, which experience appreciably colder summer temperatures, exhibit significantly lower LT_{50}'s than those populations to the south, which experience warmer temperatures. This demonstration of population-level differentiation of heat tolerance suggests this may be adaptive. Further, by repeating these experiments temporally, during which time environmental temperatures were shifting, I was able to assess the influence of short-term temperature history upon oozooid heat tolerance through developmental plasticity. I found that development at higher temperatures results in greater heat tolerance at the oozoid stage, establishing adaptive developmental plasticity. Interestingly,

populations also varied with respect to their degree of developmental plasticity, with sites experiencing more short-term temperature variability exhibiting greater levels of plasticity. This may suggest that phenotypic plasticity is locally adapted across this gradient of temperature variability, an exciting prospect.

Establishing consistent patterns of phenotypic variation across parallel environmental gradients can provide firmer support for adaptation. In Chapter 4, I extend my physiological investigation to the west coast of North America and expand its scope to include differentiation of cold tolerance. I compare heat and cold tolerance across three populations spanning a 24.3° latitudinal gradient. I found that the southernmost site displayed significantly greater heat tolerance than two populations further north, but that there was no differentiation between these two northern populations. I synthesized these heat tolerance data with those from Chapter 3 to assess the relationship between environmental temperature and LT_{50}, demonstrating that shifts in heat tolerance are coincident with differences in water temperature and are likely adaptive. To assess cold tolerance, I developed a novel method for assaying cardiac activity during a cold challenge. I compared cardiac performance across a range of temperatures to assess the potential for differentiation of cold tolerance. This investigation yielded a pattern of increasing cold tolerance with latitude, whereby the northernmost population maintained higher heart rates at 8 °C, followed by an intermediate population, and then by the southernmost population. This pattern is consistent with countergradient adaptation whereby genetic adaptation compensates for prevailing differences in seawater temperature among the sites.

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II

Population genomics of a marine invader reveals limited parallelism during local adaptation to temperature across both coasts of North America

ABSTRACT

Non-indigenous species (NIS) often face novel environmental conditions during establishment and subsequent range expansion. Evolutionary adaptation is often invoked as a mechanism by which NIS can adjust to new habitats during invasion, with many recent studies demonstrating local adaptation among populations in the invasive range of NIS. Biological invasions, then, can serve as powerful natural experiments to study the pace of adaptive evolution in natural populations. When invasions occur simultaneously across repeated environmental gradients, they provide the opportunity to investigate the potential for parallel evolution and its genetic basis. *Botryllus schlosseri* is an invasive colonial tunicate with a cosmopolitan distribution that is found in North America, where it is actively expanding northward along the Pacific and Atlantic coasts. Here we use low-coverage whole genome sequencing (lcWGS) to investigate population structure, local adaptation to temperature, and potential parallelism in genetic basis of thermal adaptation across both coasts of North America. We analyzed genome-scale data from 534 individuals from 24 populations, 12 from each coast, spanning a thermal gradient encompassing over 24˚ of latitude. Analyses of population structure revealed three main evolutionary lineages, one in the Atlantic and two in the Pacific, demonstrating multiple introductions to the west coast of North America. Genotype-environment association (GEA) analysis in two of these lineages, one on each coast, revealed thousands of loci associated with mean annual seawater temperature, suggestive of local thermal adaptation. We found little overlap in the identity of environment-associated SNPs and genes between these two lineages, illustrating the potential for parallel adaptation to temperature via contrasting molecular means. By taking advantage of parallel invasion fronts of *B. schlosseri*, we demonstrate how rapid adaptation to environmental temperature may contribute to invasion success in this pernicious NIS and provide insights into the role of local adaptation in responding to novel environmental conditions on contemporary time scales.

INTRODUCTION

How organisms will respond to changing environmental conditions is a central question in the contemporary study of ecology and evolutionary biology (Parmesan 2006). Specifically, there are questions as to whether the rate of evolutionary adaptation can keep pace with the rapid environmental changes of the Anthropocene (Martin et al. 2023). By encountering novel conditions during colonization and subsequent range expansion, invasions by non-indigenous species (NIS) provide tractable cases for studying the pace of adaptive change in nature (Huey et al. 2005; Sax et al. 2007). Furthermore, species invasions themselves are a major facet of global environmental change (Vitousek et al. 1996). While long dominated by studies examining the ecological causes and consequences of NIS introductions (Elton 1958; Williamson 1996; but see Baker and Stebbins 1965), invasion biology as a field has come to appreciate the importance of evolutionary processes in species invasions (Lee 2002). In particular, the role of natural selection in driving rapid evolutionary adaptation has emerged as a potentially important factor in mediating invasion success (Prentis et al. 2008). Common garden and reciprocal transplant studies of fitness-related traits have revealed local adaptation in the invasive ranges of a number of NIS (e.g. Maron et al. 2004; Colautti and Barrett 2013; Turner et al. 2014; van Boheemen et al. 2019), providing excellent examples of contemporary evolution and attesting to the speed at which local adaptation can arise. Studies of NIS, then, have contributed immensely to our understanding of the pace of evolutionary change and provided clues into the ability of native species to evolve in the face of rapid environmental change (Westley 2011). Genetic investigations of population differentiation and local adaptation in NIS are a logical complement to such experimental studies, as they enable investigation in a broader range of organisms and can shed light on the genetic basis of rapid adaptation in natural populations. Furthermore, the application of population genetics to species invasions can help elucidate invasion pathways, tracing introductions of NIS from their native range and tracking the spread of different lineages where they are invasive (Cristescu 2015). With the ability to resolve often complicated invasion histories and uncover loci that may be involved in adaptation to novel environments, invasion genetics can be a powerful tool for investigating

the biology of NIS and, more broadly, the potential for rapid evolution in a changing world (Bock et al. 2015).

Advances in genomics have revolutionized the field of invasion genetics, affording much greater resolution in characterizing population structure for such purposes as tracing sources of invasion, but especially for uncovering signatures of local adaptation (North et al. 2021). Approaches such as genome scans that search for regions of the genome exhibiting elevated population differentiation or genotype-environment association (GEA) analysis can reveal loci that may be involved in adaptation to distinct environments across an NIS's range (Hoban et al. 2016). For example, in an agricultural pest, the brown marmorated stink bug (*Halyomorpha halys*), population genomic investigation revealed multiple global introductions from its native range in East Asia and a genome scan uncovered signatures of adaptation near genes involved in resistance to insecticides, suggesting that the rapid evolution of insecticide resistance may contribute to its invasion success (Parvizi et al. 2023). While the genomic era has seen the expansion of such studies in certain realms, perhaps most notably in terrestrial agricultural pests (Kirk et al. 2013; Pélissié et al. 2018; Ryan et al. 2019), investigations of marine NIS have lagged behind (but see Bors et al. 2019; Chen et al. 2021; Jaspers et al. 2021; Tepolt et al. 2022 and others).

Biological invasions in the marine realm are widespread and can have devastating impacts on native ecosystems (Ruiz et al. 1999). Marine NIS receiving broad public attention such as the lionfish (*Pterois volitans*) in the Caribbean (Morris Jr et al. 2009) or *Caulerpa taxifolia* in the Mediterranean (Klein and Verlaque 2008) attest to a much broader problem in the world's ocean. Many marine NIS have broad geographic distributions in their invasive ranges and are thus exposed to a wide variety of environmental conditions. While many NIS are presumed to have intrinsically broad environmental tolerances and may thus be physiologically primed for success across broad environmental gradients (Baker 1965), it is become increasingly clear that natural selection can drive intraspecific divergence of fitness-related phenotypes on contemporary timescales (Prentis et al. 2008). While long assumed to be rare in the oceans due to the homogenizing effect of presumed high levels of gene flow, local adaptation is increasingly documented in marine species (reviewed by Sanford and Kelly 2011),

even in the face of gene flow (Butlin et al. 2014; Tigano and Friesen 2016; Barth et al. 2017). Further, high rates of dispersal are far from universal in the ocean, with some sessile species brooding their young or possessing extremely short-lived larvae that can only travel short distances. These taxa may be predisposed to local adaptation due to strong levels of larval retention and thus low gene flow among populations (Sanford and Kelly 2011). The application of genomic methods may be especially fruitful for uncovering the basis of local adaptation in such species. Additionally, the high resolution afforded by these approaches, paired with the potential for strong population genetic differentiation given short dispersal distances, makes them especially ripe for investigating demographic patterns.

Ascidians, a class of tunicates, are an especially prolific taxon of marine NIS that have invaded diverse locales around the globe (Lambert 2001). Invasive ascidians are commonly encountered in biofouling communities, growing on boat hulls, docks, and other marine infrastructure but also on natural substrates. Given their short larval duration (hours to days) (Svane and Young 1989), most long-distance dispersal is attributed to anthropogenic translocation of adults (Lambert 2001) or rafting on natural substrates (Worcester 1994). Invasive ascidians have a variety of impacts on native marine species (reviewed by Aldred and Clare 2014). For example, the invasive colonial tunicate *Didemnum vexillum* outcompetes native species for space on the benthos, often overgrowing them (Bullard et al. 2007). One species of ascidian that has become a particularly pernicious NIS is the golden star tunicate, *Botryllus schlosseri*. Found along most temperate coastlines, *B. schlosseri* is a colonial species that is often a dominant member of fouling communities (Dijkstra et al. 2007). While its native range is unknown, in its invasive range it can be found at lower latitudes in locales such as the Gulf of California (Tovar-Hernández et al. 2014) and the Indian subcontinent (Ali et al. 2009) and further north towards the subarctic in Newfoundland (Callahan et al. 2010) and Iceland (Ramos-Esplá et al. 2020). In North America, *B. schlosseri* occurs on both coasts. On the east coast, while its invasive status is unknown (i.e. cryptogenic) in the United States, it is invasive further north in the Maritime Provinces of Canada and in Newfoundland (Carver et al. 2006). On the west coast, it was first observed in 1947 in San Francisco Bay (Carlton 1979) but has since spread south towards the Gulf of California (Tovar-Hernández et al. 2014) and northwards

into Southeast Alaska (Ruiz et al. 2006; Simkanin et al. 2016; Jurgens et al. 2018). *Botryllus schlosseri* is likely a cryptic species complex (but see Reem et al. 2021), comprised of at least five lineages, only one of which (clade A) has become globally invasive (Bock et al. 2012). We thus use *B. schlosseri* to refer solely to clade A, following Brunetti et al. (2020).

Given its broad latitudinal extent in North America and beyond, *B. schlosseri* is subject to a variety of thermal regimes. Restricted larval dispersal and subsequent low levels of gene flow potentiate the emergence of strong genetic differentiation (Grosberg 1987; Yund and O'Neil 2000; Stoner et al. 2002) and highlight the potential for local adaptation to environmental temperature within its invasive range on contemporary timescales. Fast mutation rates and high levels of genetic diversity among ascidians (Leffler et al. 2012; Tsagkogeorga et al. 2012) further contribute to its strong potential for small-scale population genetic differentiation and rapid local adaptation. We previously demonstrated the potential for local adaptation to temperature in two experimental studies examining latitudinal differentiation of heat and cold tolerance along both coasts of North America (Chapters 3 and 4) (Tobias et al. 2024). However, because these studies used animals collected from the field, we could not rule out environmental effects (i.e. phenotypic plasticity) in shaping intraspecific differences phenotypic response. Genomic data, then, may provide additional evidence for local adaptation to temperature during the invasion of this marine NIS in North America and, importantly, uncover the genetic basis of thermal adaptation. Genomic data should also help resolve the complicated invasion history of *B. schlosseri* by describing patterns of population genetic structure.

Botryllus schlosseri's bicoastal distribution in North America presents a useful opportunity to investigate whether putative local adaptation to environmental temperature has a shared genomic basis on each coast. Its northward expansion along both coasts has generated parallel invasion fronts, and potentially unique evolutionary replicates, across which *B. schlosseri* is exposed to vastly different selective environments with regards to temperature. The extent to which evolution is repeatable is an open question in evolutionary biology, and how evolutionary parallelism scales across levels of biological organization is poorly understood (Bolnick et al. 2018). In some cases, the genomic regions underlying parallel evolutionary

adaptation are highly conserved among replicate population pairs or laboratory lines (e.g. Stern et al. 2022). In others, there is little parallelism at the genetic level (e.g. Therkildsen et al. 2019). The circumstances mediating the degree of observed parallelism at these lower levels of biological organization remain unclear (Bolnick et al. 2018). The parallel northward range expansions of *B. schlosseri* present a rare opportunity to investigate rapid parallel evolution of thermal tolerance and the extent to which its genetic basis is shared or divergent.

Here, we present the results of a population genomics study of *B. schlosseri* comprising the majority of its latitudinal extent in North America. We used low-coverage whole genome sequencing (lcWGS) of 534 individuals from 24 populations, 12 along each coast, to explore patterns of population structure, genetic diversity, and local adaptation to temperature. We find evidence for three main lineages within North America and report that *B. schlosseri* populations exhibit extraordinarily strong levels of genetic differentiation, even allowing us to distinguish populations within a single embayment. As our sequencing method recovers mitochondrial DNA, we also present an analysis of *COI* haplotypes and report new haplotypes for the species, placing our results in the context of prior work on the species throughout its global distribution. We employ GEA methods to investigate potential signatures of local adaptation to temperature in two of the most widespread lineages. Interestingly, we find little overlap between the two lineages in the identity of environment-associated loci or of genes containing or located near environment-associated loci, suggesting parallel adaptation to temperature via contrasting genomic routes.

METHODS

Collections and DNA Extractions

25-40 colonies of *B. schlosseri* were collected from each of 24 sites along the east and west coasts of North America (Figure 2-1, Table 2-1). Colonies were collected by hand from the underside of floating docks at recreational marinas and commercial wharves between August 2019 and September 2022. The short dispersal distances of *B. schlosseri* larvae means neighboring colonies are more prone to be close relatives (Grosberg 1987). To avoid sampling close kin, we spaced out our collections by at least two meters when possible. Approximately 1

cm^2 colony fragments were preserved in 95% ethanol and kept on ice until they could be stored in a -80 °C freezer.

Prior to DNA extraction, individual zooids were dissected out from the overlaying tunic using insulin syringes (Becton Dickinson; Franklin Lakes, NJ, USA; cat. no. 328289). Gut and brooding embryos were excised and discarded. The remaining tissue from 8-10 zooids from the same or adjacent systems was used for DNA extraction. DNA was extracted using a Qiagen DNeasy Blood and Tissue Extraction Kit (Qiagen; Hilden, Germany; cat. no. 69506) per the manufacturer's protocol for tissue extractions with the exception of eluting in 32 μl of elution buffer. DNA yields were measured using a Qubit 3.0 fluorometer (Invitrogen; Waltham, MA, USA; cat. no. Q33216) and a dsDNA Broad Range kit (Invitrogen; cat. no. Q32850).

Library preparation and sequencing

Sequencing libraries were prepared from a total of 540 specimens following Baym et al. (2015) and Therkildsen and Palumbi (2017), with some modifications. All incubation steps were performed using a BioRad T100 Thermal Cycler (BioRad; Hercules, CA, USA; cat. no. 1861096). 1-10 ng of genomic DNA was incubated in 1μl of 10 mM Tris, ph 8.5 with 0.25 μl of TDE1 tagmentation enzyme and 1.25 μl of TD buffer (Illumina; San Diego, CA, USA; cat. no. 20034197) for 5 min at 55 °C. We then performed a two-step PCR to add Illumina sequencing adapters and indexes. First, 1.25 μl of IDT for Illumina Unique Dual Index (Illumina; cat. no. 20027213) and 3.75 μl of KAPA HiFi HotStart Library Amplication Kit (Roche; Basel, Switzerland; cat. no. 7958960001) was added to each reaction. PCR conditions were as follows: 72 °C for 3 min, 98 °C for 2 min 45 sec, eight cycles of 98 °C for 15 sec, 62 °C for 30 sec, and 72 °C for 3 min, and 72 °C for 1 min. Second, 0.5 μl of primer P1 (AATGATACGGCGACCACCGA) and 0.5 μl of primer P2 (CAAGCAGAAGACGGCATACGA), each at 10uM, were added to the reaction along with 8.5 μl of KAPA HiFi. PCR conditions were as follows: 95 °C for 5 min, four cycles of 98 °C for 20 sec, 62 °C for 20 sec, and 72 °C for 2 min, and 72 °C for 2 min. Following PCR, 10 mM Tris, ph 8.5 was added to bring total volume to 30 μl prior to bead cleaning. Reactions were cleaned at a 1:1 ratio using 30 μl of KAPA Pure Beads (Roche; cat. no. KK8002). Beads were washed two times with 80% ethanol while on a magnetic stand. Library DNA was resuspended in 32 μl of 10

mM Tris, ph 8.5. Again, yields were measured using a Qubit 3.0 fluorometer, this time with a dsDNA High Sensitivity kit (Invitrogen, cat. no. Q32854).

Deviating from Baym et al. (2015) and Therkildsen and Palumbi (2017), rather than use magnetic beads for size-selection, we used an electrophoretic method. To concentrate DNA prior to size-selection, equal masses of DNA were combined into a series of pools of less than 400 μl and precipitated by sodium acetate and ethanol. A 0.1 volume of 3 M sodium acetate, ph 5.2 (Thermo Fisher; Waltham, MA, USA; cat. no. R1181) and 2.5 volumes of ice-cold 100% ethanol were added to each pool and mixed thoroughly. Pools were left overnight at -80 °C to precipitate DNA. Pools were then centrifuged at 14,000 rpm for 20 min at 2 °C, supernatant decanted, and washed twice with ice cold 70% ethanol, each followed by a 10 min centrifugation at the aforementioned speed and temperature. Pellets were left to air dry for 5-10 minutes prior to resuspension in 32 μl of 10 mM Tris, ph 8.5. Concentrated pools were quantified with Qubit and a dsDNA assay as described above. Pools were then size-selected using a Blue Pippin (Sage Science; Beverly, MA, USA; cat. no. BLU0001) with a 1.5% agarose gel cassette (Sage Science; cat. no. CDF1510). The Blue Pippin was run in the "range" mode, collecting fragments from 500-8000bp. Resulting fragment size distributions were analyzed using a Fragment Analyzer at the MIT BioMicroCenter. From these traces, pool molarities were determined and pools were combined in equimolar amounts prior to sequencing across six lanes of three NovaSeq 6000 SP flow cells in 150x150bp paired end format. Sequencing was performed by the University of Utah's High-throughput Genomics Center.

Sequence processing, alignment, and genotype likelihood estimation

All bioinformatic processing and most analyses were carried out using the Snakemake workflow manager (Köster and Rahmann 2012) and all code is available online at https://github.com/zactobias44/Botryllus and https://github.com/zactobias44/BotryllusMito. This pipeline is based on that described by Therkildsen and Palumbi (2017). From 540 initial individual libraries, one library (KWLM_3) had no assigned sequencing reads, mostly likely due to misassigned barcodes. Reads from the remaining 539 libraries were trimmed and assessed for quality using Trim Galore! v0.6.5 (Krueger 2015), a wrapper for cutadapt v3.4 (Martin 2011)

and FastQC v0.11.9 (Andrews 2010). Default parameters were used with the exception of an adapter trim stringency of 2 (--stringency 2). Reads were aligned to the *B. schlosseri* reference genome (available at http://botryllus.stanford.edu/botryllusgenome/) using the bowtie2 v2.4.4 aligner (Langmead and Salzberg 2012) with default parameters. One specimen (QUIV_13) had an extremely low mapping rate to the reference (1.53%) and was independently verified to be the related botryllid tunicate *Botrylloides violaceus* (data not shown). It was subsequently dropped from the data set. We used SAMtools v1.13 (Danecek et al. 2021) to filter out reads with mapping quality < 20. Concordant, unpaired, and discordant reads were retained for downstream analyses. We chose to keep unpaired and discordant reads due to the fragmented nature of the reference genome. To avoid artificially inflating sequencing support during downstream single-nucleotide polymorphism (SNP) calling, we soft-clipped overlaps in concordant pairs using bamUtil v1.0.15 (Breese and Liu 2013), retaining the overlapping read with the highest quality. Duplicate reads were removed using MarkDuplicates in Picard v2.26.3 (http://broadinstitute.github.io/picard/). Reads were realigned around indels using GATK's v3.8.1 IndelRealigner (McKenna et al. 2010).

The resulting realigned BAM files were further processed with the software ANGSD v0.933 (Korneliussen et al. 2014) from within a Singularity v3.7 container (Kurtzer et al. 2017) (container available at https://datasets.datalad.org/?dir=/shub/James-S-Santangelo/singularity-recipes/angsd_v0.933/2021-04-01-2e2bcdc0-c273bbe4) to compute genotype likelihoods for the entire data set of 538 individuals. We estimated genotype likelihoods using the GATK method (-GL 2) and the major allele was determined as that most frequent in the data (-doMajorMinor 1). We filtered SNPs by selecting those with data for at least 50% of individuals (-minInd 269), a minimum depth of one per individual (-setMinDepth 269), maximum depth of 3 x the number of individuals (-setMaxDepth 1614) to avoid paralogous regions, a minimum minor allele frequency of 0.05 (-minMaf 0.05), and we retained SNPs with a p-value <0.001 (-SNP_pval 1e-3). We additionally created a counts file to later parse the coverage at each SNP for each sample (-doCounts 1 -dumpCounts 2).

After this initial analysis, four samples (QUIV_26, SABS_2, SABS_7, SABS_23) were found to have low coverage at the SNP level consistent with shallow read depth at the mapping

phase. These four samples were subsequently removed, leaving 534 individuals in the full data set. We then re-ran genotype likelihood and minor allele frequency estimation as described above, with the corresponding filtering thresholds adjusted to reflect the new number of individuals (-minInd 267 -setMinDepth 267 -setMaxDepth 1602). The resulting outputs were used as the basis for the following analyses.

Population genetic structure and differentiation

We first used principal components analysis (PCA) to investigate population genetic structure across the species' North American range. Using the program PCAngsd (Meisner and Albrechtsen 2018), we constructed an individual covariance matrix and then in R v4.1.2 (R Core Team 2013) used the eigen function and the package ggplot2 (Wickham 2016) to create a PCA. Where PCA revealed distinct clusters of populations (see Results), we re-ran ANGSD using just individuals from those sites, re-calling SNPs and computing genotype likelihoods using the same filters as above, adjusting filtering thresholds based on the number of individuals. We then performed additional PCAs on these subsets of the data. This process was performed hierarchically in cases where additional sub-structure was observed.

To calculate pairwise F_{ST} values among the populations, we ran ANGSD on each population separately to generate site allele frequency likelihood (saf) files (-doSaf 1). We additionally used the -sites flag to restrict the analysis to those SNPs identified in the full data set. The saf files were then used to estimate folded, two-dimensional site frequency spectra between all possible pairs of populations using the ANGSD subroutine realSFS. From these spectra the weighted F_{ST} values were estimated using the realSFS fst stats command.
To investigate to potential for isolation by distance (IBD) along each coast of North America, we repeated this analysis independently within two of the main clusters identified by PCA, termed the Pacific Northwest cluster (PNW) and the Northwest Atlantic cluster (NWA) (see Results). We again used the -sites flag to restrict the analysis to those SNPs identified in the hierarchical running of ANGSD for these clusters of populations. We calculated the pairwise geographic distance matrices from the population coordinates using the python v3.7.4 package GeoPy's geodesic function. For analysis of isolation by distance (IBD), we used Slatkin's linearized F_{ST}

($F_{ST}/(1-F_{ST})$) (Slatkin 1995) as our measure of genetic differentiation.

Genetic diversity

To estimate population-level nucleotide diversity (π), we ran ANGSD separately for each population to generate saf files (-doSaf 1). For filtering, we selected all sites with data for at least two individuals (-minInd 2), a minimum total depth of 2 (-midDepth 2), and a maximum depth of three times the number of individuals in the population to avoid paralogous sequences. We then used realSFS to create folded site frequency spectra for each population, followed by realSFS's command saf2theta, and finally thetaStat do_stat to estimate various population genetic parameters. From this output, we estimated π by dividing the global estimate for pairwise θ (tP) by the total number of sites (nSites), following Adams et al. (2023) and https://github.com/nt246/lcwgs-guide-tutorial/blob/main/tutorial4_summary_stats/markdowns/03_diversity.md.

Linkage disequilibrium

Certain downstream analyses require physically unlinked loci. To estimate the degree of linkage disequilibrium (LD) between all sites within the PNW and NWA data sets separately, we used the program ngsLD (Fox et al. 2019). For this, we restricted our analysis only to placed chromosomes in the reference genome (excluding regions whose locations were given as "chrUn", indicating "unplaced"). To reduce computational demand, we restricted LD estimation between sites less than or equal to 50 kb apart (--max_kb_dist 50). We then used ngsLD's included utility script prune_graph.pl to return a list of loci with low levels of LD ($r^2 < 0.5$).

Genotype-environment association analyses

To investigate potential signatures of local adaptation to temperature, we used two genotype-environment association (GEA) analysis approaches: baypass v2.1 (Gautier 2015) and LFMM v1.1 (Frichot et al. 2013). Given the high level of differentiation between the PNW and NWA populations (see Results) and the potential for this to confound detection of selection, we ran these analyses separately for each cluster/region. This additionally allowed us to assess the

potential for (non-)parallelism in the genomic targets of selection between these two regions.

Environmental temperature data were collected from publicly available sources (Table S2-1). All data were recorded from *in situ* instrumentation (shore-deployed sondes, ocean-moored buoys, etc.). In most cases, we obtained data starting from January 1st, 2015, through October 19th, 2023. Data availability/quality constrained this range at some sites. Because publicly available environmental data is lacking for certain regions of British Columbia, Canada, two pairs of sites (KWLM and MSST; NCSL and REED) each share temperature readings from two buoys. From the high-frequency sampling data we calculated four summary metrics: mean number of days per year exceeding 12 °C (the approximate threshold of reproduction for *B. schlosseri*), mean annual temperature, mean winter temperature, and mean summer temperature. We also included site latitude as a potential explanatory factor. Prior to running the GEA analyses, we assessed the level of correlation between our environmental variables, removing those with Pearson coefficients > 0.65.

We converted the minor allele frequency output from ANGSD into baypass format with a custom R script (format_baypass.R, available in the aforementioned GitHub respository). This script also filters loci to select just those with data for at least half of the total individuals per population, following Mérot et al. (2021). To account for population structure, we used the unlinked SNPs to create an omega file from an initial run of baypass without environmental covariates. We then ran a final run of baypass for both the PNW and NWA clusters including the omega file and environmental covariates, centering and scaling covariates by standard deviation (-scalecov). Loci with empirical Bayesian p-values < 0.01 (eBPmc > 2) were considered to be significantly associated with environmental variables.

For LFMM, we similarly reformatted the ANGSD minor allele frequency outputs and again filtered loci to include just those with at least 50% of the total individuals per population (see LFMM.ipynb). We ran LFMM with K = 1, essentially running it without correction for population structure. At higher levels of K, LFMM returned a successively greater number of significant loci (data not shown); thus, selection of K = 1 resulted in a more conservative test. As for baypass, we centered the environmental variables around zero and scaled by standard deviation. Resulting p-values were converted to q-values using the R package qvalue and loci

were considered significant at a false discovery rate (FDR) < 0.1. Using the output of baypass and LFMM, we found their intersect for both the PNW and NWA clusters. Those identified as significant in both methods were classified as environment-associated (EA) SNPs.

Using the list of EA SNPs from each cluster, we identified which genes contain or are near EA SNPs using bedtools v2.28 (Quinlan and Hall 2010). Because the reference genome is partitioned into placed chromosomes (chrs1-13) and an unplaced chromosome (chrUn), along which positionality information is unreliable, we determined EA SNP placement separately for chrs1-13 and chrUn. For chrs1-13, we used bedtools' window function to find all genes that contain EA SNPs or are within 1000 bp of an EA SNP. The gff3 annotation file we used was downloaded from https://aniseed.fr/aniseed/download/download_data. For chrUn, we used bedtools' intersect function to find all genes that contain EA SNPs. For each cluster, we then merged these two lists to create a list of EA genes.

Test of genic enrichment

To test if EA SNPs are preferentially located in or near genes, we implemented a permutation test. First, to find out which EA SNPs were in or near genes, we repeated the same analysis in bedtools as described above for each cluster, but had the program return EA SNPs instead of genes. This returned a list of EA SNPs that were located within genes (for chrs1-13 and chrUn) or within 1000 bp of a gene (for chrs1-13). For the permutation test, we randomly sampled *n* SNPs (with *n* = # EA SNPs) from the list of SNPs included in the GEA analyses for each cluster. We then repeated the bedtools mapping procedure to determine how many randomly sampled SNPs are within or near genes. This was repeated 1,000 times to create pseudo-observed distributions of how many SNPs fall within or near genes for each cluster. For significance testing, we used numpy v1.17.4 (Harris et al. 2020) to calculate the 0.05 or 0.95 quantiles of each distribution to test for enrichment away from or close to genes, respectively.

Gene annotation and functional enrichment

Because the available annotation for the reference genome is somewhat outdated (published 2013), we performed homology-based annotation of predicted transcripts using

diamond v0.9.22 (Buchfink et al. 2015). We queried the transcripts downloaded from http://botryllus.stanford.edu/botryllusgenome/download/ against the NCBI nr protein database (downloaded November 7th, 2023).

For functional annotation, we used an orthology-based approach implemented in eggNOG mapper v2.1.12 (Cantalapiedra et al. 2021). This approach similarly uses diamond (here v2.0.15) to match query sequences to a reference database, but instead uses the reference database of orthologous groups contained in eggNOG v5.0 (Huerta-Cepas et al. 2019). This method returns both gene IDs as well as functional annotations in the form of gene ontology (GO) terms.

To determine which functional categories (GO terms) may be overrepresented among genes containing EA SNPs, we used the tool GO_MWU (https://github.com/z0on/GO_MWU). Unlike other GO term enrichment approaches, which use Fisher exact tests to determine which GO terms are overrepresented among a list of genes below an arbitrarily defined significance threshold, GO_MWU implements a Mann-Whitney U test to find GO terms that are more clustered near the top or bottom of a distribution of a gene-level measure of interest (e.g. log fold change for differential expression analyses). We used the -log10 p-values from baypass (eBPmc) as our measure of interest. To summarize this SNP-level metric to the gene level, we used bedtools intersect to find all genes that contained a SNP analyzed by baypass and assigned that gene the SNP's -log10 p-value. In cases where a gene contained more than one SNP, we used the -log10 p-value of the most significant SNP. We used a one-tailed test (Alternative="g") to test for enrichment of functions at the top of the -log10 p-value distribution. GO terms at a $p_{adj} < 0.05$ were considered significant.

Tests of parallelism

To test whether the same SNPs and/or genes are implicated in putative parallel adaptation to temperature in the NWA and PNW clusters, we asked: are SNPs/genes more or less likely to be shared between clusters than expected due to chance? To assess the true degree of overlap of SNPs/genes between the two clusters, we simply took the intersect in the SNP/gene lists generated in the steps described above. For SNPs, we implemented a

permutation test, similar to that described above. In each cluster we randomly sampled *n* SNPs (with *n* = # EA SNPs) from all SNPs included in the GEA analysis. We then assessed the degree of overlap between the randomly sampled SNP lists. This was repeated 1,000 times to create a pseudo-observed distribution of SNP overlap between NWA and PNW. We again calculated the 0.05 and 0.95 quantiles of this distribution to test for significance of the observed degree of overlap.

For genes, in each cluster we randomly sampled *n* SNPs from the list of all SNPs from chrs1-13 which were known to be within 1000bp of or within a gene (where *n* = # EA SNPs observed to be in/near genes on chrs1-13) and *m* SNPs from the list of all SNPs on chrUn which were known to be within a gene (where *m* = # EA SNPs observed to be in genes on chrUn). We then used bedtools window (for chrs1-13) or intersect (for chrUn) to identify those genes near/containing the random sample of SNPs. From these lists of genes (one for chrs 1-13 and one for chrUn), we then randomly selected *x* genes (where *x* = # of genes with/containing EA SNPs). The union of these two lists (the selection of *x* genes within 1000bp of or containing a randomly drawn SNP on chrs 1-13 or containing a randomly drawn SNP on chrUn) for NWA and PNW were then compared to assess the degree of overlap. This procedure was repeated 1,000 times to create a null distribution of the expected frequency of overlapping genes between PNW and NWA. We took the 0.95 and 0.05 quantiles of this distribution to test if the observed overlap was more or less than expected due to chance.

Variant effect prediction

We used the software snpEff v5.2a (Cingolani et al. 2012) to assess the potential effects of EA SNPs (missense, synonymous, intronic, etc.). We used a custom python script to convert the minor allele frequency files (mafs.gz) from angsd into vcf format. We created two vcfs per PCA cluster: one containing just EA SNPs and another containing all SNPs for that cluster.

Given their potential to change the coding sequence of genes, we focused primarily on missense mutations. For each cluster we identified all missense SNPs and the genes they were contained within. To assess whether these genes may be involved in thermal physiology, we searched their associated GO terms from the eggNOG annotations for the term ID GO:000926

"response to temperature stimulus", which is a parent term to other temperature-related processes ("response to heat", "response to cold", etc.).

To evaluate if our EA SNPs were enriched for non-synonymous mutations, indicative of positive selection, we calculated the ratio of missense to synonymous mutations for each of the two clusters. We again used a permutation approach to determine if this ratio was greater or less than expected due to chance. Using the vcf files for all SNPs in each cluster, we randomly selected *n* SNPs (where *n* = # of EA SNPs). We then calculated the ratio of missense to synonymous mutations. This was repeated 1,000 times to create a pseudo-observed null distribution. We took the 0.95 and 0.05 quantiles of this distribution to test if the observed ratio was greater or less than the null expectation, respectively.

Mitochondrial *COI* haplotypes

The sequencing approach we used also allows for the recovery of near-complete mitochondrial DNA. Because much of the existing population genetic literature focuses on the mitochondrial cytochrome oxidase c subunit 1 (*COI*) (López-Legentil et al. 2006; Lejeusne et al. 2011; Bock et al. 2012; Yund et al. 2015; Nydam et al. 2017), we attempted to recover *COI* haplotypes for each of our samples. Critically, because prior studies conducted more global sampling, analyzing *COI* haplotypes allows us to put our North American samples in a global context. Similar to nuclear data, our trimmed reads were mapped to a reference mitogenome (isolate sc6a-b; NC_021463.1) using bowtie2 v2.4.4, but using less stringent mapping criteria (-D 25 -R 5 -N 1 -L 15 -i S,1,0.50 -p 6 -I 0 -X 1500). We again dropped sample QUIV_13, as this was identified as *Botrylloides violaceus* (data not shown). We followed the steps outlined above for the nuclear data, filtering reads with samtools, removing duplicates with Picard's MarkDuplicates, and realigning around indels with GATK's IndelRealigner. Coverage at each position of the mitogenome was calculated using samtools depth. To retain only samples that had sufficient coverage across the commonly used 524 bp barcoding region (coordinates 145-668), we identified those with a minimum of five reads for each position across the region. For these samples, we used freebayes v.1.3.5 (Garrison and Marth 2012) to detect variants across this region, converted vcfs to fasta format using freebayes' flattenvcf and vcf2fasta tools.

To correctly match our retrieved haplotypes to those previously identified and to include *COI* haplotypes not found in our study, we retrieved all available *COI* sequences for *B. schlosseri* from GenBank on May 18th, 2023. We also retrieved a *COI* haplotype for *B. tyreus* to use an as outgroup. We aligned these sequences using muscle v3.8.31 (Edgar 2004). The resulting alignment was manually trimmed to the 524 bp barcoding region using JalView v2.11.3.2 (Waterhouse et al. 2009), removing sequences that did not span this entire region. We then removed duplicate sequences from this alignment using an online tool (https://arn.ugr.es/srnatoolbox/helper/removedup/). Because *B. schlosseri* is a cryptic species complex (Bock et al. 2012; but see Reem et al. 2021), we sought to retain just reference sequences that belong to the globally invasive clade A. Using the deduplicated alignment, we constructed a pairwise distance tree using JalView and then selected just those sequences that fell into the previously identified clade A. Using the clade A alignment, we used the software PopArt (Leigh and Bryant 2015) to construct a haplotype network using the minimum spanning algorithm. The nodes of this network were distributed to match the arrangement of the network presented by Yund et al. (2015). Pie charts of haplotype proportions at each site were created using Excel and superimposed on maps and edited using Adobe Illustrator.

RESULTS

Principal components analysis (PCA) of population structure

Of the original 540 libraries, 534 were retained for analysis (see Methods). Mean coverage among these individuals was 0.57x (range 0.19-1.21x). The mean number of samples per population was 22.25 (range 17-26) (Table 2-1). Our variant calling pipeline uncovered 1,578,760 SNPs. PCA of these variants revealed three major genetic clusters (Figure 2-2A): all twelve populations from the Northwest Atlantic (NWA), ten populations from the west coast from San Francisco Bay northward to the Gulf of Alaska (the "Pacific Northwest" cluster, PNW), and two populations from Southern California (SoCal). Each of these clusters exhibited further structure when analyzed independently. Variant calling within the NWA cluster revealed 1,402,032 SNPs, a PCA of which illustrates mostly strong genetic differentiation among

populations (Figure 2-2B), but no identifiable substructure. There was some overlap observed, particularly among members of LPCB (eastern Newfoundland) and BREK (southern Maine). Similarly, some individuals from DART (central Nova Scotia) cluster with the majority of individuals from HAWK (Strait of Canso).

We recovered 1,532,066 SNPs for the PNW cluster; PCA revealed strong genetic differentiation and pronounced substructure, with four readily identifiable "sub-clusters" (Figure 2-2C): one containing two sites from Southeast Alaska (ELSN and BHRB, "SEAK" group), a second containing two sites from San Francisco Bay (RICH and SFRA) and one from Oregon (COOS; "ORCA" group), a third containing sites from British Columbia (KWLM, MSST, NCSL, and REED; "BC" group), and a fourth containing individuals from Washington (SEAT).

We proceeded with SNP calling in two of these independent clusters to evaluate if further substructure/differentiation could be observed. For the cluster containing COOS, RICH, and SFRA, we recovered 1,619,913 SNPs and observed clear separation between all three populations (Figure 2-2G), including the two populations within San Francisco Bay (RICH and SFRA). For the British Columbia cluster (1,400,799 SNPs), we recovered two additional subclusters (Figure 2-2F), one containing individuals from sites in northern British Columbia (KWLM and MSST; "NBC" group) and another containing individuals from southern British Columbia (NCSL and REED; "SBC" group). SNPs were then re-called for each of these two clusters (NBC: 1,327,394 SNPs; SBC: 1,383,463 SNPs) and PCA revealed strong separation between REED and NCSL, and less so between KWLM and MSST. (Figure 2-2I-H).

Variant calling within the SoCal cluster returned 2,168,906 SNPs, and a PCA revealed clear differentiation between its two constituent populations (QUIV and SBRB) (Figure 2-2D).

Genetic differentiation (F_{ST}) and genetic diversity

We observed pronounced population genetic differentiation, with a mean pairwise F_{ST} of 0.178 (range 0.033-0.543) (Figure 2-3). Hierarchical clustering of the heatmap revealed three main clusters (Figure 2-3A), mirroring those indicated by PCA (see Figure S2-1 for a heatmap with just NWA and PNW sites). F_{ST} was appreciably higher for populations pairs among clusters than within them (Figure 2-3B). For the two clusters containing more than two populations

(NWA and PNW), we investigated isolation-by-distance (IBD) by plotting linearized F_{ST} ($F_{ST}/(1-F_{ST})$) against geographic distance. Populations within the NWA cluster did not exhibit a pattern of IBD ($R^2 = 0.026$, $p = 0.10$) (Figure 2-3C). Contrastingly, populations in the PNW cluster exhibited a stronger pattern of IBD, with genetic distance increasing with geographic distance ($R^2 = 0.12$, $p = 0.012$) (Figure 2-3D). Four comparisons involving population pairs between the Southeast Alaska cluster (BHRB and ELSN) and the northern British Columbia cluster (KWLM and MSST) did not fit this general pattern, with high levels of genetic differentiation despite close geographic proximity. With these population pairs removed, consistency and significance of IBD increased substantially ($R^2 = 0.48$, $p < 0.001$).

Populations generally displayed high levels of genetic diversity with a mean nucleotide diversity (π) of 0.00771 (range 0.00513-0.00937). However, π was consistently higher in some clusters than others (Figure 2-4). The NWA cluster had the highest diversity (mean π of 0.00889, range 0.00819-0.00937), followed by PNW (mean π of 0.00679, range 0.00644-0.00725), and then SoCal (mean π of 0.00518, range 0.00513-0.00522). Nucleotide diversity did not appear to be affected by the sampling depth at each site (Figure S2-2).

Genotype-environment association analysis

We performed genotype-environment association (GEA) analysis to uncover signatures of putative local adaptation to temperature, analyzing the NWA and PNW clusters separately. All potential environmental variables to be used as covariates (mean number of days per year exceeding 12 °C, mean annual temperature, mean winter temperature, mean summer temperature, and site latitude) were found to be highly correlated with one another ($r > 0.65$) (Figure S2-3). We thus proceeded with mean annual temperature as our environmental covariate.

After filtering loci to include only those with data for at least 50% of individuals within each population, we were left with 1,061,658 SNPs for NWA and 1,178,245 SNPs for PNW. Of these, 600,979 (56.6% and 51.0%, respectively) were shared between the two clusters (Figure 2-5A). LFMM identified 3,091 candidate environmentally associated SNPs for NWA and 9,392 for PNW at an FDR < 0.1. Baypass identified 13,377 candidate environmentally associated SNPs

for NWA and 20,950 for PNW with p-values < 0.01 (eBPmc > 2). We considered a SNP to be putatively environment-associated (EA) if it was below these significance thresholds for both methods, taking their intersect (Figure 2-5B-C). For NWA, this comprised 1,439 EA SNPs and for PNW this constituted 3,937 EA SNPs.

Genic enrichment

For NWA, of the 1,439 EA SNPs, 613 fell in or within 1,000 bp of a gene. For PNW, 1,677 out of 3,937 EA SNP fell in or within 1,000 bp of a gene. We implemented a bootstrap procedure to test if the proportion of EA SNPs within or near genes was greater or less than expected due to chance, indicating that EA SNPs are preferentially found near/within or away from genes, respectively. For both NWA and PNW, the observed value of EA SNPs within or near genes did not exceed the 0.95 quantile of the pseudo-observed distributions, indicating that EA SNPs are not preferentially located in genic regions (Figure S2-4).

Functional enrichment

Our test of functional enrichment among genes containing highly significant EA SNPs revealed enrichment of a handful of GO terms for the NWA and PNW clusters. For NWA, the molecular function GO terms DNA-binding transcription factor activity (p_{adj} = 0.0365) and sequence-specific DNA binding activity (p_{adj} = 0.0365) were significantly enriched. For PNW, the molecular function GO term sodium channel activity (p_{adj} = 0.0479) was the only significantly enriched GO term.

Missense mutations and potential functional consequences

Of the 1,439 EA SNPs in NWA, 136 were in coding regions, of these, 54 resulted in missense mutations and 82 were synonymous, for a ratio of 0.659. Of the 3,937 EA SNPs in PNW, 385 were coding; 100 resulted in missense mutations and 285 were synonymous, for a ratio of 0.351. Our bootstrap test indicates that in NWA the ratio of missense to synonymous mutations is greater than expected due to chance (p = 0.035) (Figure 2-5F). By contrast, in PNW the missense to synonymous mutation was not significantly different from null expectations

(Figure 2-5G) (p = 0.879).

In NWA, none of the genes affected by missense mutations had clear relationships to thermal physiology. In PNW, by contrast, a search for the GO term "response to temperature stimulus" revealed three genes potentially related to temperature stress. Xanthine dehydrogenase, on chromosome 13, contained one missense mutation, resulting in the substitution T247S. A second gene, holocarboxylase synthetase, contained six amino acid-substituting mutations (L8F, L32S, V193L, A206V, P242S, I249M). The last gene, interferon regulatory factor 4-like contained one non-synonymous mutation, resulting in the substitution A176V.

Shared EA variants among clusters

Of the 1,439 EA SNPs in NWA and 3,937 EA SNPs in PNW, three were shared between the two clusters (Figure 2-6A). This degree of observed overlap did not deviate from null expectations (p = 0.967) (Figure 2-6B), indicating that SNPs do not appear likely to be reused during putative parallel adaptation. The three shared EA SNPs are within or near genes encoding carnitine O-acetyltransferase (*CRAT*), lysozyme g, and Notch receptor 2 (*NOTCH2*) (Figure 2-6C). At the gene level (genes containing or within 1000 bp of an EA SNP), there were 769 EA genes in PNW and 325 EA genes in NWA, with 21 EA genes found in both clusters (Figure 2-6D). This degree of overlap was significantly less than expected under null expectations (p = 0.018) (Figure 2-6E).

Mitochondrial *COI* haplotypes

After filtering for sequencing depth, we recovered mitochondrial *COI* haplotypes for 445 samples. These comprised 14 unique haplotypes, four of which are new for the species. A minimum spanning haplotype network including reference haplotypes revealed a similar topology to previous studies (Yund et al. 2015; Nydam et al. 2017), with four general haplogroups (Figure 2-7A). We refer to these haplogroups by their most central haplotype, following Yund et al. (2015).

The Bs2 haplogroup, comprising the haplotypes Bs2, Bs8, Bs14, and REED_1 found in

this study is present on both coasts of North America (Figure 2-7A-C). Bs2 is strictly found on the east coast, whereas Bs8 is found on both coasts, though absent from the southern California sites of QUIV and SBRB. Bs14 was found just twice, in the east coast sites of CHRL and BREK. The new haplotype REED_1 was found in REED and appears to be intermediate between haplogroups Bs2 and HB.

The HO haplogroup, comprising the haplotypes HB, Bs1, REED_8, QUIV_21, and LBPT_16, was also distributed across both coasts. However, HB was found strictly on the east coast whereas Bs1 was found strictly on the west coast, being present in all populations there. The unique haplotypes of QUIV_21 and REED_8 are most closely related to Bs1, whereas LBPT_16 is most closely related to HB.

The HA haplogroup, comprising the haplotypes HA, Bs10, and Bs36, is similarly found on both coasts. However, its constituent haplotypes are not shared, with HA only being found on the east coast and Bs10 and Bs36 only found on the west coast. Bs10 was most commonly found in southern California, but is also present at lower frequencies at COOS, SEAT, and REED. Bs36 was found only in the British Columbia population NCSL.

The HO haplogroup, comprising the haplotypes HO and Bs13 from this study is strictly found on the east coast. Bs13 was only found at LPCB, in eastern Newfoundland.

DISCUSSION

Biological invasions present unique opportunities to investigate the pace of adaptive evolution in natural populations (Huey et al. 2005; Sax et al. 2007; Westley 2011; Moran and Alexander 2014). Here we used lcWGS to characterize the population genetic structure and detect signatures of local adaptation in the invasive golden star tunicate, *B. schlosseri*. We revealed the existence of three major lineages of *B. schlosseri* in North American waters, demonstrating multiple introductions of this NIS to the west coast. We found thousands of loci whose allele frequencies consistently vary with environmental temperature, providing evidence for rapid local adaptation to temperature during range expansion into cooler northern waters. Interestingly, despite a substantial proportion of shared standing genetic diversity, the SNPs and genes implicated in adaptation to temperature on either coast were largely distinct,

suggesting divergent means of local adaptation. Below, we discuss how our results inform our understanding of *B. schlosseri*'s invasion history in North America, the potential for rapid thermal adaptation and its molecular basis, and the possibility of parallel evolution mediated by divergent molecular means.

Invasion history of *B. schlosseri* in North America

Genomic data are often vital for reconstructing the complex invasion histories of marine NIS (Sherman et al. 2016; Viard et al. 2016). By describing variation across the entire genome of many individuals at an attainable cost, lcWGS is an especially promising approach (Lou et al. 2020), but one that, to our knowledge, has not yet been used in studying NIS, let alone one from the marine realm. While our focus on patterns within North America precludes investigation of the global origin of *B. schlosseri*, the data presented here can help unravel its complicated invasion history in the region.

We found three major lineages within our sequencing data, as revealed by PCA clustering (Figure 2-2). Most notably, the existence of two lineages on the west coast of North America demonstrates that there have been at least two independent introductions to the region. Furthermore, these two lineages (SoCal and PNW) bear little resemblance to the lineage present on the east coast (NWA) or to each other, with SoCal and PNW populations exhibiting strong genetic differentiation from populations within the NWA cluster (Figure 2-3A-B). This suggests that the east coast of North America is not the source of either introduction to the west coast, in line with the conclusions of earlier population genetic studies (Stoner et al. 2002; Lejeusne et al. 2011). This is also in accordance with physiological work that has shown that while east and west coast specimens belong to the same species, and are able to produce viable offspring, they do not fuse during allorecognition assays (Boyd et al. 1990), indicative of deep genetic divergence. Because we lack more comprehensive global sampling, we are unable to speculate as to the origins of the PNW and SoCal lineages. Prior work using allorecognition assays demonstrated the ability of colonies from Monterey Bay, California to fuse with colonies from Japan and, to a lesser extent, from Israel (Rinkevich et al. 1992), pointing to the West Pacific or Mediterranean as potential source regions for the PNW cluster. While there has been

somewhat limited population genetic investigation in the West Pacific, Lejeusne et al. (2011) found the *COI* haplotypes Bs1 and Bs10 in both Japan and Australia, but notably not in the Mediterranean. These haplotypes were present on the west coast of North America, as we also document here in both the PNW and SoCal clusters, suggesting that the West Pacific may indeed be the source of introduction to the west coast of North America. *Botryllus schlosseri*'s invasive status is unresolved (cryptogenic) in the Northwest Pacific, where some consider it native (Lee and Shin 2021) while others presume it is invasive (Ben-Shlomo et al. 2010; Lejeusne et al. 2011). In either case, the presence of haplotypes from two haplogroups (Bs1 from HB and Bs10/Bs36 from HA) in Japan (Yund et al. 2015), suggest that variants of Mediterranean origin are present in the West Pacific, as the Mediterranean contains the highest diversity of HA-group haplotypes (Yund et al. 2015). That we found the Bs10 and Bs36 haplotypes in five of our Northeast Pacific populations may suggest at least a partial Mediterranean origin, perhaps by way of Asia. The application of lcWGS to populations from these two regions would surely provide additional insights into its complex introduction history.

While our restricted global sampling leaves the question of the ultimate origin of *B. schlosseri* unresolved, our thorough sampling within North America allows for the examination of relationships among populations in the region. The strong differentiation we observe between all population pairs speaks to the sensitivity of lcWGS for describing patterns of population structure, especially in this species which has severely restricted larval dispersal and thus low potential for gene flow among populations. On the east coast, *B. schlosseri* is considered cryptogenic in the coastal waters of the United States and the Bay of Fundy, Canada. (Fofonoff et al. 2024). Long established in the Gulf of Maine (Gould 1870), *B. schlosseri* more recently expanded throughout Nova Scotia (Carver et al. 2006), Prince Edward Island (Ramsay et al. 2008), and Newfoundland (Callahan et al. 2010), where it is considered invasive (Fofonoff et al. 2024). Using analysis of mitochondrial *COI* haplotypes, Yund et al. (2015) provided evidence for the native status of at least certain lineages of *B. schlosseri* in the Northwest Atlantic. With our lcWGS data, the clustering of all Northwest Atlantic populations into a single lineage suggests that the northward spread of this species along the east coast has likely been mediated through successive introductions from further south. The lack of an

isolation-by-distance (IBD) signature (Figure 2-3C) indicates that this likely did not occur in a stepwise fashion. This is also supported by the PCA of NWA populations (Figure 2-2B), where samples from often geographically distant populations cluster together (e.g. LPCB [eastern Newfoundland] and BREK [South Portland, Maine]). The lack of IBD on the east coast could be a result of *B. schlosseri*'s longer evolutionary history there (Gould 1870; Yund et al. 2015), with the potential for long-distance dispersal events among populations to erode IBD over time.

Regarding the source of introduction to Newfoundland, the observation that our three sites from different regions of the island (BAIN, LPCB, and STPH) do not cluster together in PCA space suggests that they might have distinct origins and that there may have been independent introductions of *B. schlosseri* to the island. LPCB clusters with BREK, suggesting that the invasion of Conception Bay, Newfoundland may have originated from southern Maine or from a closely related, unsampled site. Interestingly, LPCB contains the highest diversity of *COI* haplotypes that we observed, with five unique haplotypes (Bs2, Bs8, Bs13, HA, HO) representing three haplogroups (Bs2, HA, HO). As for the remaining two populations from Newfoundland, specimens from both STPH and BAIN cluster most closely to those from HAWK, a port in the Strait of Canso, suggesting this as a possible origin for both sites, potentially followed by their differentiation after introduction. It should be noted that several samples from the site DART in central Nova Scotia cluster strongly with individuals from HAWK, suggesting that there may have been recent translocations of *B. schlosseri* from HAWK to DART, pointing to the potential importance of this port in mediating spread of *B. schlosseri* in the region. This is in line with a previous study that used *COI* and microsatellites that demonstrated that ports in this region, including HAWK, were likely beachheads for primary invasion from which secondary spread emanated (Lacoursière-Roussel et al. 2012).

That we observe strong genetic differentiation among all of our populations on the west coast speaks to the resolution afforded by lcWGS, but also the speed with which divergence arises in this species. *Botryllus schlosseri* was first detected on the west coast in 1947 in San Francisco Bay (Carlton 1979), and given that all populations from there northward fall in the same evolutionary lineage, all of this differentiation must have arisen since its introduction. The extremely restricted larval dispersal, coupled to high mutation rates in ascidians (Tsagkogeorga

et al. 2012), certainly enables the emergence of differentiation among our sampled populations. This differentiation is useful for tracing the relationships among invasive populations on the west coast, especially near the northern edge of the range.

Botryllus schlosseri's current northern range limit is in Sitka, Alaska (represented here by ELSN), where it was first detected in 2001 (Ruiz et al. 2006). Since then, it has also been documented in nearby locales such as Ketchikan, Alaska (represented by BHRB) in 2010 (Simkanin et al. 2016; Jurgens et al. 2018); Haida Gwaii, British Columbia (represented by MSST) in 2007 (Gartner et al. 2016); and Lax Kw'alaams, British Columbia (represented by KWLM) in 2018 (T. Therriault, pers. comm.). Given the relatively close proximity between these sites, it may have been anticipated that they shared a common origin or, perhaps, that Canadian populations were the source of the Alaskan populations or vice versa. To the contrary, our results demonstrate a lack of a genetic relationship between the populations in Southeast Alaska and those from northern British Columbia (Figure 2-2C). Instead, the populations in northern British Columbia of MSST and KWLM appear to be more closely allied to those in the south of the province in the Salish Sea (REED and NCSL). Similarly, while the origin of the Alaskan populations of BHRB and ELSN is not entirely clear, they do not appear to be of Canadian origin, as they are closer along PC1 to the populations of COOS, SFRA, and RICH, sites further south along the west coast in Oregon and northern California. This indicates that political boundaries, rather than strictly geography, may be governing the spread of this species in the region. As the spread of *B. schlosseri* is mostly attributed to hull fouling (Fofonoff et al. 2024), it is perhaps logical that shipping pressure is stronger between locations within national borders than across them. Another assumed vector for fouling tunicates is the transfer of shellfish aquaculture gear or spat. Given the regulations on the movement of gear or aquaculture species internationally (Muehlbauer et al. 2014), transfers within national borders may be more likely.

In contrast to the NWA cluster, the PNW cluster exhibited a strong pattern of IBD, with the exception of the four geographically close transnational population pairs involving BHRB, ELSN, KWLM, and MSST (Figure 2-3D). That we observe a stronger pattern of IBD here, but none in the NWA cluster, may be an effect of its shorter evolutionary history on the west coast.

It could also be indicative of more stepwise dispersal of *B. schlosseri* during northward range expansion from its original point of introduction in San Francisco Bay. This would be in line with the records of first observations along the west coast (see Fofonoff et al. 2024). Interestingly, while *B. schlosseri* was spreading south towards its current southern range limit near La Paz, Baja California Sur, México (Tovar-Hernández et al. 2014) in the 1960's (Lambert and Lambert 1998), apparently preceding its northward spread, this appears to have been mediated by the introduction of a distinct evolutionary lineage. It is worth noting the that the discontinuity between the PNW and SoCal lineages coincides with a broader biogeographic break at Point Conception (Briggs 1974). This break has been associated with intraspecific genetic discontinuities in native species (e.g. Burton, 1998; Eberl et al., 2013; Wares et al., 2001), often attributed genetic drift due to patterns of ocean circulation, but also potentially by selection across a sharp gradient in sea surface temperature, with the waters of the Southern California Bight being appreciably warmer than those to the north of Point Conception. It is likely that this southern California lineage of *B. schlosseri* possesses different thermal physiology than its more northern counterpart, more tolerant of warmer waters. Supporting this, we previously found that post-larvae from San Diego, California were significantly less sensitive to heat stress than those from populations in Bodega Bay, California and Sitka, Alaska (Chapter 4), suggesting the potential for genetically based, adaptive differences between the lineages present on the west coast. Through the use of genotype-environment association (GEA) analysis, we can more directly investigate patterns of adaptive evolution to environmental temperature within two of the lineages uncovered in this study, as discussed below.

Signatures of thermal adaptation along both coasts

Rapid adaptation to novel environmental conditions is often implicated in the success of NIS (Prentis et al. 2008). By examining clines of allele frequencies across environmental gradients, genotype-environment association (GEA) analyses can uncover potential signatures of local adaptation. In NIS, such analyses may be especially useful, as the non-equilibrium dynamics of invasions can confound population differentiation-based genome scan approaches (Hoban et al. 2016). In both the NWA and PNW lineages, we found thousands of loci whose

allele frequencies across populations were highly correlated with environmental temperature (Figure 2-5). These findings suggest that *B. schlosseri* populations have become locally adapted to environmental temperature across the parallel invasion fronts on both coasts of North America. This speaks to the pace at which evolutionary adaptation can proceed in invasive species, progressing since its introduction on the west coast in the mid-1900s (Carlton 1979) and its northward spread into the Atlantic Maritime provinces in the 1970's (Carver et al. 2006). We should note that our conservative approach, which takes the intersection of EA SNPs from two distinct GEA methods, means that there are likely many more loci that are involved in local adaption to temperature than we report here. In focusing on the shared proportion of SNPs, we focus on the most likely candidates of selection.

To investigate the potential functional consequences of EA SNPs, we took two different approaches. First, we used the functional enrichment tool GO_MWU to assess whether certain molecular function and biological process gene ontology (GO) terms were overrepresented among genes containing highly significant EA SNPs (using baypass' Bayesian p-values [eBPmc]). This analysis recovered few significant categories. In the NWA cluster, the recovered biological process terms "DNA-binding transcription factor activity" and "sequence-specific DNA binding activity" suggest selection on processes concerning the regulation of DNA transcription, a category of activity that of course may be important for adaptation to temperature, but one that is so general that, without a more detailed picture of the specific transcription factors and their targets, it precludes a more detailed discussion. For PNW, the enrichment of the molecular function GO term "sodium channel activity" suggests that processes mediating the ion permeability of cell membranes may be a target of temperature adaptation. This may be especially relevant to cells whose function relies on membrane excitability, such as neurons and muscle. Several genes (g19033, g02087, g26527) containing EA SNPs that contribute to the enrichment of sodium channel activity are orthologous to the vertebrate gene *SCN4A*, which encodes the voltage-gated sodium channel $Na_v1.4$. This protein has been implicated in temperature adaptation of cardiac activity in fish, with a "faster" isoform being expressed in the cold-adapted rainbow trout (*Oncorhynchus mykiss*), in contrast to the "slower" isoform expressed in the warm-adapted zebrafish (*Danio rerio*) (Haverinen et al. 2021). Furthermore,

several other genes contributing to the enrichment of sodium channel activity in PNW are also orthologous to vertebrate genes for voltage-gated sodium channels, suggesting their potential importance in temperature adaptation in *B. schlosseri*. For example, the voltage gated sodium channel $Na_v1.8$ has been implicated in sensing cold and the propagation of neuronal action potentials at cold temperatures in other species across the evolutionary tree (Zimmermann et al. 2007; Kiss et al. 2014; Bagriantsev and Gracheva 2015).

As a second means of inferring the potential functional outcomes of EA SNPs, we identified genes with non-synonymous mutations at EA loci. We then queried the GO terms of these genes for the function "response to temperature stimulus." In the NWA lineage, we found no genes that matched this description. However, in the PNW lineage we observed three genes with missense mutations associated with environmental temperature that had this functional annotation. These three genes are orthologous to those encoding the proteins holocarboxylase synthetase (HCS) (g52126), xanthine dehydrogenase (XDH) (g36797), and interferon regulatory factor 4 (IRF4) (g66813). HCS is a chromosomal protein that is associated with heat tolerance in *Drosophila melanogaster*; HCS-deficient flies have a 55% reduction in survival at 34 °C relative to flies kept at room temperature (Camporeale et al. 2006). In contrast to HCS' role in heat tolerance, XDH appears to be involved in cold tolerance. In *Caenorhabditis elegans*, XDH knockouts exhibited severely reduced survival at 2 °C compared to wild-type, and exogenous expression of XDH in neurons rescued this phenotype, suggesting that XDH's function in mediating cold tolerance is restricted to neural tissue (Takagaki et al. 2020), again highlighting the potential importance of neurological processes in cold adaptation. XDH also appears to play a role in cold tolerance in *D. melanogaster* (Duncker et al. 1995). As its name suggests, IRF4 is a protein involved in the regulation of interferons, a type of cytokine important in immune responses. Given the potential for higher diversity and virulence of parasites and pathogens at higher temperatures (Marcogliese 2008), populations spanning a thermal gradient may face differential selection pressures from these tiny foes. Indeed, other studies investigating local adaptation to temperature have demonstrated the potential role for immune-related genes, particularly in fishes (Dionne et al. 2007; Narum et al. 2013; Amish et al. 2019).

While the functional annotations of the EA SNPs can offer some corroborative evidence of their relevance to thermal adaptation, ultimately more detailed, experimental investigation would be required to definitively demonstrate their role. Nonetheless, that we observed thousands of variants whose frequencies strongly correlated with environmental temperature suggests the strong likelihood of local adaptation to temperature in this species. This offers an additional line of support for local adaptation's role in driving differentiation of thermal tolerance across latitude on both coasts, which we have also demonstrated experimentally (Chapters 3 and 4). A previous population genomic study using restriction site-associated DNA sequencing (RADseq) found evidence for local adaptation to a variety of environmental variables in *B. schlosseri*, including temperature (Gao et al. 2018, 2022). By investigating genomic variation across nine sites across several ocean basins, Gao et al. (2022) provided important insights into how adaptive variation may be partitioned across *B. schlosseri* global range. Critically, by focusing primarily on regions where *B. schlosseri* is known to be invasive and is currently expanding northward, our data provide a much more nuanced test of the pace at which thermal adaptation can proceed. Indeed, the clines in allele frequency we observed at EA SNPs must have been established since the species' introduction to the west coast in 1947 (Carlton 1979) and, on the east coast, since its northward spread into the Atlantic Maritime provinces in Canada in the 1970's (Carver et al. 2006).

Other population genomic studies of marine NIS have similarly revealed patterns suggestive of rapid adaptation to a variety of environmental variables (Tepolt and Palumbi 2015; Lin et al. 2017; Chen et al. 2021, 2024). In the invasive European green crab (*Carcinus maenas*), rapid selection at a putative inversion polymorphism appears to underlie adaptation across a temperature gradient in its invasive range on the west coast of North America (Tepolt et al. 2022). Allele frequency at this locus is strongly correlated with cold tolerance across its native and invasive ranges, suggesting that standing genetic variation from the native range fueled rapid adaptation to cold temperatures during northward expansion in its invasive range (Tepolt and Palumbi 2020). *Carcinus maenas*, like many marine invertebrates with highly dispersive larvae, experiences high levels of gene flow (Tepolt et al. 2022). Variation at regions of suppressed recombination, like inversions, which can contain few loci but of large effect,

may be especially important for adaptation in highly dispersive species (e.g. Barth et al. 2017; Samuk et al. 2017; Akopyan et al. 2022). In contrast, we found no evidence for extended regions of elevated linkage disequilibrium (Figure S2-5) and instead found many thousands of SNPs associated with environmental temperature distributed across the genome (Figure 2-5D). This suggests that highly polygenic, dispersive architectures of local adaptation may be favored in situations of low gene flow (Griswold 2006; Shi et al. 2023), as opposed to the evolution of genomic islands of divergence and/or inversions in regimes of high gene flow (Tigano and Friesen 2016; Schaal et al. 2022; Jay et al. 2024).

Parallel adaptation via contrasting genomic routes?

The degree to which evolutionary adaptation is repeatable, and at which levels of biological organization, is an open question in contemporary evolutionary biology (Bolnick et al. 2018). The parallel thermal gradients across both coasts of North America afforded us the opportunity to interrogate whether the mechanisms putatively underlying adaptation to environmental temperature are shared versus divergent across these invasion fronts. The substantial overlap in the identity of overall SNPs between the NWA and PNW lineage (Figure 2-5A) suggests a large portion of standing genetic variation is segregating on both coasts. Nonetheless, we observed minimal overlap in the identity of EA loci between the two lineages, with just three SNPs that fit our criteria as EA in both clusters (Figure 2-6A). Our permutation test indicated that this degree of shared putatively adaptive variation at the SNP level is not significantly different from null expectations (Figure 2-6B). Given that putatively adaptive loci are no more likely to be shared among coasts than would be expected due to chance, we may conclude that parallel adaptation to temperature along these bicoastal temperature gradients is not underlain by parallel genomic shifts at the SNP level.

It should be noted that our approach for identifying EA SNPs may have affected our ability to detect parallel signatures of local adaptation, thus influencing our conclusion that adaptation has occurred primarily by divergent molecular routes. Because we used a rather conservative criterion for determining whether a SNP was associated with habitat temperature (using the intersection of two distinct methods), it is possible that some SNPs that were

marginally significant in one or both methods and displayed parallelism were excluded from the EA SNP set. If this were the case, our approach would favor detection of non-parallel SNPs and thus skew our interpretations. This could be explored further by proceeding with each GEA method independently, rather than taking the intersection, or by using less stringent significance thresholds. While it is possible that our permutation test would behave differently with a larger set of EA SNPs identified by a less conservative approach, the results presented here do indeed point toward limited parallelism during putative local adaptation to temperature on either coast.

While few in number, the identity of shared EA SNPs can provide some insights in the potential conserved mechanisms underlying thermal adaptation. The EA SNP chr13_12950991 is within the gene *NOTCH2*, which encodes a Notch receptor which are widely implicated in developmental processes (Mumm and Kopan 2000). The EA SNP chr6_2480339 is downstream of the gene *CRAT*, which encodes for the protein carnitine O-acetyltransferase. This protein is involved in the transport of fatty acids into mitochondria for subsequent β-oxidation (Ramsay et al. 2001). Changes in lipid metabolism, and specifically those involving carnitine transferases, have been implicated in adaptation and acclimation to cold temperatures (Lu et al. 2019; Mast et al. 2022); it is thus possible that this variant downstream of the *CRAT* gene may be involved in its regulation during cold stress. The EA SNP chr8_1041275 is an intronic variant within the gene *LYG2*, which encodes a lysozyme g protein. Lysozymes are a component of the innate immune system that are responsible for lysing potentially pathogenic bacteria (Saurabh and Sahoo 2008). The appearance of another immune-related gene further suggests the involvement of immune processes in mediating adaptation across a thermal gradient, not adaptation to temperature *per se*, but rather to a covarying selective pressure from pathogens.

Despite the large proportion of shared variation, the lack of substantial overlap in EA SNP identity between the two coasts (Figure 2-6A) indicated a lack of SNP reuse (i.e. no significant parallelism at the SNP level). This was again reflected at the gene level (Figure 2-6D). The extent to which parallel phenotypic evolution is mediated by parallel changes at lower levels of biological organization (SNPs, genes, biochemical pathways, etc.) is highly variable among systems (reviewed by Bolnick et al. 2018). Because of many-to-one mapping and the

consequent redundancy afforded by the often highly polygenic genetic architecture underlying complex traits, there are potentially many different genomic trajectories to a parallel phenotypic outcome (Barghi et al. 2020). The large proportion of standing genetic variation that is shared between both coasts should perhaps increase the likelihood that SNPs/genes would be reused during parallel adaptation to temperature. Interestingly, we did not observe any evidence of excessive parallelism at these levels; the two clusters apparently developed largely idiosyncratic genomic responses to selection across parallel thermal gradients. Other studies have demonstrated how parallel phenotypic adaptation can be mediated by divergent molecular means (e.g. Elmer et al. 2014; Barghi et al. 2019; Fischer et al. 2021; Whiting et al. 2022; Szukala et al. 2023). For example, in an experimental evolution experiment in the Atlantic silverside (*Menidia menidia*), changes in body size in response to artificial size-selective fishing pressure were accompanied by mostly distinct genomic changes among replicate lines (Therkildsen et al. 2019). The inverse has also been observed, where the genetic mechanisms underlying adaptive evolution are highly parallel (e.g. Colosimo et al. 2005; Chan et al. 2010; Soria-Carrasco et al. 2014; Alves et al. 2019). For example, an experimental evolution study investigating the molecular basis of adaptation to low salinity in the copepod *Eurytemora affinis* found that nearly 80% of selected alleles were shared among ten replicate lines (Stern et al. 2022). The factors influencing whether parallel phenotypic evolution is mediated by parallel changes at the molecular level are varied and complex (see Bolnick et al. 2018), highlighting the need for more studies of parallelism and its molecular basis.

As for rapid adaptation more generally, biological invasions present excellent opportunities for investigating parallelism of evolutionary responses (Lin et al. 2015; Stuart et al. 2022). The aforementioned copepod *E. affinis* has repeatedly invaded freshwater habitats across its range (Lee 1999; Lee et al. 2011). These invasions are associated repeated evolution of specific molecular responses, at both the genomic (Stern and Lee 2020) and gene expression levels (Posavi et al. 2020). In contrast, Hodgins et al. (2016) found minimal reuse of orthologous genes during evolution among 35 native and invasive species of Asteraceae. In the present study, our observation of minimal SNP and gene reuse in putative thermal adaptation between the NWA and PNW lineages suggests that the molecular mechanisms are highly non-parallel.

Why we observed this degree of non-parallelism in unclear. One the one hand, the large proportion of shared genetic variation between the two regions, allowing selection to draw on an overlapping pool of variants, should potentiate parallel evolution. On the other hand, the large number of loci identified as adaptive, even with our conservative approach, suggests a highly polygenic basis of thermal tolerance, adding genetic redundancy and thus the potential for divergent molecular routes. Further investigations should explore how various factors such as the extent of standing genetic variation, genetic architecture, demographic history, and their interactions influence the observed level of parallelism.

CONCLUSION

By applying lcWGS in a widespread marine NIS, we explore the potential for rapid evolutionary adaptation to temperature during invasion and subsequent range expansion. We also apply this genome-scale data towards the resolution of *B. schlosseri*'s invasion history in North America. That we found thousands of loci that are associated with environmental temperature suggests that thermal tolerance in this species has evolved rapidly and has a highly polygenic basis. Furthermore, the existence of several evolutionary lineages across both coasts of North America allowed us to evaluate the potential for parallel evolutionary adaptation on distinct genomic backgrounds. Despite a high proportion of shared standing genetic variation between two lineages, we found that the genetic targets of selection are largely distinct, adding valuable evidence to the potential for parallel evolution via contrasting molecular routes.

While study of NIS is valuable in its own right, it can also provide insights into broader problems in ecology and evolutionary biology (Huey et al. 2005; Sax et al. 2007). Specifically, by encountering novel environmental conditions during invasion and range expansion, NIS present useful opportunities to investigate the pace and mode of adaptive change in response to global change. By investigating genomic patterns of local adaptation in *B. schlosseri*, we demonstrate how a high-profile marine NIS has been able to adapt to environmental temperature on contemporary time scales. While NIS may represent an upper bound on the pace of adaptive change (Moran and Alexander 2014), they provide useful case studies for examining the potential of all species to adapt to a changing environment.

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TABLES

Table 2-1. Site information, including number of specimens and nucleotide diversity (π).

Code	Locality	Latitude	Longitude	*n*	π
BAIN	Baine Harbour, NL, Canada	47.3634	-54.8984	20	0.00840
BHRB	Ketchikan, AK, USA	55.3506	-131.6837	22	0.00650
BREK	South Portland, ME, USA	43.6500	-70.2319	22	0.00907
CHRL	Boston, MA, USA	42.3770	-71.0487	24	0.00907
COOS	Charleston, OR, USA	43.3462	-124.3268	24	0.00648
DART	Dartmouth, NS, Canada	44.7004	-63.6120	23	0.00937
DEAD	New York City, NY, USA	40.5859	-73.9008	22	0.00905
ELSN	Sitka, AK, USA	57.0581	-135.3542	26	0.00644
FALM	Falmouth, MA, USA	41.5483	-70.6027	24	0.00928
HAWK	Port Hawkesbury, NS, Canada	45.6136	-61.3652	23	0.00928
INRV	Delaware Seashore State Park, DE, USA	38.6130	-75.0737	24	0.00878
KWLM	Lax Kw'alaams, BC, Canada	54.5609	-130.4316	22	0.00689
LBPT	Virginia Beach, VA, USA	36.9038	-76.0736	20	0.00843

LPCB	Conception Bay South, NL, USA	47.5222	-52.9680	24	0.00919
MSST	Masset, BC, USA	54.0082	-132.1407	23	0.00659
NCSL	Nanaimo, BC, USA	49.1801	-123.9293	23	0.00725
QUIV	San Diego, CA, USA	32.7624	-117.2380	21	0.00513
REED	Port Moody, BC, Canada	49.2925	-122.8844	22	0.00704
RICH	Richmond, CA, USA	37.9135	-122.3521	22	0.00700
SABS	St. Andrews, NB, Canada	45.0825	-67.0849	21	0.00819
SBRB	Santa Barbara, CA, USA	34.4050	-119.6921	17	0.00522
SEAT	Seattle, WA, USA	47.6289	-122.3923	24	0.00663
SFRA	San Francisco, CA, USA	37.8066	-122.4447	17	0.00712
STPH	Stephenville, NL, Canada	48.5141	-58.5382	24	0.00859

FIGURES

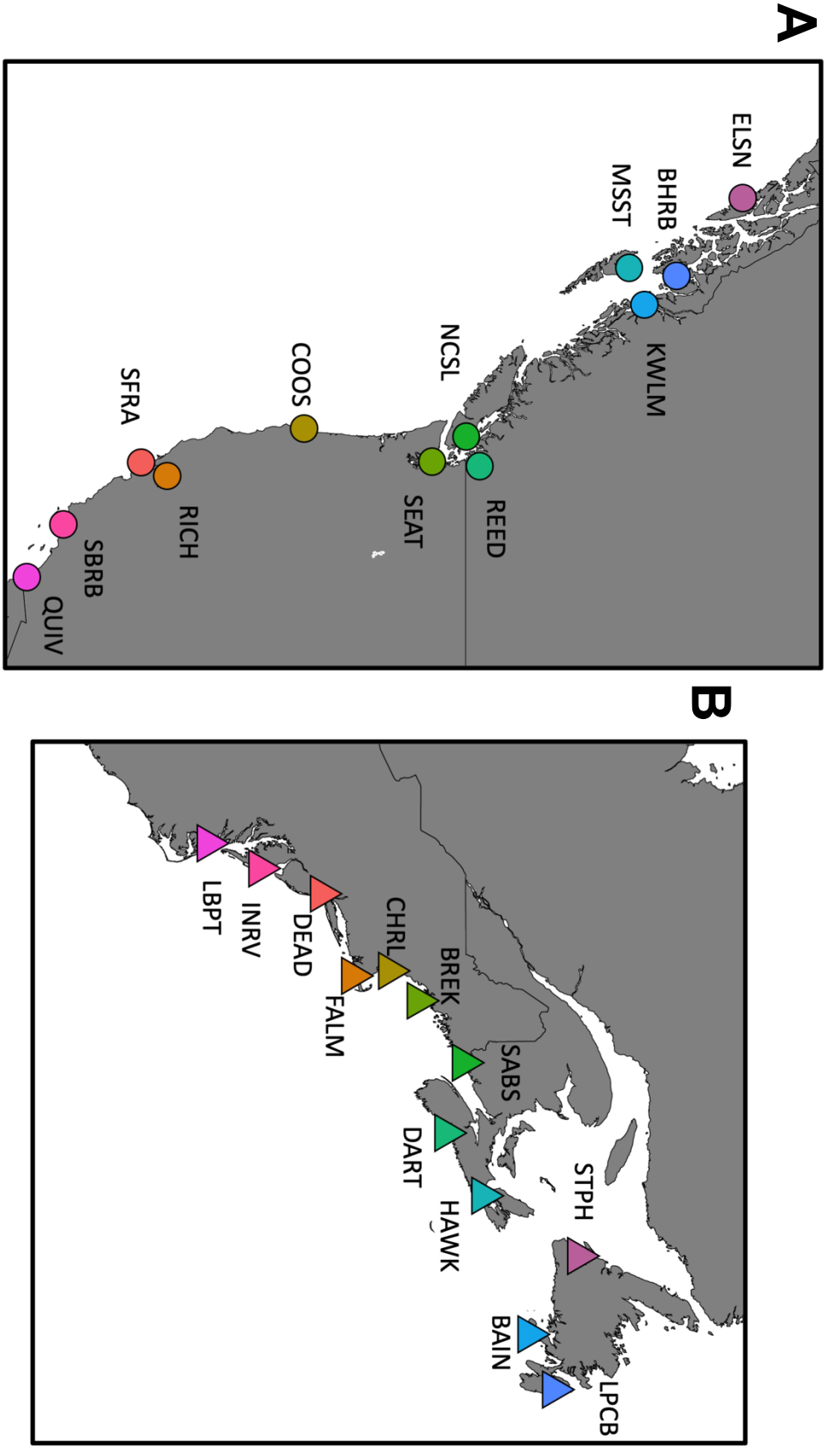

Figure 2-1. Map of study sites on the west (A) and east (B) coasts of North America. Refer to Table 2-1 for full site names and coordinates.

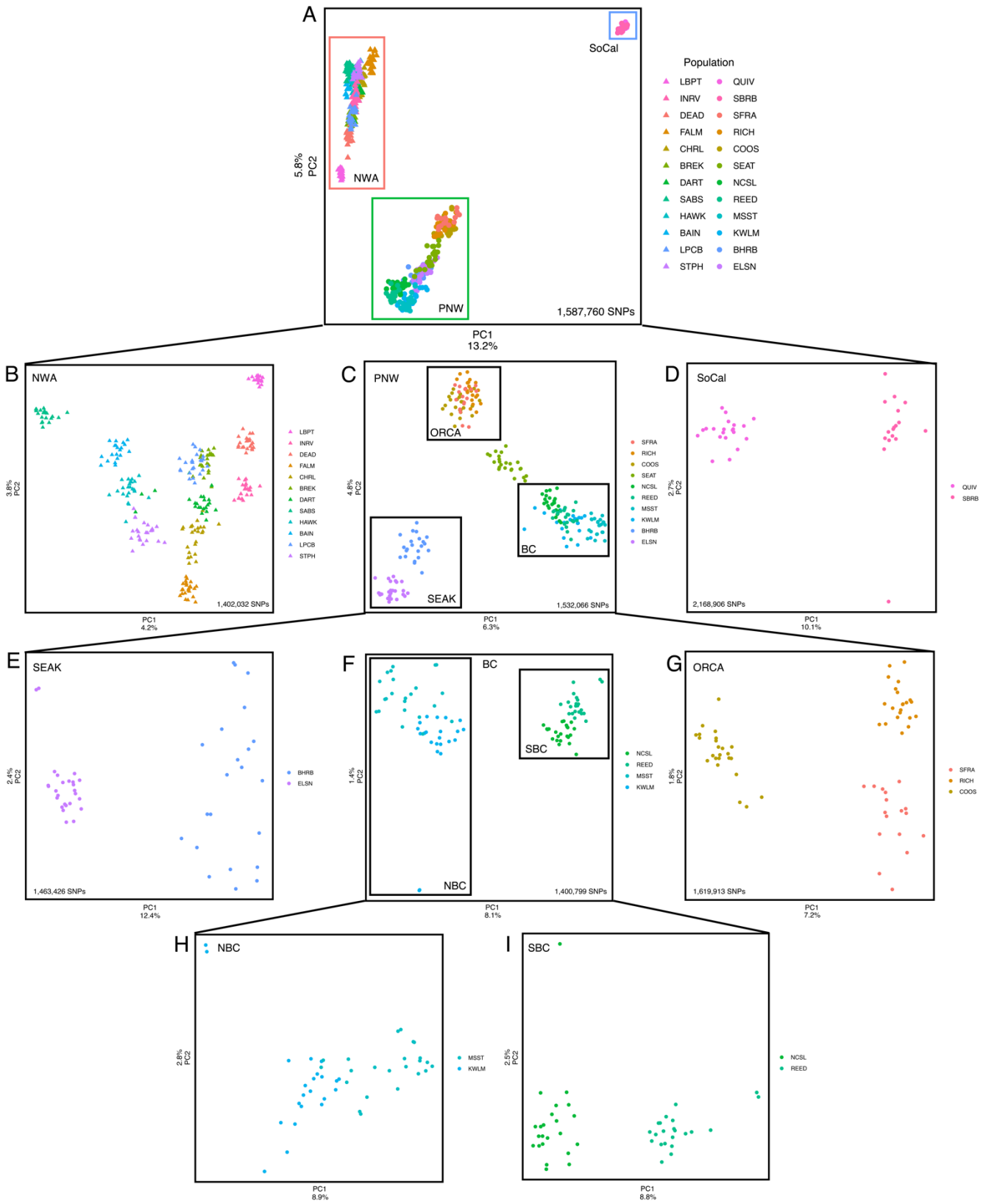

Figure 2-2. Principal components analysis (PCA) reveals hierarchical population structure in *B. schlosseri*. NWA = Northwest Atlantic, PNW = Pacific Northwest, SoCal = Southern California, SEAK = Southeast Alaska, ORCA = Oregon and northern California, BC = British Columbia, NBC = northern British Columbia, SBC = southern British Columbia.

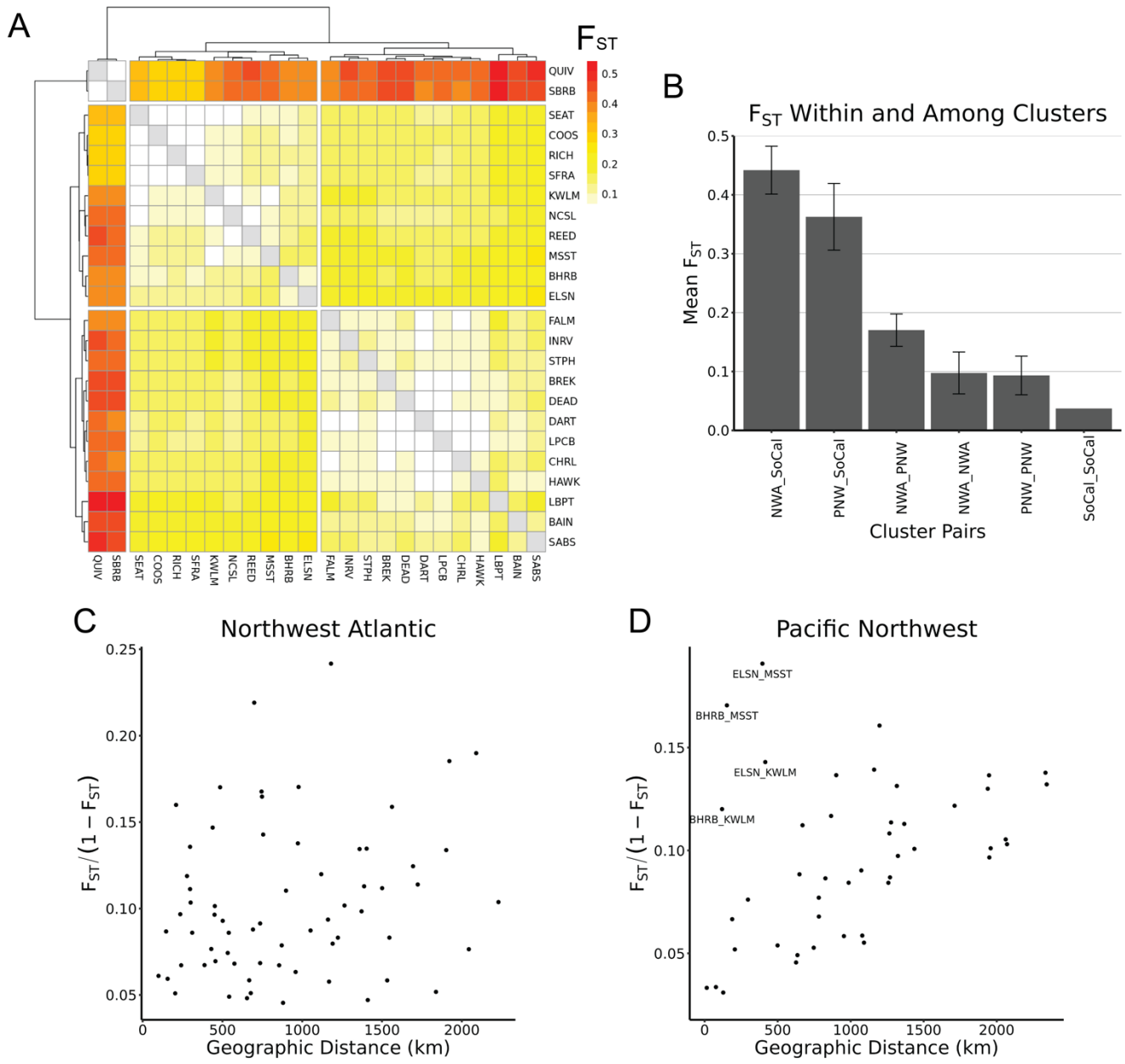

Figure 2-3. lcWGS reveals strong genetic differentiation among populations of *B. schlosseri*. A) Heatmap of pairwise F_{ST} among populations. Hierarchical clustering reveals identical groupings to those in Figure 2A. (See Figure S2 for heatmap without SoCal populations). B) Mean pairwise F_{ST} within and among clusters. Error bars represent standard deviations. C) No signal of isolation-by-distance (IBD) in NWA cluster ($R^2 = 0.026$, $p = 0.10$). D) PNW cluster exhibited strong IBD ($R^2 = 0.12$, $p = 0.012$; $R^2 = 0.48$, $p < 0.001$ with labelled outliers removed).

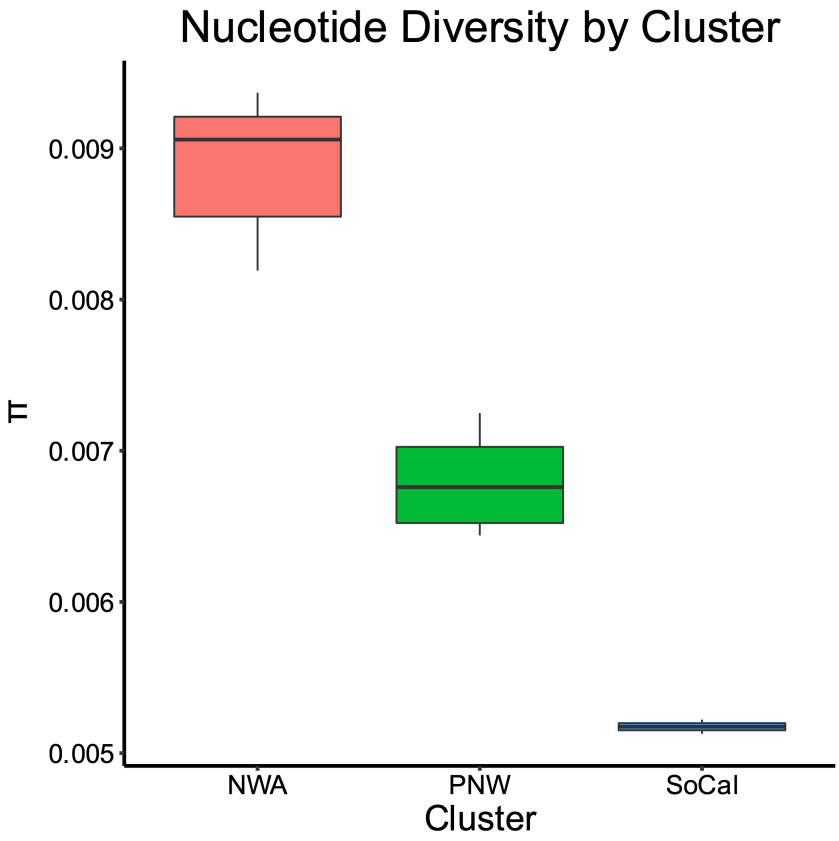

Figure 2-4. Nucleotide diversity (π) systematically differs by cluster. Bold lines within boxes represent the median. Edges of boxes represent the interquartile range. Whiskers represent the maximum and minimum values within each set.

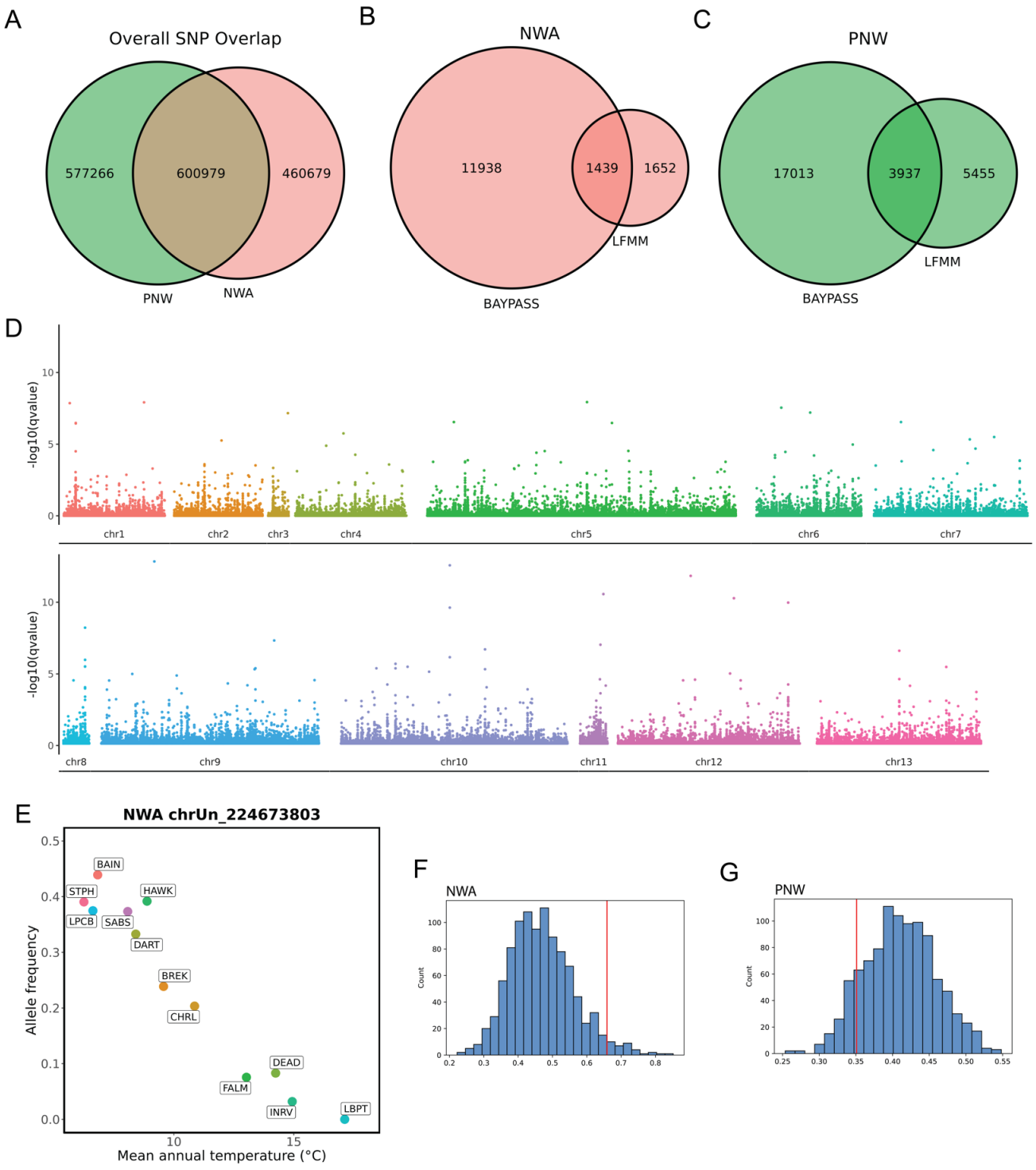

Figure 2-5. A) Venn diagram showing the overlap of all SNPs between NWA and PNW clusters. B-C) Overlap between BAYPASS- and LFMM-identified environmentally associated (EA) SNPs reveals 1,439 EA SNPs in NWA and 3,937 EA SNPs in PNW, respectively. D). Manhattan plot of LFMM q-values in NWA across chromosomes 1-13 of the *B. schlosseri* reference genome. E) Plot of allele frequency vs. environmental tempearture at the most significant LFMM locus in NWA cluster. F-G) Histograms of null distribution of ratio of missense to synonymous mutations for NWA and PNW, respectively. Red lines represent observed value. NWA had a significantly higher ratio than expected due to chance (p = 0.035). No significant difference in PNW (p = 0.879).

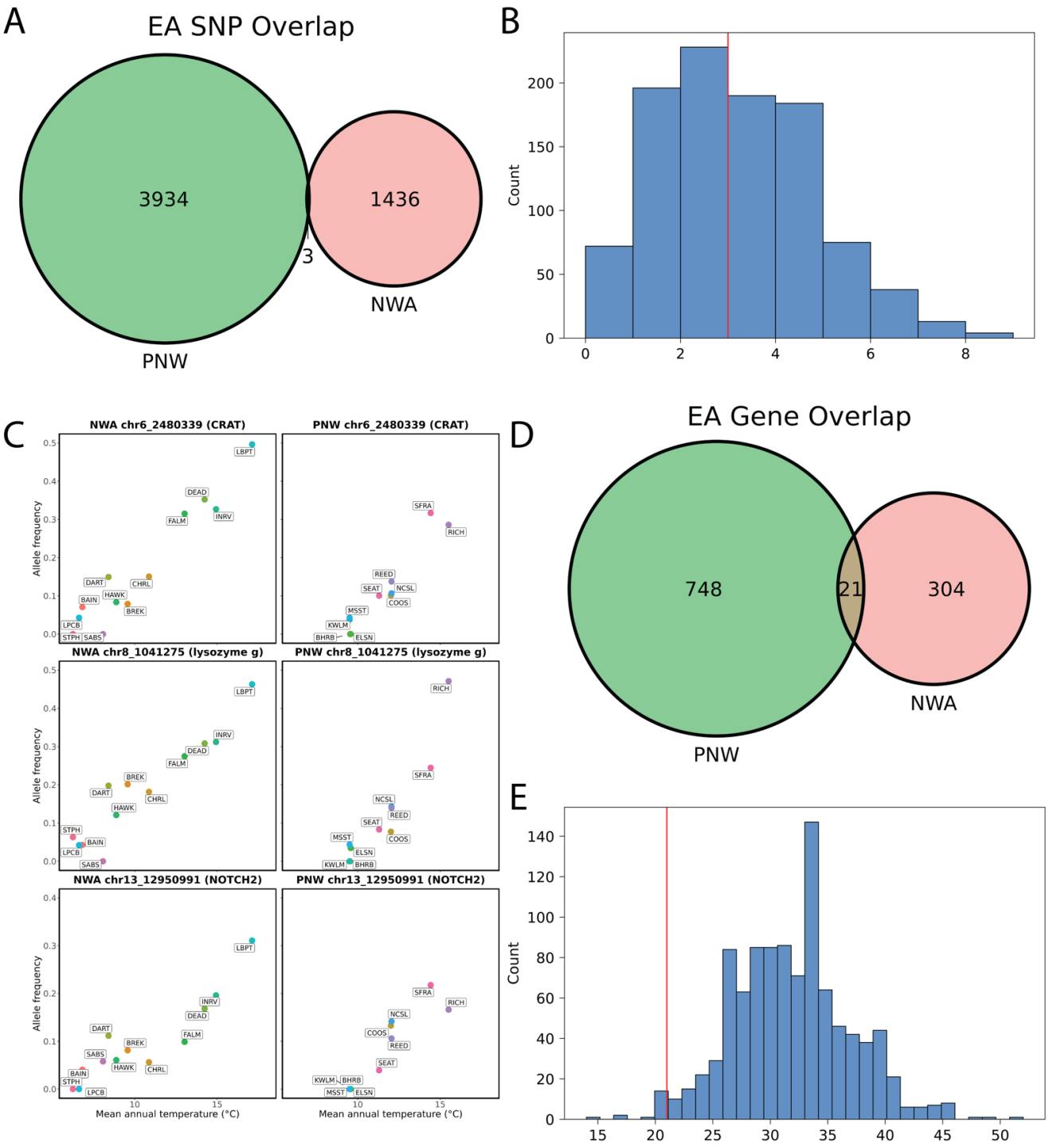

Figure 2-6. Putative parallel evolution of thermal tolerance is mediated by divergent molecular means across the NWA and PNW clusters. A) Venn diagram showing the overlap in EA SNPs between NWA and PNW. B) Histogram showing the distribution of EA SNP overlap under null expectations. Red line represents the observed value of 3. C) Allele frequency vs. mean annual environmental temperature for the three EA SNPs found in both clusters. D) Venn diagram showing overlap in EA genes between PNW and NWA. E) Histogram of expected degree of EA gene overlap from permutation test. Red line represents observed overlap. There is significantly less overlap at the gene level than expected due to chance (p = 0.018).

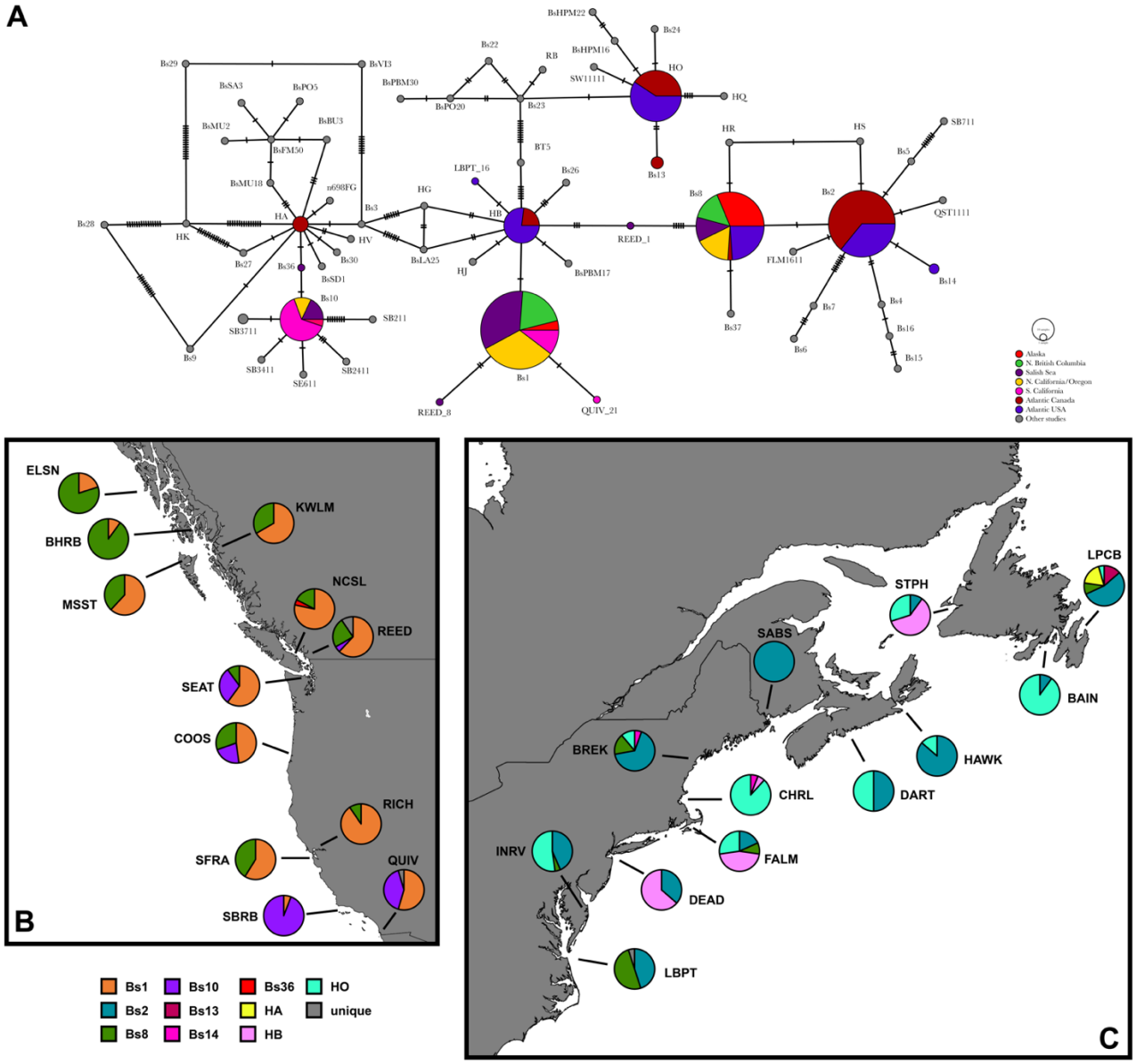

Figure 2-7. Mitochondrial *COI* haplotype analysis. A) Minimum spanning haplotype network reveals four primary haplogroups, all of which are represented in our sampling. Grey circles represent haplotypes found in other studies but not in our own. B) Map of haplotype frequencies at each population.

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Table S2-1. Environmental temperature source metadata (continued on next page).

Station name	Site(s)	Lat	Lon	Agency	ID	Start
Hog Island Channel, NY	DEAD	40.6088	-73.6561	USGS	1311143	5/5/15
West Chedabucto Bay, NS	HAWK	45.4869	-61.1410	Smart Atlantic	44489	9/16/20
Sewells Point, VA	LBPT	36.9467	-76.3300	NOAA	8638610	1/1/15
Hollyrood 2, NL	LPCB	-53.1081	-53.1081	Smart Atlantic		8/29/18
San Diego, CA	QUIV	32.7142	-117.1736	NOAA	9410170	1/1/15
Richmond, CA	RICH	37.9231	-122.4097	NOAA	9414863	1/1/15
Stearns Wharf, CA	SBRB	34.4107	-119.6874	SCCOOS		9/16/05
Massey Ditch, DE	INRV	38.6249	-75.0992	USGS	1484680	11/2/11
Menauhant Yacht Club, MA	FALM	41.5526	-70.5485	NERRS	WQBMHW	1/1/15
Boston, MA	CHRL	42.3539	-71.0503	NOAA	8443970	1/14/07
Portland, ME	BREK	43.6581	-70.2442	NOAA	8418150	1/1/15
Eastport, ME	SABS	44.9046	-66.9829	NOAA	8410140	1/1/15
Halifax Herring Cove Buoy, NS	DART	44.5559	-63.5445	Smart Atlantic		11/7/13
Channel/Port Aux Basques Buoy, NL	STPH	47.5628	-59.1004	Smart Atlantic		8/29/13
Pilot Boarding Station/Red Island Shoal Buoy, NL	BAIN	47.3184	-54.1222	Smart Atlantic		7/6/10
Charleston Bridge, OR	COOS	43.3377	-124.3205	NERRS	SOSCHWQ	1/1/15
Seattle, WA	SEAT	47.6019	-122.3392	NOAA	9447130	1/1/15
Halibut Bank, BC	NCSL, REED	49.3400	-123.7300	Environment CA	46146	5/5/15
Central Dixon Entrance Buoy, BC	KWLM, MSST	54.3700	-132.4400	Environment CA	46145	5/5/15
Ketchikan, AK	BHRB	55.3319	-131.6261	NOAA	9450460	1/1/15
Sitka, AK	ELSN	57.0517	-135.342	NOAA	9451600	1/1/15

End	URL
10/19/23	http://erddap.sensors.ioos.us/erddap/tabledap/gov_usgs_waterdata_01311143.html
10/19/23	https://www.smartatlantic.ca/erddap/tabledap/eccc_opp_44489_west_chedabucto_bay.html
12/31/21	http://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_8638610.html
10/19/23	https://www.smartatlantic.ca/erddap/tabledap/SMA_Holyrood_Buoy2.html
10/19/23	https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_9410170.html
10/19/23	http://erddap.cencoos.org/erddap/tabledap/noaa_nos_co_ops_9414863.html
10/19/23	https://erddap.sccoos.org/erddap/tabledap/autoss.html?station=%22stearns_wharf%22
10/19/23	https://erddap.sensors.ioos.us/erddap/tabledap/gov_usgs_waterdata_01484680.html
10/19/23	https://cdmo.baruch.sc.edu/aqs/index.cfm
6/16/17	https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_8443970.html
10/19/23	https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_8418150.html
10/19/23	https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_8410140.html
10/19/23	https://www.smartatlantic.ca/erddap/tabledap/SMA_halifax.html
4/18/23	https://www.smartatlantic.ca/erddap/tabledap/SMA_port_aux_basques.html
2/9/23	https://www.smartatlantic.ca/erddap/tabledap/SMA_red_island_shoal.html
10/19/23	https://cdmo.baruch.sc.edu/aqs/index.cfm
10/19/23	https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_9447130.html
10/19/23	https://erddap.sensors.ioos.us/erddap/tabledap/ca_weather_46146.html
10/19/23	https://erddap.sensors.ioos.us/erddap/tabledap/ca_weather_46145.html
10/19/23	https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_9450460.html
10/19/23	https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_9451600.html

SUPPLEMENTARY FIGURES

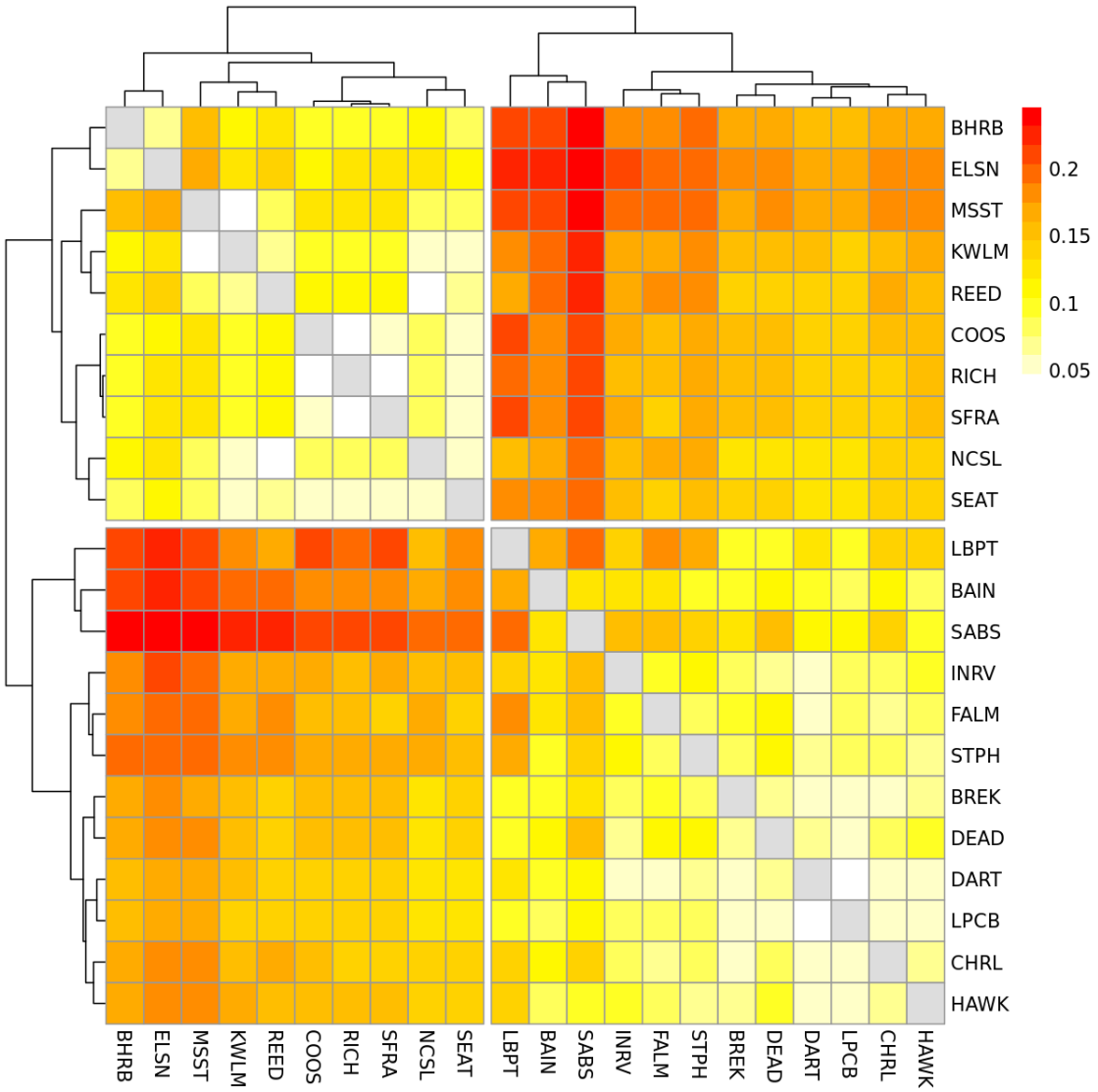

Figure S2-1. Heatmap of pairwise F_{ST} with SoCal populations removed.

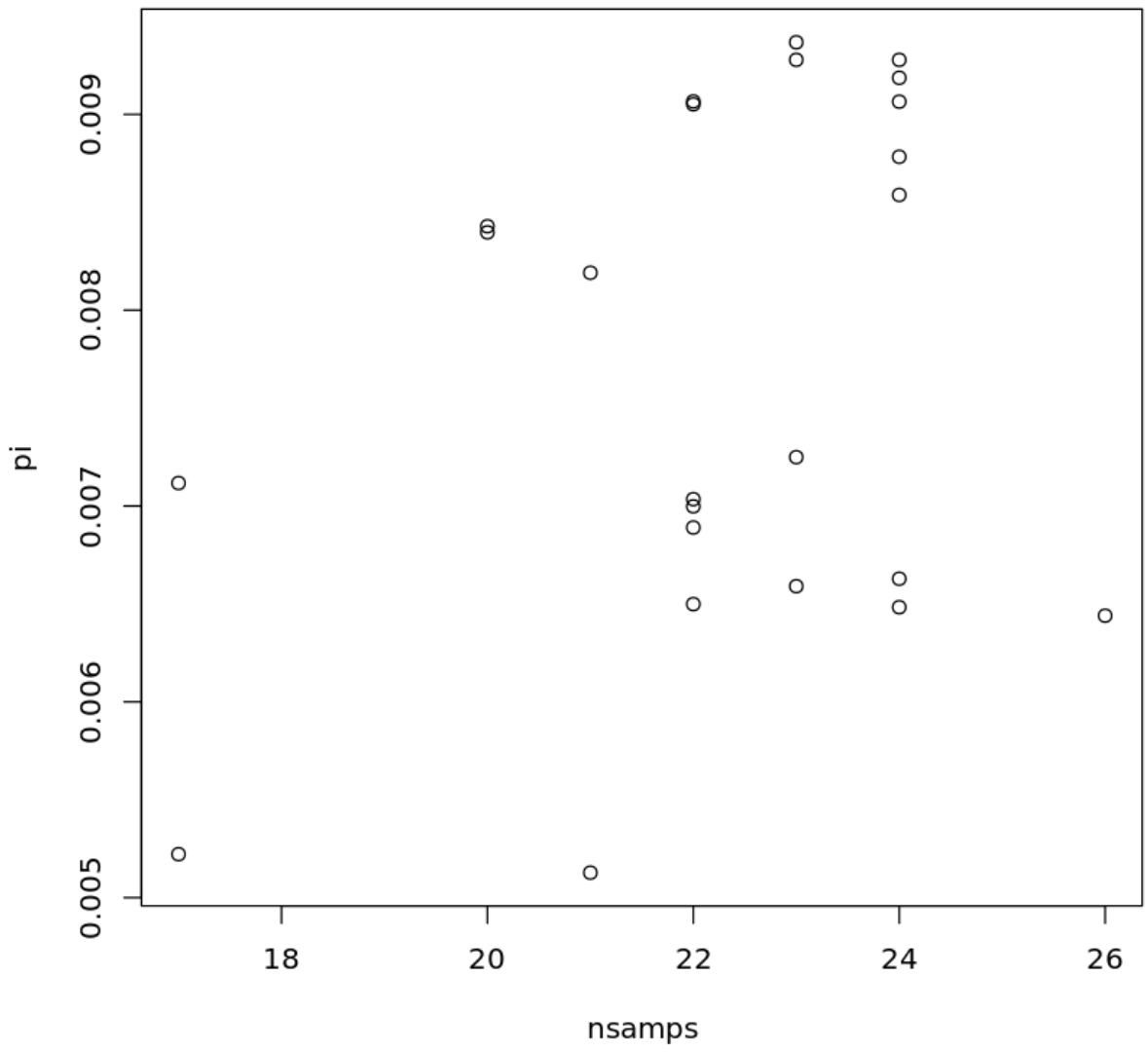

Figure S2-2. Sampling depth at each site does not affect nucleotide diversity (π).

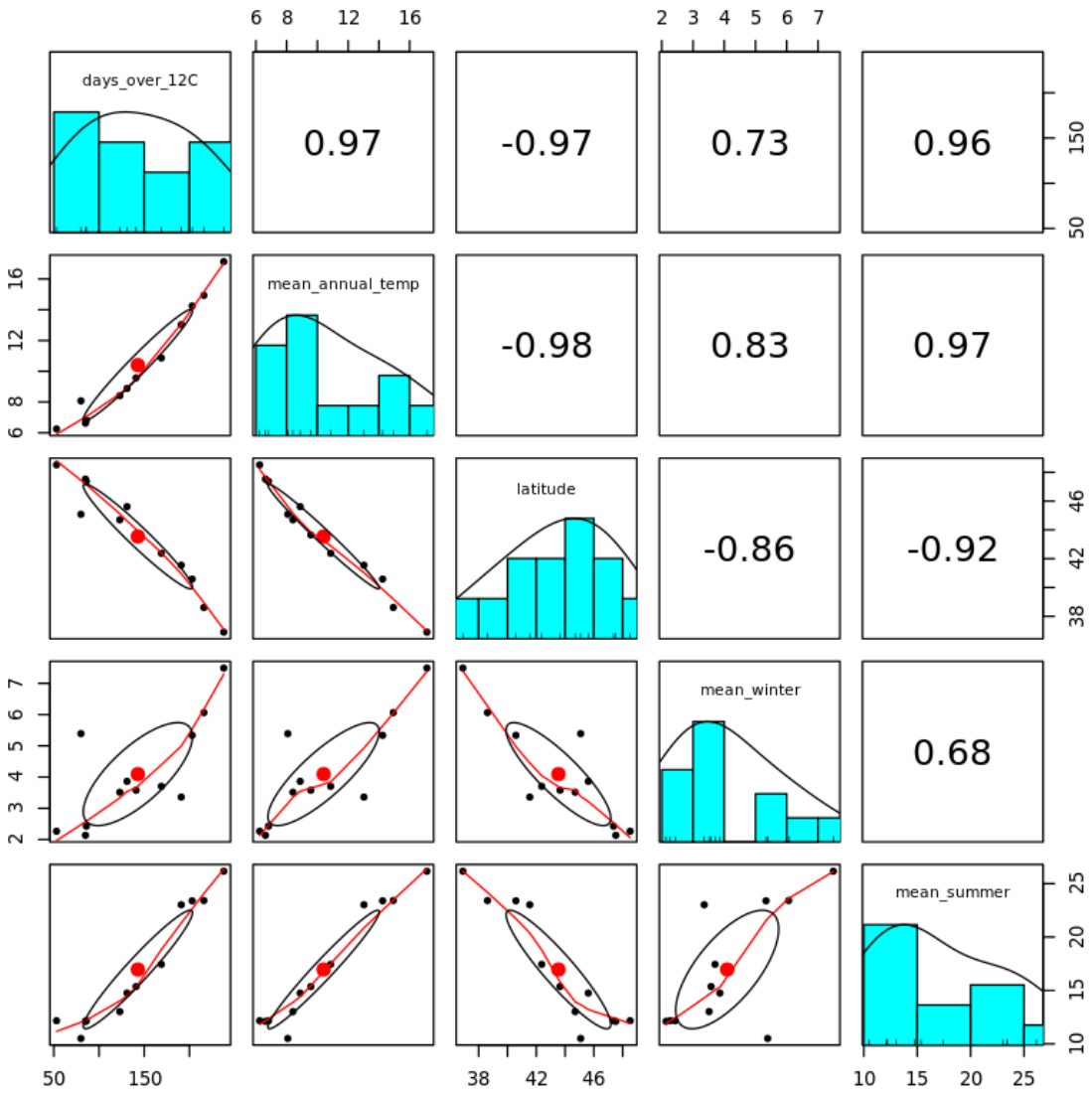

Figure S2-3. Temperature variables across sites in NWA are highly correlated with one another ($r > 0.65$). Upper triangle shows Pearson correlation coefficients. Diagonal shows distribution of metrics. Lower triangle shows Loess regressions of environmental covariates on one another.

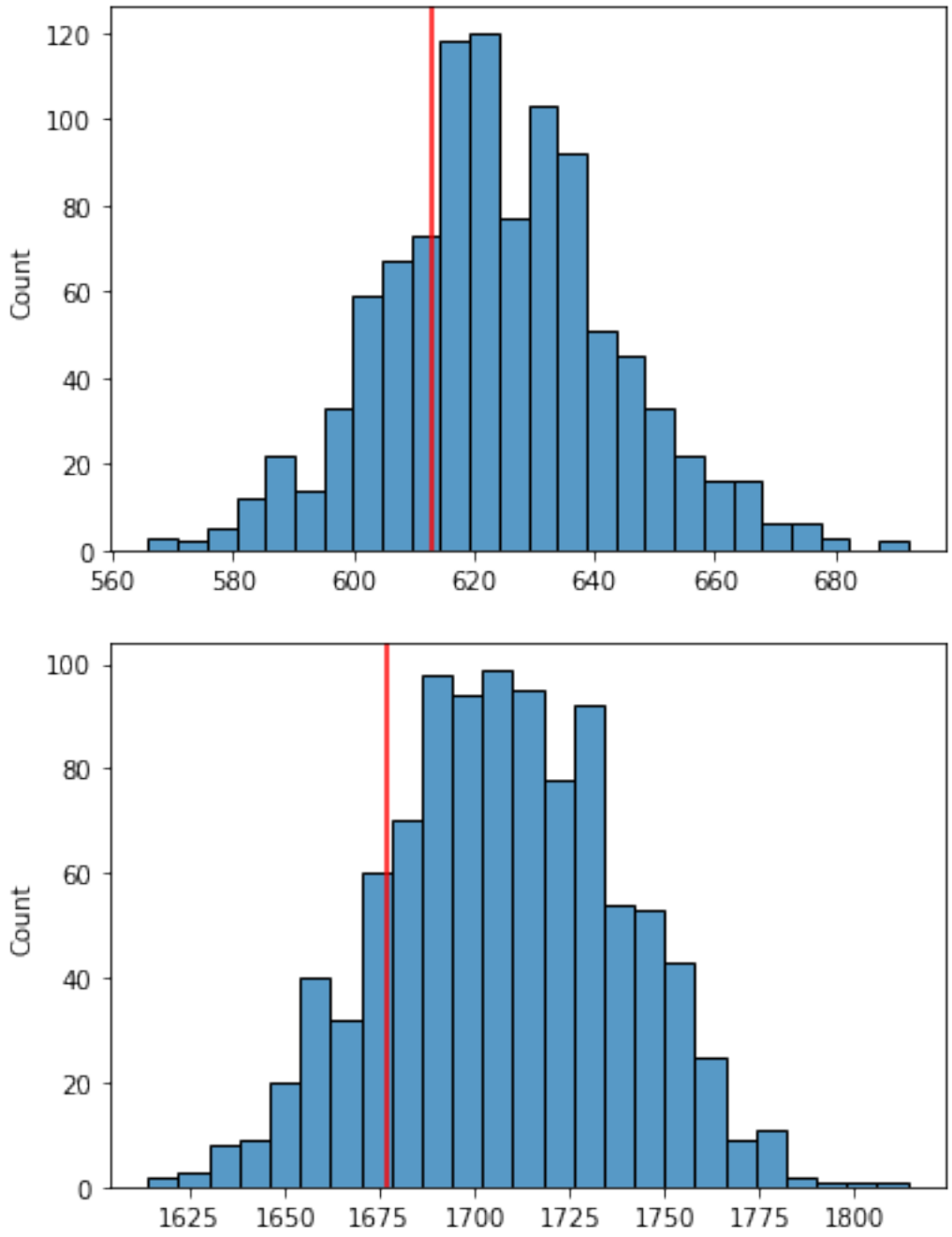

Figure S2-4. Histograms of expected number of EA SNPs to fall within 1000bp of a gene (for chrs1-13) or within a gene (for chrUn) in NWA (top) and PNW (bottom). Red line represents the observed value.

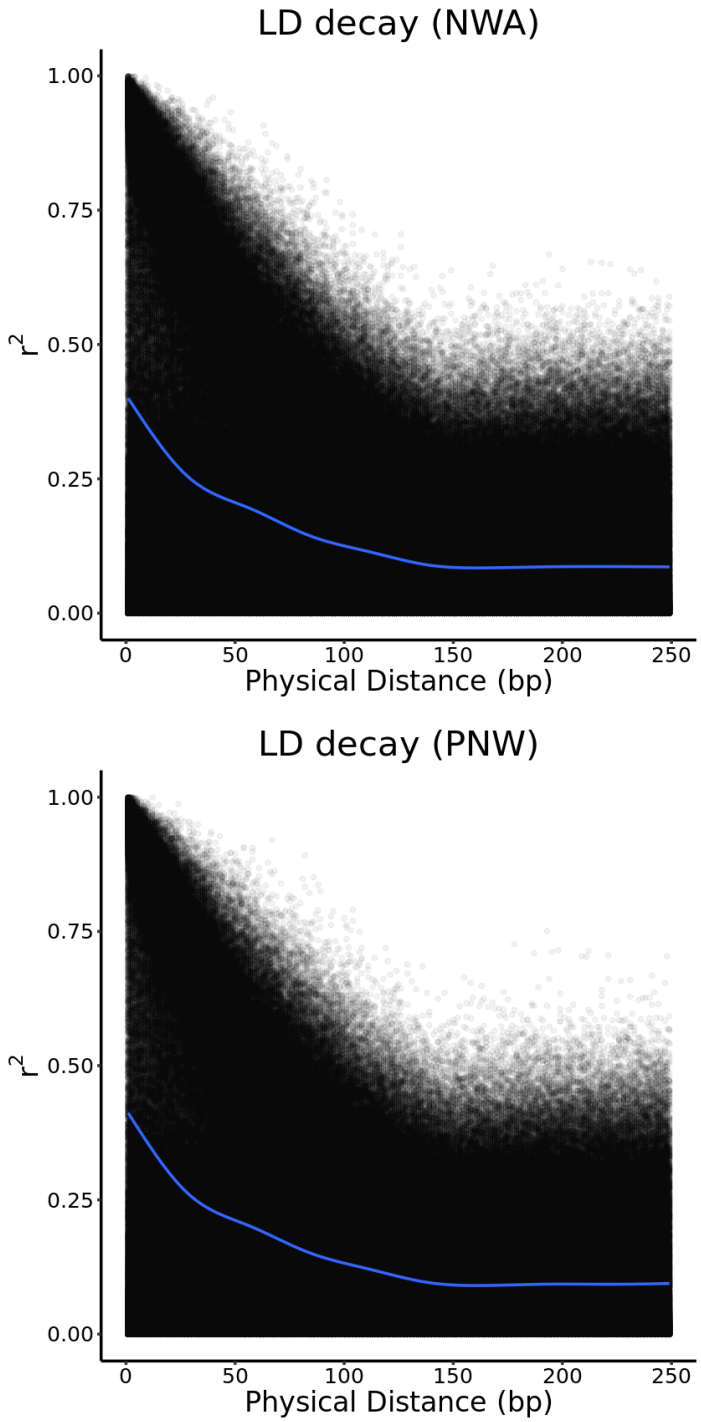

Figure S2-5. Linkage disequilibrium (LD) decays quickly in both the NWA (top) and PNW (bottom) clusters. Black points are individual locus pairs, with x-axis illustrating their physical distance along the chromosome and the y-axis the level of LD (in r^2). Blue lines are Loess regressions of r^2 vs. distance in bp.

III

Geography and developmental plasticity shape post-larval thermal tolerance in the golden star tunicate, *Botryllus schlosseri*[1]

[1] This chapter has been published as:

Tobias Z, Solow A, Tepolt CK (2024). Geography and developmental plasticity shape post-larval thermal tolerance in the golden star tunicate, *Botryllus schlosseri*. Journal of Thermal Biology, 119, 103763.

As original authors of the work, we have permission to reproduce this material in this dissertation. Minor formatting changes slightly alter the presentation of this work.

ABSTRACT

Local adaptation and phenotypic plasticity play key roles in mediating organisms' ability to respond to spatiotemporal variation in temperature. These two processes often act together to generate latitudinal or elevational clines in acute temperature tolerance. Phenotypic plasticity is also subject to local adaptation, with the expectation that populations inhabiting more variable environments should exhibit greater phenotypic plasticity of thermal tolerance. Here we examine the potential for local adaptation and developmental plasticity of thermal tolerance in the widespread invasive tunicate, *Botryllus schlosseri*. By comparing five populations across a thermal gradient spanning 4.4° of latitude in the northwest Atlantic, we demonstrate that warmer populations south of the Gulf of Maine exhibit significantly increased (~0.2 °C) post-larval temperature tolerance relative to the colder populations within it. We also show that *B. schlosseri* post-larvae possess a high degree of developmental plasticity for this trait, shifting their median temperature of survival (LT_{50}) upwards by as much as 0.18 °C per 1 °C increase in environmental temperature. Lastly, we found that populations vary in their degrees of developmental plasticity, with populations that experience more pronounced short-term temperature variability exhibiting greater developmental plasticity, suggesting the local adaptation of developmental plasticity. By comparing the thermal tolerance of populations across space and through time, we demonstrate how geography and developmental plasticity have shaped thermal tolerance in *B. schlosseri.* These results help inform our understanding of how species are able to adjust their thermal physiology in new environments, including those encountered during invasion and under increasingly novel climate conditions.

INTRODUCTION

Temperature exerts broad influence on biological systems, from setting rates of biochemical reactions to impacting the geographic distribution of species (Hochachka and Somero 2002). Understanding how species cope with changes in temperature is a fundamental concern of ecophysiology and has broad implications in an era of global climate change (Chown et al. 2010; Somero 2010). For sessile marine ectotherms, which have limited capacity for behavioral thermoregulation, the physiological effects of temperature can be especially

pronounced (e.g. Hofmann and Somero, 1995). Nonetheless, many marine invertebrates have extensive geographic distributions and persist over broad spatial gradients in temperature. Many such organisms, especially those in coastal and intertidal environments, are subject to wide swings in temperature across a range of temporal scales, including tidal, diel, seasonal, and interannual (Hochachka and Somero 2002; Deser et al. 2010). The persistence of marine ectotherms in the face of such spatiotemporal variation demonstrates their ability to tune their physiological performance to the prevailing temperature conditions.

Geographic variation in temperature is often reflected by clines in the acute thermal tolerance of a focal species across its range (Fangue et al. 2006; Kuo and Sanford 2009; Zippay and Hofmann 2010; Kelly et al. 2012; Tepolt and Somero 2014; Pereira et al. 2017; Sasaki and Dam 2019). For example, populations of the intertidal copepod *Tigriopus californicus* exhibit increasing thermal tolerance with decreasing latitude across a gradient spanning British Columbia to Baja California (Pereira et al. 2017). Such patterns can arise through two general mechanisms. First, provided sufficient heritable variation and selective pressure, separate populations of a given species may evolve different thermophysiological phenotypes (Angilletta 2009). This type of local adaptation, while historically considered to be rare in the marine environment due to the assumption of widespread connectivity, has now been demonstrated in a variety of systems (reviewed in Sanford and Kelly, 2011). By contrast, individual organisms are also often capable of altering their phenotypes depending on environmental conditions. Phenotypic plasticity, the ability of an individual genotype to produce multiple phenotypes depending on its environment (West-Eberhard 2003), is pervasive in the ocean and is a key modality for coping with environmental variability (Somero 2010; Padilla and Savedo 2013; Foo and Byrne 2016). Phenotypic plasticity is itself subject to natural selection, leading to the expectation that populations experiencing more variable environments should exhibit greater acclimatory capacity (Bradshaw 1965; Levins 1968).

Investigating how thermal tolerance varies within a species both across space and through time can inform us about how local adaptation and phenotypic plasticity are operating in natural populations. However, most studies of intraspecific variation of thermal tolerance in marine invertebrates focus on either the spatial or temporal dimension, but not both (but see

Crickenberger et al., 2015; Morley et al., 2012). When the temporal scale of environmental temperature variation is less than the generation time of the focal species, temporal variation in thermal tolerance can generally be attributed to phenotypic plasticity. Spatial variation in thermal tolerance, however, can be shaped by both local adaptation and plasticity. By studying different populations both across space and through time, one can assess how thermal tolerance is shaped by both long-term temperature trends coincident with latitude (potentially *via* local adaptation) and short-term variation due to more rapid bouts of heating and cooling (*via* phenotypic plasticity). While definitively disentangling the relative contributions of local adaptation and plasticity to variation in a particular phenotype requires a reciprocal transplant or common garden approach, ideally across multiple generations, a comparison among wild individuals from populations along a latitudinal gradient can inform us of the *potential* for local adaptation. A spatiotemporal approach can also reveal how the capacity for phenotypic plasticity differs among populations, testing the prediction that populations experiencing more variable environments should exhibit greater acclimatization potential.

Temperature tolerance is often not fixed throughout the life of an individual, but rather can shift across ontogeny (Komoroske et al. 2014; Pincebourde and Casas 2015; Klockmann et al. 2017; Ruthsatz et al. 2022). In general, it is early life history stages of marine species that are most vulnerable to temperature stress (Collin et al., 2021; Pandori and Sorte, 2019; Pineda et al., 2012; but see Peck et al., 2013; Tangwancharoen and Burton, 2014). Given the biphasic life history of many marine organisms and the importance of larval dispersal, studying thermal tolerance at early stages in development is critical for assessing the sensitivity of marine species to climatic changes (Pankhurst and Munday 2011; Przeslawski et al. 2015; Pandori and Sorte 2019). Importantly, temperature tolerance of later life stages can be affected by the thermal environment experienced by individuals earlier in ontogeny. Developmental plasticity is a form of phenotypic plasticity and is a critical means of coping with variability in the thermal environment, especially for marine and aquatic organisms (Pottier et al. 2022). Studies that simultaneously investigate how local adaptation and developmental plasticity affect thermal tolerance in natural populations of marine animals are rare (but see Pereira et al., 2017; Sasaki and Dam, 2020, 2019) but important for assessing species' ability to cope with increasing

environmental stress. Here we use cross-population comparisons and natural temporal variability in environmental temperature to investigate the potential for local adaptation and developmental plasticity of thermal tolerance in post-larvae of the colonial tunicate *Botryllus schlosseri*.

Botryllus schlosseri (Pallas, 1776) is an invasive ascidian that has successfully colonized diverse habitats around the globe (invasions.si.edu/nemesis/species_summary/159373). In the northwest Atlantic, where it is considered cryptogenic, *B. schlosseri*'s range extends from Virginia, USA to Newfoundland, Canada. *Botryllus schlosseri* is thus subject to a wide gradient of temperatures across this range, exceeding 30 °C in the summer in the south and reaching sub-zero temperatures during the northern winters. Its persistence across this broad temperature gradient suggests the potential for local adaptation and/or phenotypic plasticity of thermal tolerance. *Botryllus schlosseri* larvae are incredibly short-lived, spending mere hours in the water column, which severely restricts their capacity for larval dispersal (Grosberg 1987). Instead, most long-distance dispersal is thought to be through relatively infrequent anthropogenic translocation events or rafting on natural substrates (Worcester 1994; Thiel and Gutow 2005; Lacoursière-Roussel et al. 2012). This results in limited gene flow and a high degree of genetic differentiation among populations (Grosberg 1987; Chapter 2), thus contributing to the high potential for local adaptation in this species. Furthermore, ascidians are known to be extremely fast-evolving at the molecular level, exhibiting high mutation rates (Vinson et al. 2005; Tsagkogeorga et al. 2010, 2012), which may further potentiate adaptive divergence of thermal tolerance among populations. In addition to its high potential for local adaptation, *B. schlosseri* exhibits a high degree of physiological flexibility. Prior studies have demonstrated pronounced plasticity for traits including growth and reproductive effort in response to changes in temperature (Rinkevich et al. 1998; Newlon et al. 2003). *Botryllus schlosseri* can also establish in extremely thermally variable environments, for example, at the mouths of large estuaries, where temperatures can fluctuate in excess of 10 °C over the six hours of a tidal period (pers. obs.). This suggests that individuals must be capable of tuning their physiology over short temporal scales. This, coupled with its high potential for adaptive divergence, make *B. schlosseri* an excellent system for testing predictions about the evolution

of phenotypic plasticity by comparing populations experiencing differing levels of short-term temperature variability.

In the present study, we conducted experiments across a latitudinal and temporal gradient in temperature to address three questions: 1) how does local adaptation potentially shape thermal tolerance across populations?, 2) how does short-term temperature history affect subsequent thermal tolerance through developmental plasticity?, and 3) how does the degree of developmental plasticity vary among populations, potentially through local adaptation of phenotypic plasticity?

METHODS

Sites

Lethal tolerance of 50% survival (LT_{50}) experiments were conducted during the summer of 2022 at five sites in the northeastern USA: Rutgers University Marine Field Station, Tuckerton, NJ (RUTG), Falmouth Harbor, Falmouth, MA (FALM), Sandwich Marina, Sandwich, MA (SAND), Chatham Fish Pier, Chatham, MA (CHAT), and Darling Marine Center, Walpole, ME (DARL) (Figure 3-1A, Table 3-1). For the three Massachusetts sites, all experiments were conducted at Woods Hole Oceanographic Institution. For RUTG and DARL, experiments were conducted over two weeks at the each field station.

Environmental temperature

Short-term environmental temperature data from all sites were collected with HOBO loggers while experiments were being run. Long-term field temperatures were obtained with HOBO loggers (for FALM and SAND) or from publicly available sources (for CHAT, DARL, RUTG) (see Supplementary Materials).

Experimentation

The day prior to each experiment, 24 adult *B. schlosseri* colonies were collected by hand from the underside of floating docks and other substrates. Colonies were transported back to the laboratory and placed into individual 3.5 x 2 x 2” wells of polycarbonate compartment

boxes with ~200 ml of local seawater. Each well was fitted with five 3 x 2” extra-thick microscope slides (Fisherbrand, Pittsburgh, PA, USA) along the bottom and side walls. Colonies were left overnight at room temperature and allowed to release larvae. Like in all colonial ascidians, fertilization of ova in *B. schlosseri* occurs internally and embryos are brooded for roughly one week before being released as tadpole larvae. Thus, larvae used in this experiment experienced field temperatures throughout the majority of their development.

The following morning, each well was checked for larval release and settlement on the slides. To facilitate counting, oozooids (settled and metamorphosed larvae) were thinned to a maximum of 35 individuals per slide. Oozooids were then censused under a Stemi dissecting stereomicroscope (Zeiss, Oberkochen, Germany) at 50x magnification and examined for a heartbeat and typical development. All those showing slowed (tail still present) or abnormal development were removed prior to the experiment.

The five slides from each clutch (offspring of a single clonal colony) were partitioned across five temperature treatments, consisting of set temperatures of 30.0, 30.6, 31.1, 31.7, and 32.2 °C. These temperatures were selected based on pilot experiments run in the summer of 2021 in Woods Hole, MA and were chosen to yield approximately 100% survival at the lower end, 100% mortality at the higher end, and intermediate survivorship in between. The heat exposures were performed in polycarbonate 4”-deep 1/3 pans (Cambro, Huntington Beach, CA, USA) equipped with programmable 25 W glass aquarium heaters (YOFOTHS, Shenzhen, Guangdong, China) and HOBO Pendant temperature loggers (Onset, Bourne, MA, USA). While temperatures were set using the programmable aquarium heaters, the temperatures used for downstream analysis were those recorded by the temperature loggers.

Heat exposures lasted for 20 hours, starting from the ambient temperature of local seawater with a ramp speed of approximately 4°C/hr, which approximates the maximum rate of temperature change at our most variable site of CHAT (Figure 3-1C). After exposure, survivorship was assessed through a second census. All individuals without a detectable heartbeat were considered dead.

LT_{50} experiments were repeated through time across the summer at each site with the exception of CHAT. Because environmental temperature varied across the summer, even during

the course of the two-week-long field excursions at RUTG and DARL, we used temporal variation in environmental temperature as a proxy for developmental temperature (see *2.4. Data analysis*), essentially providing pseudo-developmental temperature treatments. While our design does not directly manipulate developmental temperature, by investigating how heat tolerance varies across short-term changes in environmental temperature we are able to make inferences about how developmental plasticity is operating in natural populations.

Data analysis

All data analysis was performed in R v. 4.1.2 (R Core Team 2013). To investigate differences in thermal tolerance among sites, we first used the R package *drc* v. 3.0.1 (Ritz et al. 2015) to derive LT_{50} estimates for each population. Two-parameter (slope and midpoint [i.e. LT_{50}]) log-logistic curves were fit to the binomial survivorship data. To test for significance of population-level differences in LT_{50}, a model with separately estimated LT_{50} values for each population was compared to a jointly-estimated LT_{50} model using a likelihood ratio test. Post-hoc comparisons between population pairs were made using the same approach, using Bonferroni-corrected p-values to account for multiple comparisons.

Model

While the package *drc* allows for a comparison of response curves among categorical variables (i.e. population), it does not contain an accessible functionality for investigating the effect of continuous variables (i.e. environmental temperature). In order to explore the effect of short-term temperature history on thermal tolerance through developmental plasticity, we created a separate statistical model, reparameterizing a conventional logistic regression model in terms of LT_{50}.

We defined $p_{jk}(x)$ as the survival probability at experimental temperature *x* of individuals collected at site j (j = 1, 2, ... , J) on day k (k = 1, 2, ... , n_j). The analysis was performed under the general model:

$$p_{jk}(x) = \frac{e^{a_{jk}\cdot\left(1-\frac{x}{g_{jk}}\right)}}{1+e^{a_{jk}\cdot\left(1-\frac{x}{g_{jk}}\right)}} \qquad (1)$$

where g_{jk} is the LT_{50} at site *j* for individuals collected on day *k*. Under this model, the parameters a_{jk} and g_{jk} represent the slope and LT_{50}, respectively (Figure S3-1). This is analogous to the model used above in the R package *drc*. Indeed, both approaches yield near identical LT_{50} values (Figure S3-2).

To assess the effect of environmental temperature on survival probability through developmental plasticity, we introduced a linear dependence of *g* (LT_{50}) on environmental temperature:

$$g_{jk} = c_{0j} + c_{1j} \cdot T_{jk} \qquad (2)$$

such that g_{jk} is modulated by a constant c_{0j}, a plasticity parameter c_{1j}, and environmental temperature T_{jk}. c_1 is referred to as the plasticity parameter, as it relates the environmental temperature experienced by oozooids as embryos during development to heat tolerance post-settlement. Greater values of c_1 indicate greater levels of plasticity, with each increment in developmental temperature inducing a greater upward shift in thermal tolerance. c_0 has a limited biological interpretation (i.e. basal thermal tolerance at a developmental temperature of 0°C) and is not a focus of our study. We defined T_{jk} as the mean temperature of the day prior to collection and chose this rather than an earlier day or longer time period because it would be less likely to impose biases in developmental stage among sites/collection days due to the temperature-dependence of development. Thus, the full model can be written as:

$$p_{jk}(x) = \frac{e^{a_{jk}\cdot\left(1-\frac{x}{c_{0j}+c_{1j}\cdot T_{jk}}\right)}}{1+e^{a_{jk}\cdot\left(1-\frac{x}{c_{0j}+c_{1j}\cdot T_{jk}}\right)}} \qquad (3)$$

We used a maximum likelihood approach to fit this model, estimating the parameters a_{jk}, c_{0jk}, and c_{1jk} for each combination of site and collection day. Because clutches born from

adults collected on the same day experienced the same field temperatures, grouping by collection day provides the most granular perspective. We used the R package *bbmle* (Bolker 2022) to perform maximum likelihood estimation, using its core function *mle2* with default parameters, apart from using Nelder-Mead as the optimization algorithm (Nelder and Mead 1965).

Clutches from adults collected from CHAT and a small number of additional clutches were excluded from these analyses. Because CHAT is only represented by a single collection date, oozooids from this site all experienced the same field temperatures, precluding an analysis of how thermal tolerance is influenced by developmental temperature. Oozooids belonging to one clutch from an adult collected at RUTG on June 5th exhibited 100% survival across the experimental temperatures, precluding fitting a survivorship curve. Two clutches from adults collected at DARL on August 29th were also excluded, as there was only one experimental temperature of the five with intermediate survivorship (not 100% survival/mortality). This results in an inability to accurately estimate the *a* and *g* parameters. See Figures S3-3 and S3-4 for plots of raw data for these two collection dates. With these clutches excluded from the dataset, we used three separate tests to evaluate 1) the potential for local adaptation of thermal tolerance among sites, 2) whether short-term temperature history affects temperature tolerance through developmental plasticity, and 3) how the degree of developmental plasticity varies among sites. See Supplementary Materials and Figure 3-2 for detail on null models used for these tests.

RESULTS

Environmental temperature

While data was missing from SAND for 2022, sites mostly followed the expected latitudinal trend in environmental temperature, with more southern sites exhibiting higher mean daily temperatures over the course of the summer (Figure 3-1B). The exception to this pattern is RUTG showing lower daily mean temperature than expected, falling in between two more northern populations on Cape Cod, FALM and CHAT.

Sites also differed in their degree of short-term (tidal or diel) temperature variability (Figure 3-1C). Both CHAT and RUTG exhibit pronounced tidal fluctuations in temperature across the summer months, with mean daily ranges of 5.24 and 4.05 °C, respectively. DARL showed some tidal variation in temperature, though to a lesser extent (mean daily range of 1.84 °C). FALM exhibited the least short-term variability, fluctuating slightly over a diel period (mean daily range of 1.05 °C). SAND, while missing from the 2022 temperature data, showed pronounced tidal variability in the summer of 2021 (mean daily range of 3.42 °C), a pattern that is likely to hold across years (Figure S3-5).

Thermal tolerance experiment

In total across the five sites, 3,728 oozooids from 93 clutches were included in 24 experiments across the summer (Table 3-1, Figure S3-6). Using the R package *drc*, the LT_{50} estimates at each site ranged from 32.01 to 32.24 °C. A model including separate LT_{50} estimates for each population explained the data significantly better than a model with a jointly estimated LT_{50} value ($\chi^2(4) = 77.30$, $p = 6.66 \times 10^{-16}$), indicating a significant effect of site. Post-hoc comparisons revealed that sites in the Gulf of Maine (DARL and SAND) exhibited significantly lower LT_{50} values than sites further to the south (CHAT, FALM, and RUTG) (Figure 3-3, Table S3-1).

After removing clutches from CHAT and from adults collected on June 5th at RUTG and August 29th at DARL, 2,907 oozooids from 80 clutches across 21 collection days at four sites remained for investigating the effect of environmental temperature history on thermal tolerance using our statistical model. Plotting the estimated LT_{50} for each collection day against the mean environmental temperature the day prior to collection demonstrated a clear relationship between thermal tolerance and the temperature experienced during development (Figure 3-4). All sites exhibited some degree of developmental plasticity, with warmer field temperatures prior to collection resulting in higher LT_{50} values. However, the degree of developmental plasticity, represented by the c_1 plasticity parameter in the model, varied by site (Figure 3-5). Sites that experienced more short-term temperature variability exhibited higher estimates for c_1 (RUTG $c_1 = 0.184$; SAND $c_1 = 0.187$) and thus greater developmental plasticity

for thermal tolerance. FALM, the site with the least short-term temperature variability (c_1 = 0.070), correspondingly exhibited the least developmental plasticity. DARL, with intermediate temperature variability, showed intermediate plasticity (c_1 = 0.142), though this estimate is subject to great uncertainty due to small sample size and a small range of field temperatures during the experimental period. In addition to LT_{50} being sensitive to environmental temperatures, survivorship curves also became steeper with increasing developmental temperatures, as indicated by upward shifts in the *a* parameter (Figure 3-6).

A likelihood ratio test comparing our full model against a "no differentiation" null model (Figure 3-2, upper left) in which c_0 and c_1 are fixed across populations revealed a significant effect of site ($\chi^2(6) = 227.43$, $p = 2.71 \times 10^{-46}$), indicating that each population's thermal tolerances responded differently to environmental temperature. A second likelihood ratio test comparing our full model against a "no plasticity" null model (Figure 3-2, lower left) in which c_0 is allowed to vary by population but c_1 is fixed at zero revealed a significant effect of environmental temperature history on thermal tolerance ($\chi^2(4) = 1{,}072.58$, $p = 3.33 \times 10^{-231}$), indicating the presence of developmental plasticity. A third likelihood ratio test comparing our full model against a "no difference in plasticity" null model (Figure 3-2, upper right) in which c_0 is allowed to vary by population but c_1 is shared among sites revealed that sites significantly differ in their degree of developmental plasticity ($\chi^2(3) = 373.24$, $p = 1.28 \times 10^{-80}$).

It is possible that our experimental design allowed for some pre-selection of embryos to occur. If elevated environmental temperatures were lethal to some heat-sensitive embryos during development, this would have biased our study to include more heat-tolerant individuals. Thus, it is conceivable that the observed pattern of higher thermal tolerance in response to greater environmental temperatures could be a result of environmentally imposed selection prior to experimentation. However, we find this unlikely, as we typically observed 100% survivorship at our lowest experimental temperature (~30.5 °C), which was significantly warmer than field temperatures.

DISCUSSION

How local adaptation and phenotypic plasticity contribute to species' ability to cope with spatiotemporal variation in temperature has broad implications for persistence in a rapidly changing environment (Somero 2010; Hoffmann and Sgró 2011; Gunderson and Stillman 2015; Seebacher et al. 2015; Donelson et al. 2019). Here, we demonstrate that post-larvae (oozooids) of the invasive tunicate *Botryllus schlosseri* exhibit differentiation of thermal tolerance among populations and possess pronounced developmental plasticity. We also show that populations vary in their degrees of developmental plasticity and that this trend coincides with the extent of short-term environmental temperature variability, with populations in more variable environments exhibiting greater developmental plasticity. This observation demonstrates the potential for local adaptation of phenotypic plasticity in this system.

Species persistence in new or changing environments relies in large part upon an ability to shift ecologically relevant phenotypes through local adaptation, phenotypic plasticity, or both (Somero 2010). However, how local adaptation and phenotypic plasticity interact to promote adaptive responses to novel conditions remains poorly understood (Ghalambor et al. 2007). Given the importance of thermal tolerance in shaping the biogeographic distributions of species (Sunday et al. 2012), our study highlights the importance of understanding the potential contributions of thermal adaptation and plasticity in the context of species invasions and global change.

Spatial variation of thermal tolerance

We found that populations differed in their thermal tolerance, and that these differences were broadly consistent with latitude. Populations south of the Gulf of Maine (CHAT, FALM, and RUTG) possessed higher LT_{50} values than those within it (SAND and DARL), matching prevailing environmental temperatures: the Gulf of Maine, bounded by Cape Cod and Nova Scotia, harbors cooler waters than those further south (Figure 3-1). That we observe differentiation in thermal tolerance across this break, with populations clustering according to their latitudinal position relative to Cape Cod, may suggest local adaptation to temperature among populations of *B. schlosseri*. By comparing the results in Figure 3-4 to the null models in

Figure 3-2, we can observe vertical differentiation of reaction norms, further bolstering support for local adaptation. However, because individuals were taken from the wild and not lab-reared in a common garden, we cannot rule out an effect of environment. Indeed, our temporal data demonstrate a high degree of phenotypic plasticity for this trait (see below). Thus, the differences in the environmental temperatures at the times of collection at each site have likely also contributed to the differences we observe in temperature tolerance among populations.

There has been somewhat limited investigation of the thermal tolerance of *B. schlosseri*, with most studies focusing on a single population and time of year (Brunetti et al. 1980; Epelbaum et al. 2009). Sorte et al. (2011) found that adult colonies of *B. schlosseri* from Massachusetts had greater heat tolerance than those from California, consistent with higher summer temperatures in the northwest Atlantic. Interestingly, for both of their populations, adult LT_{50} values were lower than those reported here (Sorte et al. 2011). This suggests that adults may be more sensitive to heat than oozooids, which is contrary to expectations (Pandori and Sorte 2019). However, methodological differences, such as the length of heat exposure, which was slightly shorter in our study (20 vs. 24 hours), could also contribute to the observed differences.

While the geographic differentiation of thermal tolerance could be an effect of the environment, *B. schlosseri* and ascidians more generally possess several characteristics that promote local adaptation. First, tunicates are considered to have extremely high rates of molecular evolution (Vinson et al. 2005; Denoeud et al. 2010; Tsagkogeorga et al. 2010, 2012; Berna and Alvarez-Valin 2014). Second, ascidian populations tend to harbor remarkable levels of genetic diversity, even in putatively bottlenecked invasive populations (Rius et al. 2008; Reem et al. 2013; Zhan et al. 2015). Such standing genetic diversity can serve as a pool of adaptive variation upon which natural selection can act to drive local adaptation (Barrett and Schluter 2008). Third, ascidians tend to exhibit extremely restricted larval dispersal, with *B. schlosseri* larvae typically only settling on the scale of a few meters from their parent colony (Grosberg 1987). This can result in markedly high levels of genetic differentiation across small spatial scales (Grosberg 1987; Yund and O'Neil 2000), contributing to the high potential for evolutionary divergence of thermal tolerance in *B. schlosseri*.

Despite historical assumptions of the limited potential for local adaptation in marine systems, many recent studies have demonstrated local adaptation in marine organisms (DuBois et al., 2022; Kuo and Sanford, 2009; Pereira et al., 2017; reviewed in Sanford and Kelly, 2011). For example, in a multigenerational common garden experiment using the intertidal snail *Nucella canaliculata*, Kuo and Sanford (2009) demonstrated evolutionary divergence of upper thermal limits among populations. Beyond this single example, recent work has shown that the evolutionary differentiation of thermal limits is more pervasive in the marine environment than on land (Sasaki et al. 2022). Marine organisms, especially those that are sessile, generally have more limited capacity for behavioral thermoregulation and more restricted access to thermal refugia than terrestrial species (Sunday et al. 2015; Pinsky et al. 2019; Antão et al. 2020). This likely renders them more susceptible to natural selection, driving local adaptation of thermal physiology (Sasaki et al. 2022). This greater potential for thermal adaptation in the ocean has implications for marine species persistence under a changing climate. Evolutionary differences in thermal physiology among populations can confound predictions of distributional shifts under future climate scenarios, and render highly locally adapted species more susceptible to climatic changes (Bennett et al. 2019). Conversely, local adaptation to heterogeneous environments can promote persistence by preserving adaptive variation that can be redistributed via natural dispersal or programs of assisted gene flow (Garant et al. 2007; Aitken and Whitlock 2013). Clearly, the consequences of local adaptation in the ocean for species persistence are complex and rely heavily on the potential for species to adapt on contemporary time scales.

Developmental plasticity of thermal tolerance

In addition to differences among sites, we found that the mean temperature of the day prior to collection had a strong effect on the measured thermal tolerance of *B. schlosseri* oozooids (Figure 3-4). This demonstrates a clear effect of environment on thermal tolerance and suggests the presence of developmental plasticity in this system. Developmental plasticity may thus be a key mechanism by which *B. schlosseri* oozooids cope with temperature stress.

Developmental plasticity of thermal tolerance has been demonstrated in a wide variety of taxa and is especially pervasive in marine and aquatic organisms (Pottier et al. 2022). In a recent meta-analysis, Pottier et al. (2022) synthesized the findings of 150 experimental studies investigating developmental plasticity of thermal tolerance. Using acclimation response ratios (ARRs), or the change in thermal tolerance for a 1°C increase in developmental temperature, they found that marine and aquatic animals tend to be much more plastic than terrestrial animals (mean ARR of 0.209 vs. 0.051). Our estimates of c_1, which is analogous to ARR, were 0.184 and 0.187 for RUTG and SAND, respectively, slightly lower but comparable to the mean ARR for marine and aquatic organisms found by Pottier et al. (2022). Given the pervasiveness of developmental plasticity of temperature tolerance in the marine realm, it is not altogether surprising that we observe this in *B. schlosseri*. However, our study represents an important first step in understanding this phenomenon in ascidians.

To our knowledge, our study comprises the first investigation of developmental plasticity of tolerance to any abiotic stressor, including temperature, in an ascidian. However, previous studies have investigated other types of phenotypic plasticity in ascidians (Chadwick-Furman and Weissman 1995; Newlon et al. 2003; Renborg et al. 2014). For example, Renborg et al. (2014) demonstrated that sensitivity to salinity in embryos of *Ciona intestinalis* depended on the salinity of the parental environment prior to embryogenesis, establishing transgenerational plasticity of salinity tolerance. Given the long-standing consideration of the role for phenotypic plasticity in species invasions (see below) and the remarkable success of ascidians as an invasive taxon (Zhan et al. 2015), further study of phenotypic plasticity in ascidians is warranted.

Phenotypic plasticity has long been thought to play a major role in the success of invasive species (Baker 1965; Richards et al. 2006; Davidson et al. 2011). Generalist species with broad ecological tolerances and pronounced phenotypic plasticity have been assumed to be ideal invaders (Daehler 2003; Higgins and Richardson 2014). This extends to thermophysiological phenotypes. For example, the widespread invasive European green crab, *Carcinus maenas*, has extreme thermal breadth, with limits far surpassing those of co-occurring native species (Tepolt and Somero 2014). Additionally, it exhibits pronounced thermal phenotypic plasticity, shifting its upper and lower limits substantially in response to short-term

acclimation. While its invasive status in unclear in the northwest Atlantic, *B. schlosseri* is an invasive species elsewhere and has established in waters spanning a vast thermal gradient. Its persistence across this broad gradient suggests a pronounced ability to cope with spatially variable environmental temperatures, whether through local adaptation, phenotypic plasticity, or both. Given our observations, it is likely that developmental plasticity has played some role in its ability to thrive across diverse habitats. Further study is warranted to investigate how other types of phenotypic plasticity, for example, acclimatization in adults or transgenerational plasticity, may also shape the thermal tolerance of this species.

While we ascribe temporal variation in thermal tolerance to phenotypic plasticity, it is worth noting that there may be a hereditary component as well. Fluctuating selection by environmental temperature may result in shifts in the genotypic composition of *B. schlosseri* populations, with more heat-sensitive genotypes produced earlier in the summer and more heat-tolerant genotypes later in the summer. While this may contribute to the patterns described at sites sampled over many months (e.g. FALM), because in some cases we are describing variation across limited duration (e.g. less than two weeks at RUTG), we consider it more likely that phenotypic plasticity plays a dominant role.

Potential for local adaptation of developmental plasticity

Theory predicts that populations in more variable environments should evolve greater phenotypic plasticity (Bradshaw 1965; Levins 1968; Schlichting 1986). While evidence for this prediction for thermal tolerance is equivocal (see meta-analysis by Barley et al., 2021), we found that sites experiencing more short-term temperature variability harbored populations with greater degrees of developmental plasticity, consistent with local adaptation of phenotypic plasticity. Again, because the individuals used in these experiments were born from wild-collected adults, it is impossible to disentangle the effects of environment from heredity with our data. Nonetheless, given the high potential for local adaptation in this system (see above), it is likely that it plays some role in generating the patterns observed here.

Despite predictions for the evolution of phenotypic plasticity in variable environments, there are surprisingly few studies demonstrating local adaptation of phenotypic plasticity

(Hendry 2016). Many, like ours, relate differentiation in phenotypic plasticity to the degree of environmental heterogeneity experienced by populations (De Meester 1996; Gianoli and González-Teuber 2005; Richter-Boix et al. 2015; Phillips et al. 2016; Reger et al. 2018; Diaz et al. 2021). Others use experimental evolution approaches, comparing lineages reared in constant conditions to those reared in a variable environment (Reboud and Bell 1997; Leggett et al. 2013; Condon et al. 2014). While not definitive, our results add to a growing body of literature demonstrating the potential for local adaptation of phenotypic plasticity in natural populations.

Notably, plasticity in one life stage can affect plasticity at later life stages (Beaman et al. 2016). For example, Healy et al. (2019) demonstrated that developmental temperature can affect acclimatory capacity at later life stages in the intertidal copepod *Tigriopus californicus*. Similarly, this cascading effect can also apply across generations through transgenerational plasticity (Donelson et al. 2018). If the adult *B. schlosseri* colonies from which larvae were collected experienced a warmer or more variable thermal environment during their lifetime, it may be advantageous for their larvae to exhibit developmental plasticity for temperature tolerance, generating a pattern like that which we found in our study. Reaction norm shape in offspring has been shown to depend in part on parental environment (Salinas et al. 2013; Donelson et al. 2016; Stein et al. 2018; Wadgymar et al. 2018; Cavieres et al. 2019; Sturiale and Bailey 2021). Despite these examples, the relationship between transgenerational plasticity and within-generation plasticity remains understudied (Donelson et al. 2018; Donelan et al. 2020), but interactions between the two could play some role in our observation of increased developmental plasticity at sites with more short-term temperature variability. Manipulative multi-generational experiments would have to be performed to test this potential in more detail.

Steepening of survivorship curves and the limits of thermal tolerance

While the main metric of interest in our study was LT_{50}, we have also shown that the steepness of the survivorship curves, as contained within the *a* parameter in our model, increases with greater developmental temperature (Figure 3-6). This steepening effect suggests that 1) development at elevated temperatures reduces inter-individual variation in thermal

tolerance and 2) there may be relatively hard upper limits for thermal tolerance in this system. There is undoubtedly variation for thermal tolerance within populations of *B. schlosseri*: some oozooids survive at a particular experimental temperature while others die. It is apparent from this steepening observation that the capacity for developmental plasticity is not equal across individuals. Higher developmental temperatures seem to allow those oozooids with lower basal thermal tolerance to shift their probability of survival upwards, while those with higher basal thermal tolerance are more constrained, leading to a steepening of the survivorship curve. This observation is consistent with studies that demonstrate a negative relationship between basal thermal tolerance and thermal tolerance plasticity (Faulkner et al., 2014; Kelly et al., 2017; Morgan et al., 2020; Phillips et al., 2016; Sasaki and Dam, 2021, 2019; Stillman, 2003; but see Calosi et al., 2008), which may be indicative of a trade-off (van Heerwaarden and Kellermann 2020; Barley et al. 2021).

Constraints on the plasticity and evolution of upper thermal limits appear to be common across taxa, while lower limits appear to be more labile (Gaston and Chown 1999; Munoz et al. 2014; Phillips et al. 2016; van Heerwaarden et al. 2016). For example, in the tsetse fly, *Glossina pallidipes*, acclimation temperature had a pronounced effect on tolerance to cold temperatures, but upper thermal limits were less flexible (Terblanche and Chown 2006). The meta-analysis by Pottier et al. (2022) found that while marine and aquatic organisms exhibit greater levels of developmental plasticity for heat tolerance, this shift is unlikely to perfectly compensate for anticipated levels of warming (Gunderson and Stillman 2015). On an evolutionary scale, there may be strong phylogenetic constraints on upper thermal limit evolution (Kellermann et al. 2012). Several meta-analyses have demonstrated that while lower thermal limits vary with latitude, as would be expected through directional selection on temperature tolerance, upper thermal limits appear to be rather inflexible, again pointing towards the potential importance of evolutionary constraints on upper thermal limits (Araújo et al. 2013; Hoffmann et al. 2013; Sunday et al. 2019).

While we are not aware of studies that explicitly discuss this pattern of survivorship curve steepening, several have demonstrated this effect as a component of plasticity and/or adaptation of heat tolerance (Pereira et al. 2017; Sasaki and Dam 2019, 2021; Rebolledo et al.

2021). For example, Sasaki and Dam (2019) compared thermal tolerance and its plasticity across populations of the coastal and estuarine copepod *Acartia tonsa*. At increased developmental temperatures, not only did LT_{50} increase, but so did the steepness of the survivorship curves (p. 4153, Figure 2). Correspondingly, they showed that thermal limits (as reflected in LT_{10}, or the temperature inducing 10% survival) were less labile than LT_{50} (their Figure S2); if limits shift less than midpoints in response to higher developmental temperatures, curves necessarily become steeper. This pattern was also observed in a related study investigating adaptation of thermal tolerance in *A. tonsa* via experimental evolution (Sasaki and Dam 2021), suggesting that both plasticity and evolutionary adaptation can have this effect. While it seems there has been minimal attention on this phenomenon on survivorship curve steepening, these examples indicate that it may be widespread. Given its potential implications for the evolution and plasticity of upper thermal limits, further investigation is warranted.

CONCLUSION

We have shown that temperature tolerance in *B. schlosseri* oozooids varies by population across a latitudinal gradient, that tolerance is influenced environmental temperature through developmental plasticity, and that the degree of developmental plasticity varies according to the level of short-term temperature variability at each site. Together, these results demonstrate how local adaptation and phenotypic plasticity may underly spatiotemporal variation of temperature tolerance in this system. Importantly, our findings suggest the potential for local adaptation of developmental plasticity, with populations inhabiting more thermally variable sites exhibiting greater levels of plasticity. While further investigation using a multigenerational approach would be necessary to fully disentangle the relative contributions of heritable versus environmental effects, by taking advantage of natural spatiotemporal variability in temperature, our study documents important patterns of temperature tolerance that could have implications for species invasions and population persistence in an era of global change.

Organisms in all habitats are under increasing threat of climatic changes, but it is marine ectotherms that are considered most at risk with an increase in global temperatures (Pinsky et

al. 2019). Many marine species are living near their upper thermal limits (Pinsky et al. 2019), and the rate of warming may exceed the ability of evolution and/or phenotypic plasticity to buffer species against its effects (Gunderson and Stillman 2015; Bennett et al. 2021). Despite some ability to shift thermal tolerance upward in response to elevated temperatures, the steepening of survivorship curves observed here and in other studies suggests that there may be strong constraints on the plasticity and evolution of upper thermal limits in the ocean. In the absence of widespread adjustments to thermal niches, we may expect to see increasing rates of marine extirpation and range shifts (Sunday et al. 2012; Donelson et al. 2019). Indeed, it has been demonstrated that distributional shifts in the ocean track climate velocities more closely than on land (Lenoir et al. 2020). Understanding the interplay between evolutionary adaptation, phenotypic plasticity, and range shifts in response to climate warming is key to making predictions about species persistence in the modern ocean (Donelson et al. 2019).

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TABLES

Table 3-1. Collection site information, with LT_{50} and the lower and upper bounds of 95% confidence intervals.

Site	Code	Lat	Lon	Oozooids	Clutches	LT_{50} (°C)	Lower	Upper
Tuckerton, NJ	RUTG	39.509	-74.325	900	24	32.21	32.15	32.28
Falmouth, MA	FALM	41.548	-70.603	769	18	32.21	32.15	32.27
Chatham, MA	CHAT	41.689	-69.951	731	10	32.24	32.17	32.32

Sandwich, MA	SAND	41.772	-70.503	1003	22	32.01	31.95	32.06
Walpole, ME	DARL	43.935	-69.581	325	19	32.06	32.00	32.12

FIGURES

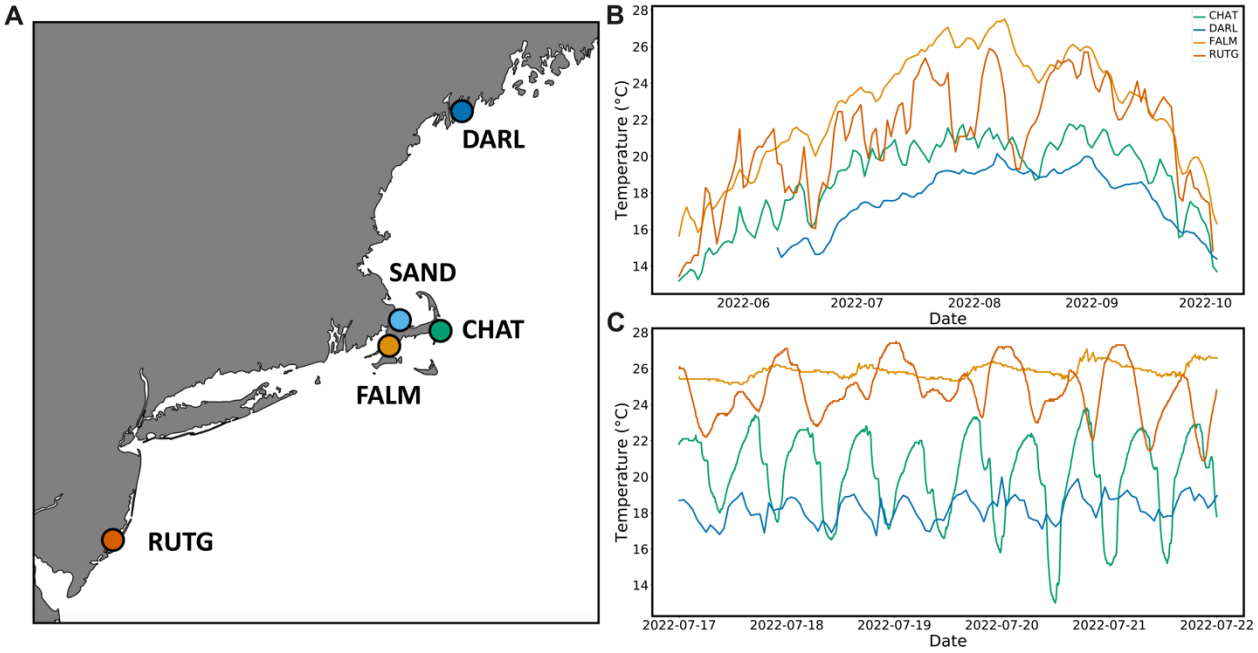

Figure 3-1. Sites differ in mean summer temperature trends and the extent of daily variability. (A) Map of sites. (B) Mean daily environmental temperatures at study sites during the late spring, summer, and early fall of 2022. (C) Environmental temperature data during a five-day period from July 17-22, 2022. Note the differences in temperature variability among sites. Temperature data for SAND is missing due to temperature logger failure.

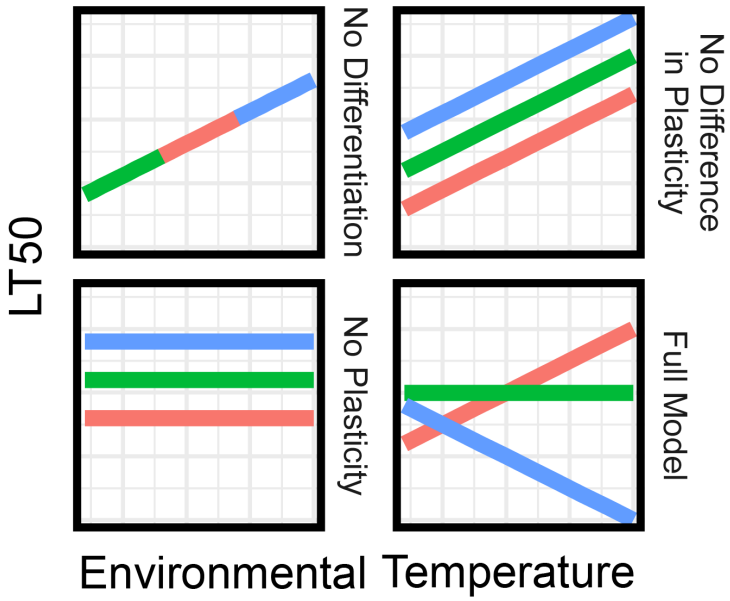

Figure 3-2. Schematic of null and general statistical models. Here the lines represent the response of thermal tolerance (LT_{50}) to environmental temperature, with color indicating population. Upper left represents the null hypothesis that there is no differentiation among populations (i.e. single, global estimates of c_0 and c_1), and is depicted by three identical, overlapping lines. Lower left represents the null hypothesis that there is no phenotypic plasticity for thermal tolerance (i.e. population-specific estimates of c_0, c_1 globally fixed at 0). Upper right represents the null hypothesis that there is no difference among populations in their degree of phenotypic plasticity (i.e. population-specific estimates of c_0, a single, global estimate of c_1). The lower right depicts the general model, which allows each population to vary with respect to model parameters (population-specific estimates of c_0 and c_1). See Supplemental Materials for additional information on null model design.

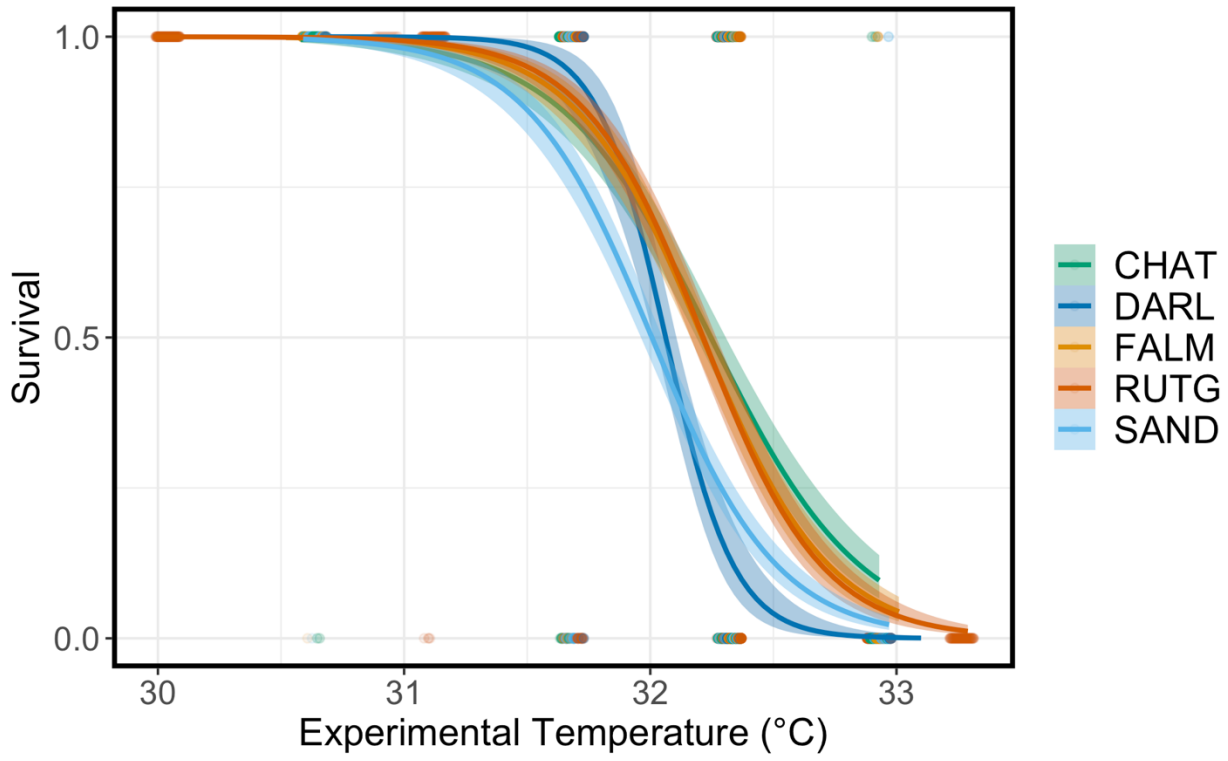

Figure 3-3. Sites differ in thermal tolerance. Survivorship curves are displayed for each population, aggregated across all collection days. Curves derived by logistic regression. Shaded areas represent standard error.

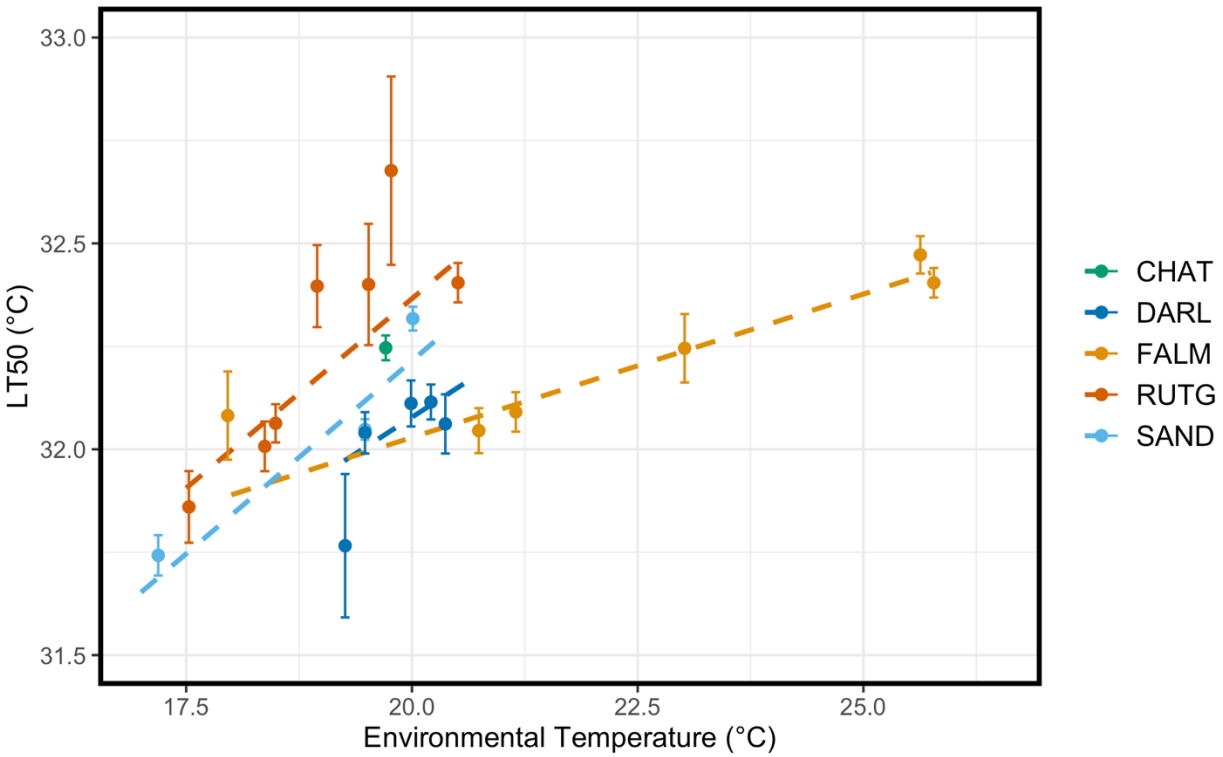

Figure 3-4. Developmental plasticity results in upward shifts in thermal tolerance in response to higher environmental temperatures. LT_{50} is plotted against mean temperature of day prior to collection. Points represent the LT_{50} estimate across all clutches born on a particular day. Error bars represent 95% confidence intervals. For each population except CHAT, dashed lines represent the response of LT_{50} to environmental temperature, as given by $g = c_{0jk} + c_{1jk}*T_{jk}$, where j represents population, k represents collection day, and T_{jk} represents environmental temperature at site j on day k (see 2. Materials and Methods).

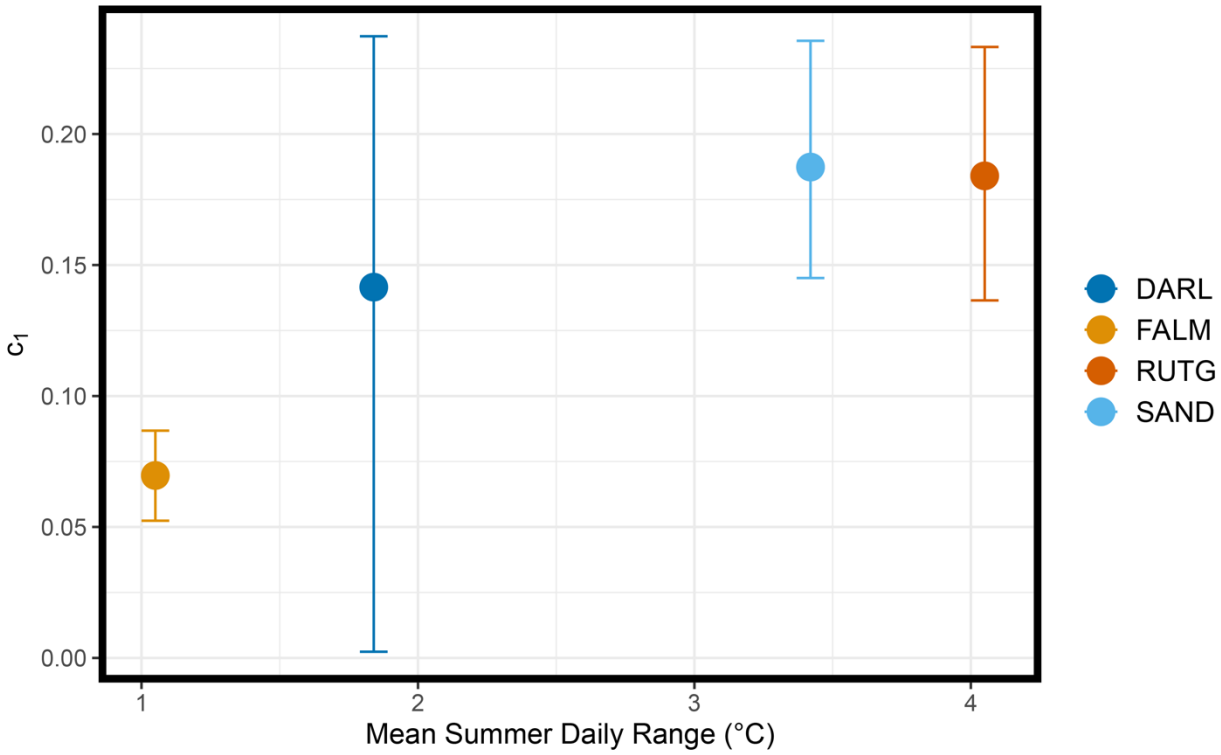

Figure 3-5. The degree of developmental plasticity varies across sites according to degree of temperature variability. Estimates for c_1 parameter (plasticity) plotted as a function of mean daily range of summer temperatures at each site. Error bars represent 95% confidence intervals.

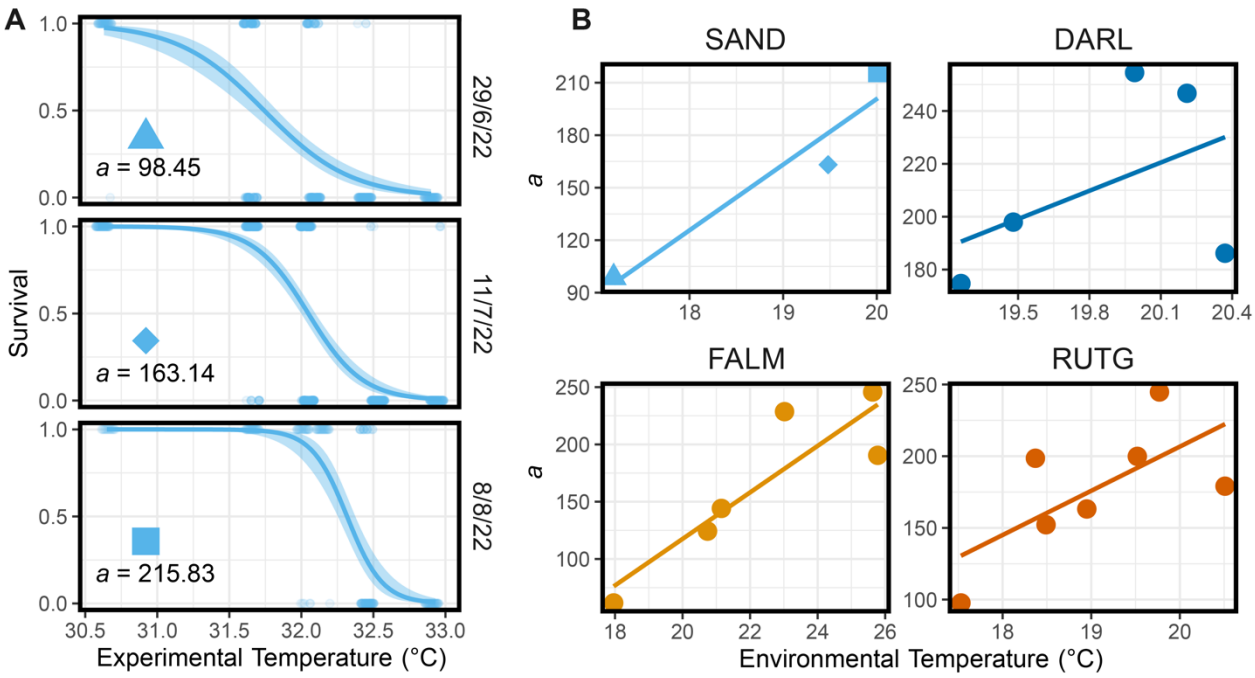

Figure 3-6. Survivorship curves become steeper with increasing environmental temperature, as represented by upward shifts in the *a* parameter, which encapsulates curve steepness. (A) Survivorship curves for the three days of collection at SAND. As the summer progresses and temperatures rise, the curves steepen. Symbols (triangle, diamond, and square) denote each day of collection and are repeated in panel B. (B) Estimates for *a* increase with increasing environmental temperatures. Line represents linear regression of *a* against environmental temperature. Different axis scales have been used for each panel to highlight relationships within each site.

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SUPPLEMENTARY METHODS

Environmental temperatures:

For CHAT, data were obtained from the National Oceanic and Atmospheric Administration's (NOAA's) Center for Operational Oceanographic Products and Services station no. 8447435 (https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_8447435.html), which is located at the same dock used for *B. schlosseri* collection. For RUTG, data were obtained from the United States Geological Survey's National Water Information System station no. 01409335 (https://erddap.sensors.ioos.us/erddap/tabledap/gov_usgs_waterdata_01409335.html), located within the Rutgers University Marine Field Station boat basin, the collection site. For DARL, data were obtained from the University of Maine's Land/Ocean Biogeochemical Observatory Lower Damariscotta buoy (http://maine.loboviz.com/loboviz/), which is located 470 m south of the collection site at the Darling Marine Center dock.

The HOBO loggers at SAND failed prior to the experimentation period, resulting in no usable temperature record. However, we collected temperature data in 2021 for a preliminary experiment. With these data, we performed a multiple regression, using 2021 daily mean temperature data from both the nearby NOAA National Buoy Data Center station no. 44090 (https://erddap.sensors.ioos.us/erddap/tabledap/edu_ucsd_cdip_221.html) and CHAT as predictor variables. The model was fitted using 80% of the time series and tested on the remaining 20%. Despite marginal explanatory power ($r^2 = 0.215$, see Figure S3-7), the resulting

model was then used to predict 2022 daily mean temperatures at SAND for use in analyses investigating the effect of short-term environmental temperature on LT_{50}.

Experimentation:

Initial collections at DARL resulted in minimal release of larvae. Accordingly, some days' collections were carried out at South Bristol harbor, 8 km south of DARL, where oozooid collection was more successful. Rather than treat this as a separate site, results from oozooids collected at South Bristol were pooled with those from DARL, as sample size was quite low for each of the two collection sites. Water temperatures from South Bristol were similarly recorded using a HOBO logger. For assessing the effect of short-term temperature history on thermal tolerance on days where oozooids were collected from South Bristol, we used temperature data from South Bristol rather than from DARL.

Model:

Differences among sites

To evaluate the potential for local adaptation, we compared our full model with separate a, c_0, and c_1 parameters for each collection day against a null model in which a was permitted to vary according to collection day but c_0 and c_1 were fixed at the global estimate.

$$H_0^1: c_{0j} = c_0, c_{1j} = c_1, j = 1,2,\dots,J \qquad (4)$$

This model represents a situation in which there is no difference in temperature tolerance among sites, or a "no differentiation" model. For a graphical representation of this null model, refer to Figure 3-2 (upper left). We used a likelihood ratio test to assess significance.

Effect of short-term temperature history

To examine how short-term temperature history affects temperature tolerance through developmental plasticity, we compared our full model against a null model in which a and c_0 were permitted to vary according to collection day, but c_1 was constrained at a value of zero.

$$H_0^2: c_{1j} = 0, j = 1,2, \dots, J \qquad (5)$$

This model reflects a situation without any dependence of temperature tolerance upon temperature history, or a "no plasticity" null model (Figure 3-2, lower left). We used the one-sided alternative hypothesis $H_1^2: c_{1j} > 0$ to test for a positive effect of developmental temperature upon temperature tolerance (adaptive developmental plasticity). Again, we used a likelihood ratio test to assess significance.

Differences in developmental plasticity among sites

To assess whether sites exhibited differences in their degree of developmental plasticity, we compared our full model against a null model in which a and c_0 were permitted to vary according to collection day, but sites possessed a shared, jointly-estimated c_1 parameter.

$$H_0^3: c_{1j} = c_1, j = 1,2, \dots, J \qquad (6)$$

This model represents a situation in which sites exhibit plasticity but do not differ in their degree of plasticity, or a "same plasticity" model (Figure 3-2, upper right). Significance was assessed using a likelihood ratio test.

SUPPLEMENTARY TABLES

Table S3-1. Matrix showing significance testing for comparisons of LT_{50} values among sites. Values are Bonferroni-adjusted p-values.

	DARL	FALM	RUTG	SAND
CHAT	1.089e-05	1	1	1.394e-09
DARL		1.092e-4	9.744e-05	.7435
FALM			1	1.350e-08
RUTG				6.747e-09

SUPPLEMENTARY FIGURES

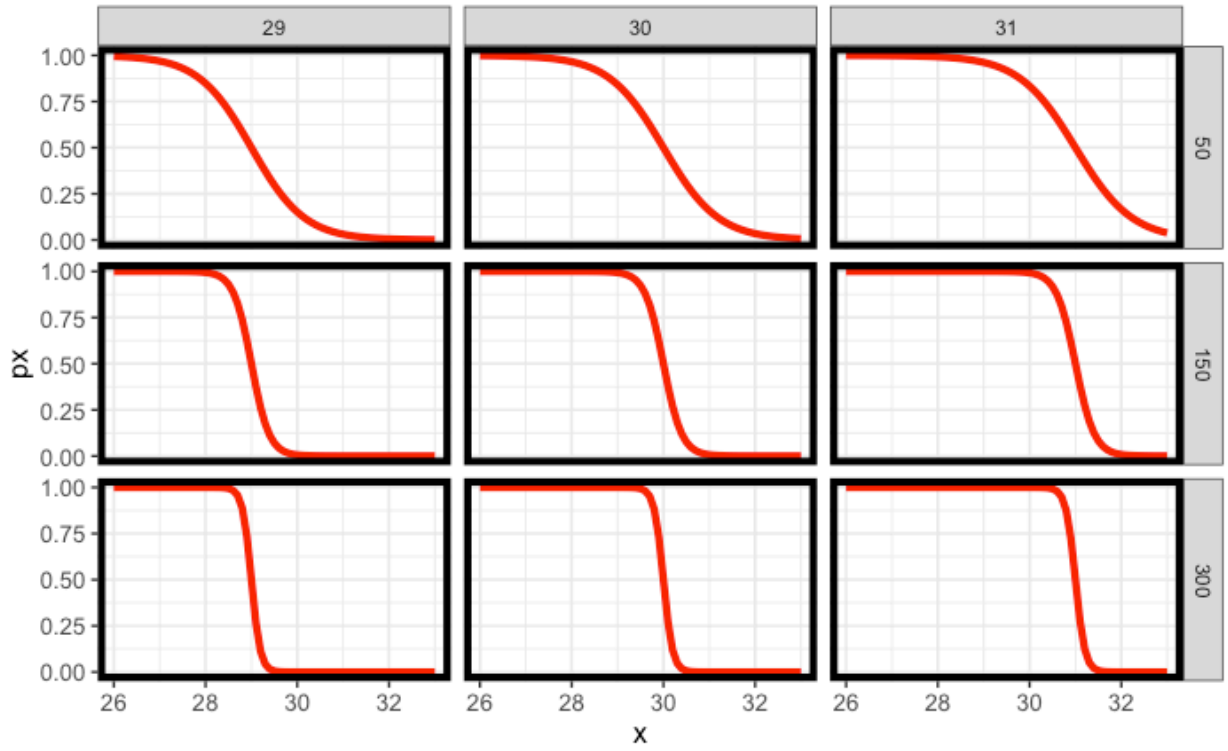

Figure S3-1. Illustration of varying *a* and *g* parameters. x represents the experimental temperature and px represents the probability of survival. Horizonal facets vary *g* (LT_{50}) from 29-31 °C. Vertical facets vary *a* from 50-300. Note that varying *g* results in the survivorship curve shifting horizontally along x-axis, whereas varying *a* changes the steepness of the curve.

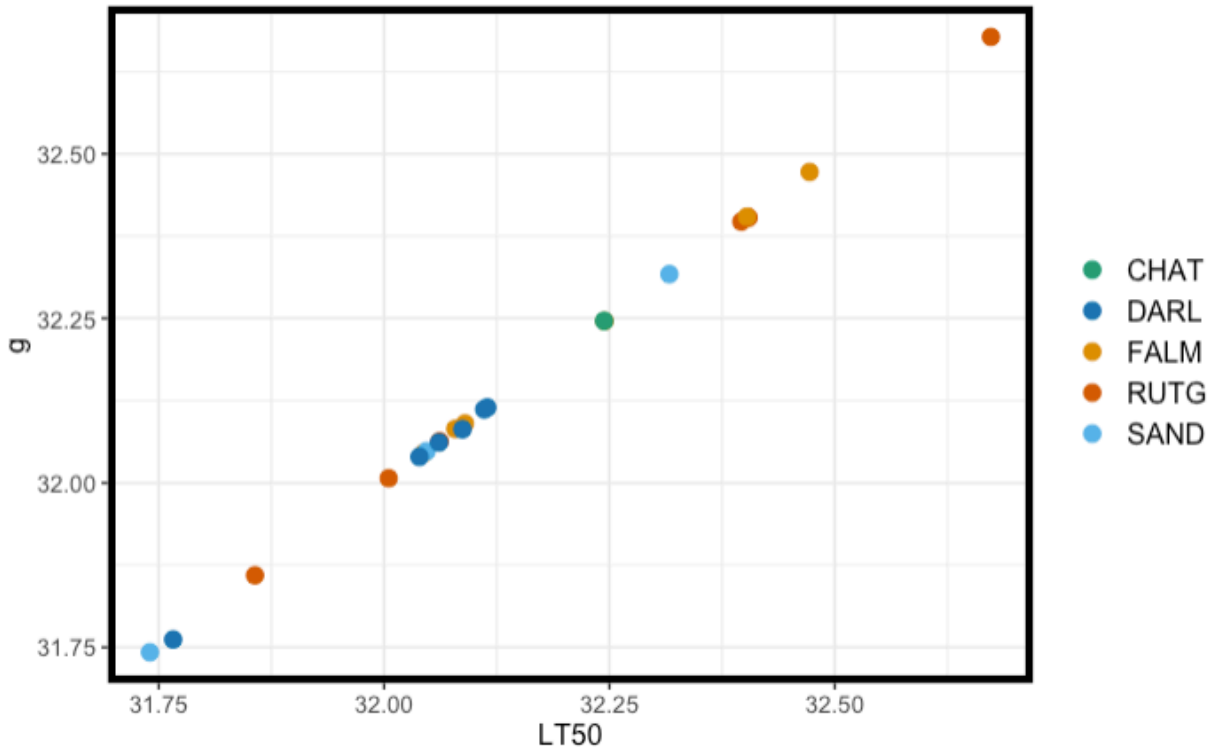

Figure S3-2. Estimates of *g* (LT_{50}) from our model vs. estimates of LT_{50} from package *drc* for each collection day. Note that both methods result in near identical values.

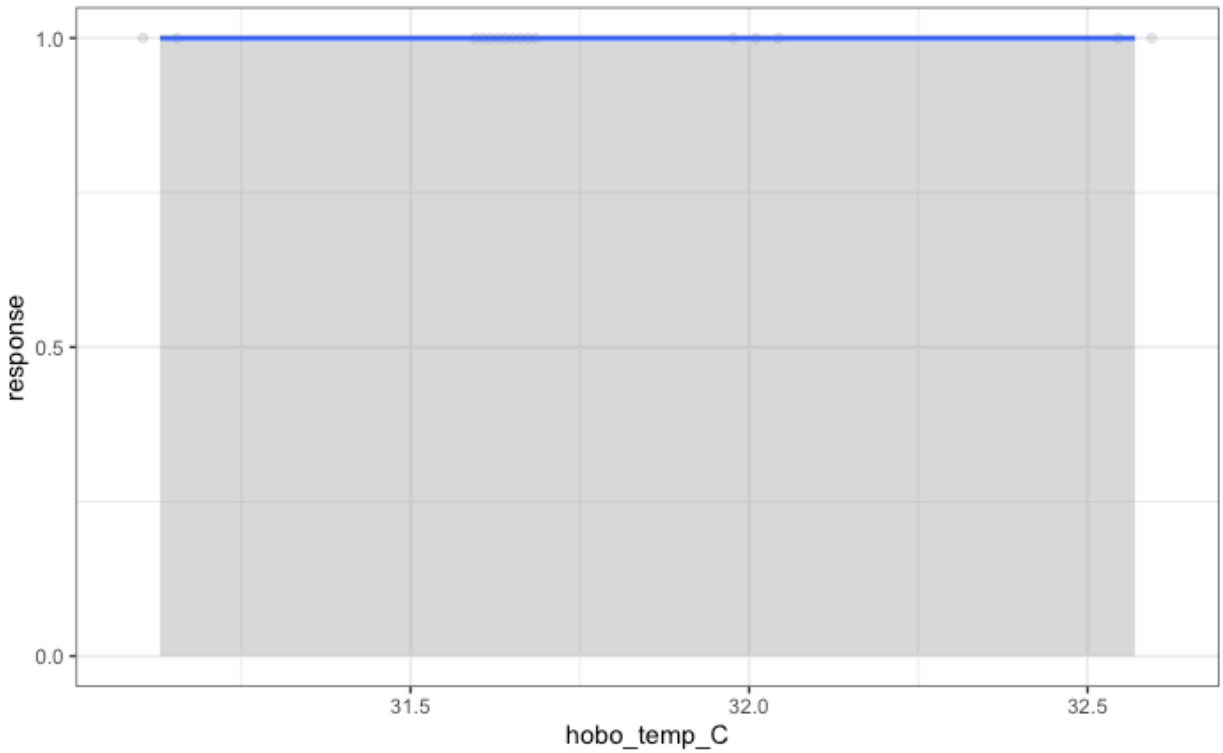

Figure S3-3. June 5th RUTG experiment. Once clutch with 100% survival across all four temperature treatments. This day's data was removed from subsequent analyses focusing on effect of environmental temperature.

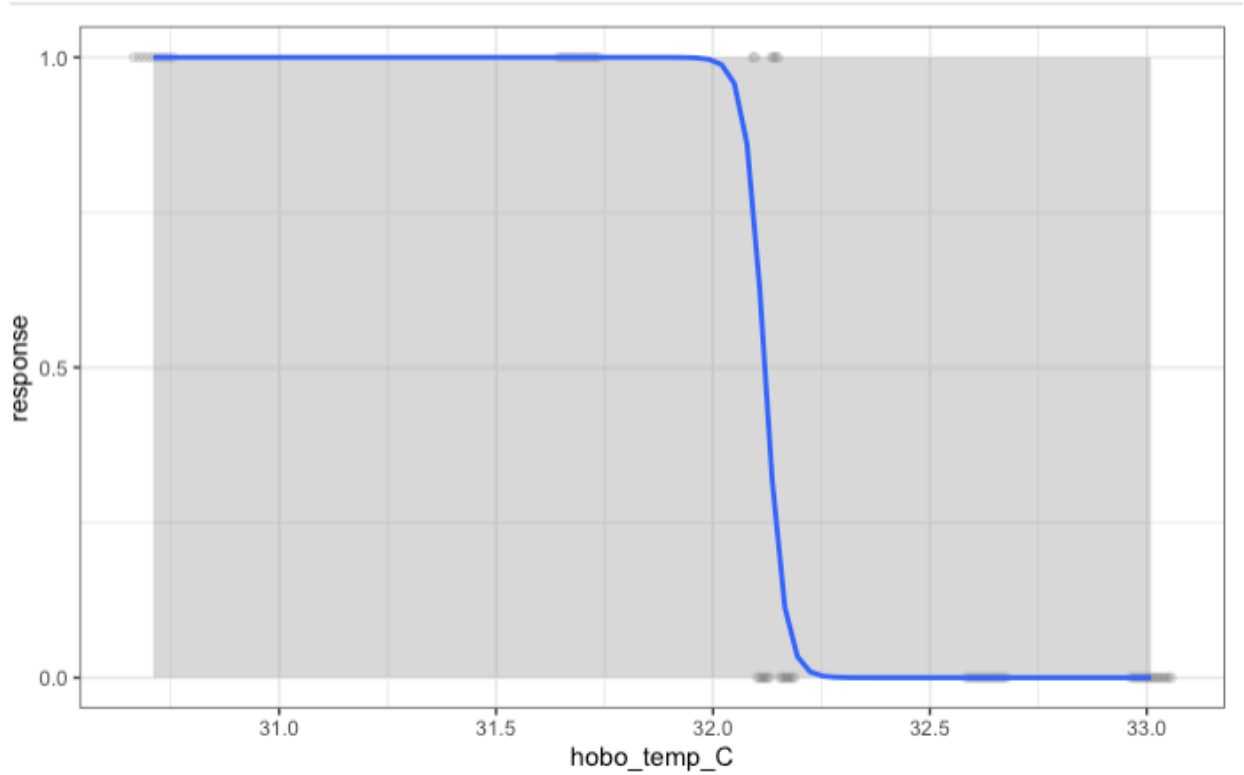

Figure S3-4. August 29th DARL experiment. Two clutches with intermediate survivorship at only one temperature, precluding the estimation of *a*. This day's data was removed from subsequent analyses focusing on effect of environmental temperature.

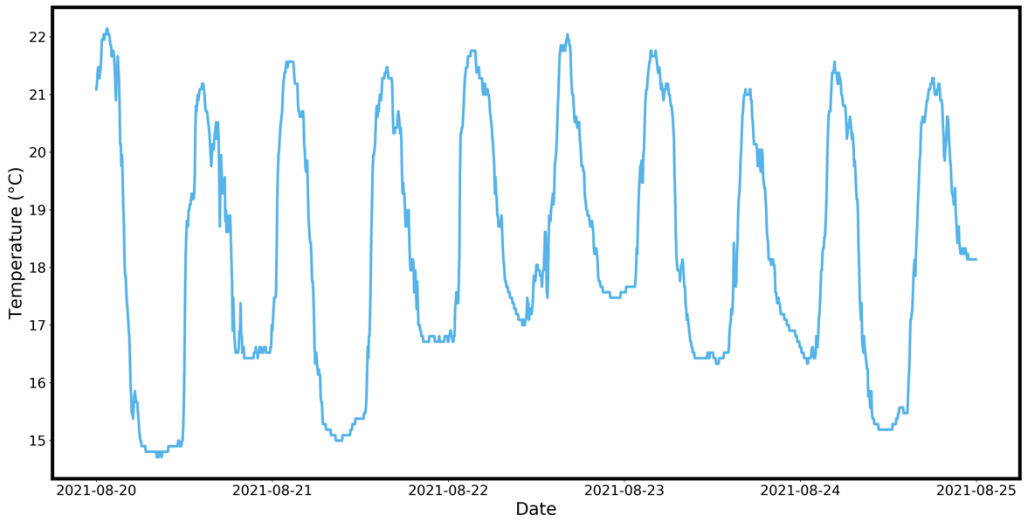

Figure S3-5. SAND temperature over six-day period during the summer of 2021.

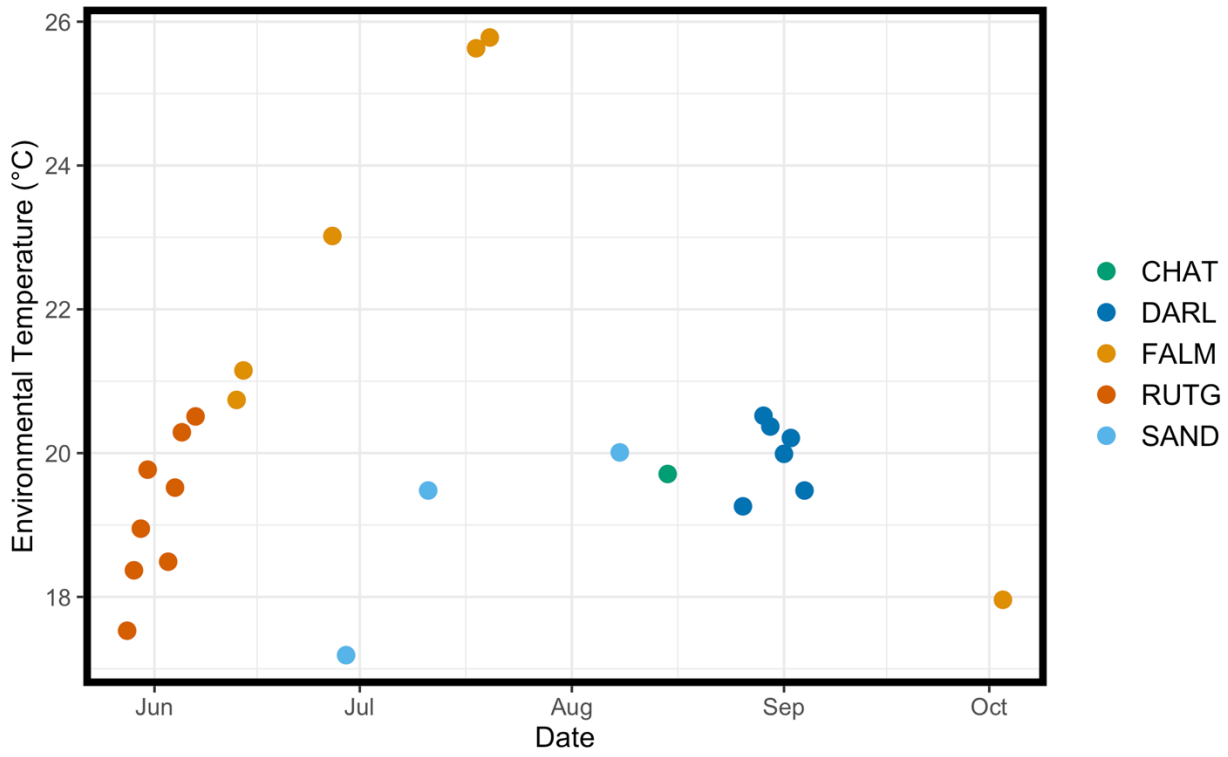

Figure S3-6. Collection dates for the 24 experiments conducted over the summer and fall of 2022, and the corresponding environmental temperatures of the day prior to collection.

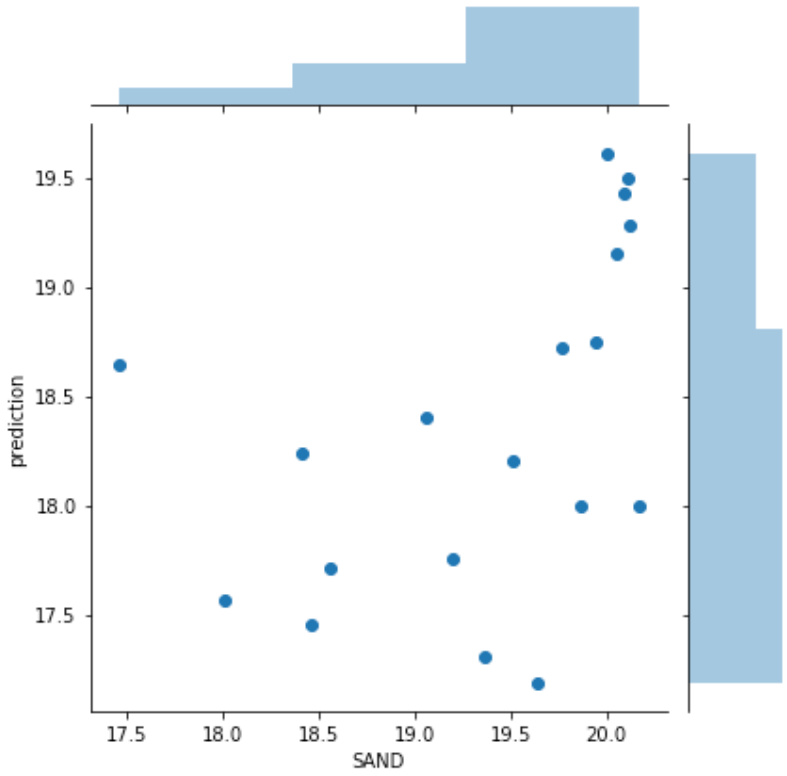

Figure S3-7. Predicted 2021 SAND temperatures vs. true temperatures. Model trained on contiguous 80% of data earlier in the summer. Data presented here represent the 20% testing set from later in the summer.

IV

Clinal variation of thermal tolerance during northward range expansion in the invasive golden star tunicate, *Botryllus schlosseri*

ABSTRACT

Populations can differ with respect to their thermal physiology, with clinal variation often observed across gradients in environmental temperature with latitude or elevation. The tempo at which phenotypic plasticity and/or local adaptation are able to shape variation in thermal tolerance has implications for species persistence under an increasingly volatile climate. Having encountered novel environments during introduction and subsequent range expansion, non-indigenous species (NIS) present useful case studies for examining thermal tolerance differentiation on contemporary time scales. Here we test for differentiation of heat and cold tolerance among three populations of the invasive golden star tunicate, *Botryllus* schlosseri spanning a 24.3° latitudinal gradient in the Northeast Pacific. We observed differentiation of post-larval heat tolerance among our sites, with our southern, putatively warm adapted population exhibiting a significantly higher LT_{50} than the two more northern populations. We synthesize these results with previous data for this species from the Northwest Atlantic, demonstrating consistent adaptive shifts in heat tolerance coincident with environmental temperatures. We also found that adult cardiac performance at cold temperatures is progressively greater at colder, higher-latitude populations. This pattern of countergradient variation of cold tolerance suggests compensatory genetic adaptation to colder environmental temperatures. By examining both heat and cold tolerance simultaneously among populations of an invasive ascidian, we document how a marine ectotherm is capable of shifting its physiology to novel environmental conditions over compressed time scales, with implications for species persistence in an era of global change.

INTRODUCTION

Biogeographic ranges often encompass a wide variety of environmental conditions, across which species must tune their physiology to match the local environment. Within species, phenotypic variation is often observed along climatic gradients with latitude or elevation (Hall et al. 2007; Gonzalo-Turpin and Hazard 2009; Keller et al. 2013; Pereira et al. 2017). Such variation can be shaped by both genetic and environmental factors, through local adaptation and phenotypic plasticity, respectively (West-Eberhard 2003; Kawecki and Ebert

2004). Understanding how these processes interact to produce clinal variation in phenotypes is a central goal of many evolutionary studies and can provide insights into the potential for species persistence under an increasingly volatile climate (De Frenne et al. 2013). On contemporary timescales, for example, in the case of species invasions or range expansions, the establishment of population-level differentiation in key phenotypes such as environmental tolerances hints at the ability for these traits to shift rapidly (e.g. Maron et al. 2004; Monty and Mahy 2009; Leal and Gunderson 2012; Carbonell et al. 2021; Neu and Fischer 2022). This rapid phenotypic divergence across space also speaks to the potential for species to alter their physiology in response to rapid environmental change (Somero 2010). Until recently, however, intraspecific variation of environmental tolerances, specifically thermal tolerance, has often been overlooked in macrophysiological studies that forecast species responses to climate warming (Valladares et al. 2014; Bennett et al. 2019). An appreciation of intraspecific variation of thermal tolerance and its ability to shift rapidly, then, is critical to understanding species responses to environmental change.

In the oceans, population-level divergence of thermal tolerance is often observed across latitudinal clines. For marine ectotherms, heat tolerance often increases with decreasing latitude (Zippay and Hofmann 2010; Sanford and Kelly 2011; Pereira et al. 2017; Sasaki and Dam 2019; Sasaki et al. 2022), a presumably adaptive response to warmer environmental temperatures, though invariance of heat tolerance or more complex patterns have also been documented (Kuo and Sanford 2009; Gaitán-Espitia et al. 2014). While there are comparatively fewer data for latitudinal variation of cold tolerance in marine ectotherms, some studies have revealed similar latitudinal patterns, with more cold-tolerant populations at northern latitudes (Wallace et al. 2014; Chiba et al. 2016; Thyrring et al. 2019). Divergence of cold tolerance can also arise rapidly, as illustrated by Tepolt and Somero (2014), who demonstrated the maintenance of higher heart rates in the cold after a common acclimation temperature in northern populations within the invasive range of the European green crab, *Carcinus maenas*.

Northern, more cold-exposed populations are often driven to evolve adaptations to compensate for the countervailing environmental effects of cold temperatures and/or a shorter growing season (Schultz et al. 1996; Yamahira et al. 2007; Hong and Shurin 2015). For example,

in the Atlantic silverside, *Menidia menidia*, whose range extends from Florida to the Gulf of St. Lawrence, body size does not appear to vary with latitude despite a drastic gradient in environmental temperature. However, in multigenerational common garden experiments, Conover and Present (1990) uncovered a genetic basis for faster growth rates in more northern, colder populations, demonstrating the presence of countergradient variation and genetic compensation to a shorter growing season. First observed by Levins (1968, 1969), countergradient variation has since been documented in a diverse array of organisms and is especially common as a consequence of adaptation to temperature (e.g. Schultz and Conover 1997; Laugen et al. 2003; Skelly 2004; Fangue et al. 2009; reviewed by Conover et al. 2009). As temperatures decrease with increasing latitude or elevation, local adaptation in traits such as growth or metabolic rate can counter the plastic response to colder temperatures, such that when animals are brought into a warmer common garden, rates of those from colder environments actually outpace those of conspecifics from warmer climes.

Understanding how variation in fitness-related phenotypes is partitioned across space is vital for assessing the capacity for populations and species to adapt in the face of global change (Hoffmann and Sgró 2011). In recent decades, an increasing number of studies have demonstrated the potential for rapid evolutionary adaptation (e.g. Reznick et al. 1990; Barrett et al. 2011), begging the question of whether this can keep pace with the rate of environmental change (Jump and Peñuelas 2005; Bradshaw and Holzapfel 2006). Many such studies have relied on the investigation of non-indigenous species (NIS) (Gilchrist et al. 2004; Maron et al. 2004; Colautti and Barrett 2013; reviewed by Colautti and Lau 2016). As NIS by their very nature encounter new habitats during introduction, they present useful "natural experiments" for studying species responses to novel environmental conditions on contemporary timescales (Huey et al. 2005; Sax et al. 2007). For example, in the common Puerto Rican anole, *Anolis cristatellus*, cold tolerance in the more cold-exposed invasive population in Florida is significantly greater than in the native range, with this differentiation arising on the order of just ~35 generations (Leal and Gunderson 2012). The pace at which NIS are able to adapt to new habitats thus provides insights into how native species may respond to various axes of anthropogenic change (Moran and Alexander 2014).

One consequence of global climate change is the observed poleward range shift/extension of many species (Sorte et al. 2010a; Chen et al. 2011; Pinsky et al. 2020). While this is perhaps most obviously driven by an increase in temperatures at higher latitudes, there are also more complex drivers. For example, in the marine realm there is an increasing concern about introductions of NIS to northern latitudes because of a predicted increase in global shipping in the Arctic due to retreating sea ice (Ware et al. 2016; Chan et al. 2019). Due to this and other modes of dispersal, northward spread of NIS may outpace the rate of warming, provided that these species have the physiological capacity to deal with colder northern temperatures. Many NIS exhibit intrinsically broad physiological tolerances (Zerebecki and Sorte 2011; Higgins and Richardson 2014) and may thus be primed for success at northern latitudes (Kelley et al. 2013; Tepolt and Somero 2014).

One high-profile marine NIS that is currently expanding its range northward is the golden star tunicate, *Botryllus schlosseri*. *Botryllus schlosseri* is a colonial ascidian and a common member of fouling communities, typically growing on the bottoms of docks, boat hulls, and other substrates within harbors and marinas. While its native range is unknown, *B. schlosseri* is invasive across much of the global ocean, including parts of the North Atlantic, South Pacific, and Northeast Pacific (Fofonoff et al. 2024). In North America it is actively expanding northward along both coasts. On the east coast, it is considered cryptogenic in the United States but invasive further north in the Maritime Provinces of Canada and Newfoundland. Its current northern range limit on the east coast of North America is in Newfoundland, where it was first detected in 2006 (Callahan et al. 2010). On the west coast of the United States, *B. schlosseri* was first detected in San Francisco Bay in 1947 (Carlton 1979) and has since spread southward as far as San Diego, CA, where it was detected in 1965 (Lambert and Lambert 1998), and northward as far as Sitka, AK, where it was detected in 2001 (Ruiz et al. 2006). Globally, its most recent northern range expansion was reported in Iceland, where it was found in 2011 (Ramos-Esplá et al. 2020). This global northward range expansion into cooler waters, along with its persistence at lower latitudes (Ali et al. 2009; Tovar-Hernández et al. 2014), demonstrates that *B. schlosseri* is capable of inhabiting a broad range of thermal habitats.

There has been limited exploration of the potential for population-level divergence of thermal tolerance in *B. schlosseri*. We previously studied differentiation of heat tolerance among populations along the east coast of North America, where its invasive status is unknown (Tobias et al. 2024). Here we extend our investigation of *B. schlosseri* thermal tolerance to the Northeast Pacific, where it is definitely invasive, focusing on three populations spanning a thermal gradient encompassing 24.3° of latitude. Using this system, we explore the potential for rapid differentiation of thermal tolerance since its introduction to the west coast about eight decades ago. We assess population-level differentiation of both heat tolerance, through LT_{50} experiments on newly settled post-larvae, and cold tolerance, through a cardiac performance assay during a cold challenge in adult *B. schlosseri*. We find that populations differ in their sensitivity to heat, with northern populations being more susceptible to heat stress than their southern counterpart. For cold tolerance, we observed a pattern of countergradient variation, with more northern populations maintaining progressively higher heart rates in the cold after acclimation at a common temperature. By comparing thermal tolerance across populations, we illustrate that this species is capable of tuning its physiology to local habitats on a contemporary timescale, providing important insights into the capacity of marine organisms to adapt to changing environmental conditions.

METHODS

Sites

We conducted LT_{50} and cardiac performance experiments on *B. schlosseri* individuals collected from three sites spanning 24.3° of latitude in the Northeast Pacific: Eliason Harbor, Sitka, AK, USA (SITK); Bodega Harbor, Bodega Bay, CA, USA (BDGA); and Sea World Marina, San Diego, CA, USA (SDGO) (Figure 4-1A, Table 4-1). Experiments at SITK were performed at the Sitka Sound Science Center, BDGA at the University of California at Davis' Bodega Marine Lab, and SDGO at San Diego State University's Coastal and Marine Institute Laboratory.

Environmental temperature

In situ temperatures were recorded at the collection sites during the 2-week experimentation periods using HOBO Pendant temp/light loggers (Onset; Bourne, MA, USA; cat. no. UA-002-64). Long-term temperature for stations near our collection sites were obtained from publicly available sources for SDGO (https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_9410170.html) and SITK (https://erddap.sensors.ioos.us/erddap/tabledap/noaa_nos_co_ops_9451600.html). For BDGA, temperature data for 2020-21 were generously shared by Dr. Jay Stachowicz.

LT_{50} experiments

We conducted LT_{50} experiments as described previously (Tobias et al. 2024), with some modifications. Two days prior to each experiment approximately 50 *B. schlosseri* colonies were collected by hand from the underside of floating docks. Colonies were transported to the laboratory and placed in groups of 2-3 in 3.5 x 2 x 2" wells of polycarbonate compartment boxes with ~200 ml of local seawater. Five extra-thick microscope slides (Fisherbrand; Pittsburgh, PA, USA; cat. no. 22-267-013) were placed along the bottom and side walls of each compartment, to allow for larval settlement upon the slides. Colonies were then kept at room temperature over two nights, exchanging seawater twice during the first day after collection and once the second day after collection. Once slides were observed to be covered in settled larvae, we transferred them to a separate holding tank with aeration. Two days after collection, all oozooids (metamorphosed settled larvae) were censused under a Stemi dissecting stereomicroscope (Zeiss; Oberkochen, Germany), and examined for a heartbeat and normal development. Those with slowed (tail still present) or abnormal development were removed, as well as those settled on the extreme margin of the slides. Slides were further thinned of oozooids to retain a maximum of 35 individuals.

Up to five slides from each clutch (offspring of a single clonal colony, as we never observed multiple colonies from one compartment simultaneously release larvae) were partitioned across five temperature treatments. In SITK and BDGA, the set temperatures were 29.4, 30.0, 30.6, 31.1, and 31.7 °C, whereas at SDGO the set temperatures were 30.0, 30.6, 31.1, 31.7, and 32.2 °C. We selected these temperatures based on the results from Tobias et al.

(2024), with the expectation that SDGO would exhibit a higher LT_{50}, thus the use of a slightly higher temperature range. The heat tolerance assays were performed in polycarbonate 4”-deep 1/3 pans (Cambro; Huntington Beach, CA, USA; cat. no. 34CW) equipped with programmable 25 W glass aquarium heaters (YOFOTHS; Shenzhen, Guangdong, China) and HOBO pendant light/temp temperature loggers. Temperatures used for downstream analysis were those recorded by the HOBO loggers rather than the set temperatures on the heaters.

Heat exposures lasted 20 hours, starting from the ambient temperature of local seawater. The ramp speed was approximately 4°C/hr, chosen to conform with previously conducted experiments of *B. schlosseri* populations in the Northwest Atlantic (Tobias et al. 2024). After 20 hours, the oozooid slides were removed and censused again. Oozoids without a detectable heartbeat were considered dead.

Cardiac performance experiment

To assess the potential for variation in cold tolerance among the three populations, we conducted an assay of adult *B. schlosseri* cardiac performance during a cold challenge. We used the same colonies as collected for the LT_{50} experiment. If kept undisturbed, *B. schlosseri* colonies will often begin to attach to and grow out along the bottom slide within each well of the compartment box. We treated the colonies as described above, performing two water exchanges the day after collection and one water exchange two days after collection. If two days after collection a colony was firmly attached to the slide, we transferred it to a holding tank of room temperature seawater with aeration, placing it vertically in a slide storage box. We left the colonies in this holding tank for an additional night. On the third day after collection, we placed colonies in a portable incubator (IVYX Scientific; Seattle, WA, USA) set to 18 °C for 24 hours. By acclimating individuals from different populations to a common temperature we sought to minimize the potential effect of short-term phenotypic plasticity on the outcome of the cardiac performance experiment. We chose 18 °C as an acclimation temperature, as this was near the upper limit of temperatures experienced in the field (Figure 4-1C) and would afford a broader range across which to measure heart rate (see below).

After the 24-hour acclimation (on the fourth day after collection), colonies were subjected to a cold challenge experiment. Slides with colonies were placed in a specialized imaging chamber designed and 3D printed at Woods Hole Oceanographic Institution on an Objet350 Connex3 3D printer (StrataSys; Eden Prairie, MN, USA). Seawater was pumped to this chamber from an insulated sump tank (Coleman Party Stacker cooler; Chicago, IL, USA; cat. no. 3000005591) through 3/8" vinyl tubing using a small aquarium pump (Hygger; Shenzhen, China; cat. no. HG-939). An additional pump (Hawthorne Hydroponics EcoPlus; Vancouver, WA, USA; cat. no. HGC728310) fed water to a 1/13 hp seawater chiller (AquaEuro USA; Los Angeles, CA, USA; cat. no. AC13H) through 1/2" vinyl tubing. We converted the chiller to on/off operation by soldering together two circuits in the control panel to bypass the chiller's temperature controller. Temperature was controlled using an external programmable temperature controller (Bayite; Zhongshan, China; cat. no. BTC201). In case ambient seawater was lower than 18 °C, we also included a 300 W aquarium heater (Finnex; Countryside, IL, USA; cat. no. TH-300S) in the sump. We included a HOBO pendant temp/light logger in the sump to record experimental temperatures.

Starting from 18 °C, we ramped down the temperature to 2 °C over the course of an hour, for a ramp speed of ~16 °C/hour. During this ramp, we recorded a video of one to several zooids' (individuals' within the colony) hearts under a M80 stereomicroscope (Leica; Wetzlar, Germany) fitted with a microscope camera (AmScope; Irvine, CA, USA; cat. no. MU1803-HS). Videos were acquired using the AmLite software (AmScope), adding a timestamp. To prevent condensation on the imaging chamber at cold temperatures, we added a thin layer of fresh water to the upper surface of the outside of the chamber. At the end of each experiment, *B. schlosseri* colonies were discarded.

Heart rates over time were extracted from the videos manually. Starting from the first full minute according to the video timestamp, we counted the number of heart beats per minute at five-minute intervals. If the heartbeat was arrhythmic or there were issues with the video quality (out of focus, out of frame, etc.), we skipped that minute and proceeded to subsequent minutes until conditions improved. For videos that contained footage of multiple hearts, we counted each heart separately but kept track of colony membership, to account for

potential colony effects during analysis. We excluded all zooids in blastogenetic stage D (i.e. those not actively siphoning), as these are known to exhibit aberrant physiology (pers. comm., A. Voskoboynik).

Data analysis

For the LT_{50} experiment, analyses were performed in R v. 4.1.2 (R Core Team 2013). We used the R package *drc* v. 3.0.1 (Ritz et al. 2015) to estimate LT_{50} values for each population, fitting two-parameter (slope and midpoint) log-logistic models to each population's binomial survivorship data. To test if there was a significant effect of population on LT_{50}, we compared a model with separately estimated LT_{50} values for each population to a null model with a jointly-estimated LT_{50} value for all populations using a likelihood ratio test. Post-hoc pairwise comparisons among populations were conducted using the same approach, with p-values adjusted using the Bonferroni method to account for multiple comparisons.

To put our results in the context of previous work on heat tolerance of *B. schlosseri*, we synthesized our LT_{50} data with those for five populations from the Northwest Atlantic collected with the same approach (Tobias et al. 2024). To investigate the potential for local adaptation and/or adaptive phenotypic plasticity of heat tolerance, we performed a linear regression of the eight population-level LT_{50} estimates against mean summer temperature, using the long-term environmental temperature data presented here or in Tobias et al. (2024). With the exception of BDGA and Sandwich, MA (SAND), mean summer temperatures were obtained for the year in which the respective experiments were conducted (2023 for the present study, 2022 for those from Tobias et al. (2024). For BDGA, long-term temperature data were only available for 7/21/2020-6/28/21. We thus took the mean of all summer dates within this series. For SAND, we lacked temperature records for 2022 and instead used temperature data from 2021.

For the cardiac performance experiment, analyses were performed in Python v. 3.11.5 (Van Rossum and Drake 2009). Heart rates over time were converted to heart rate over temperatures using the temperature recorded the sump tank HOBO logger at the start of each minute. We compared the heart rate at 18 °C, the heart rate at 8 °C, and the Q_{10} in heart rate between these two values among all populations. Because many heart rate records did not

include values for heart rate at the exact temperatures of 18 °C and 8 °C, we used scikit-learn v. 1.2.2 (Pedregosa et al. 2011) to perform linear interpolation, extracting predictions for these values. To ensure we were not extrapolating outside the bounds of the available data, we excluded all records that did not span the full 8-18 °C range.

To test for differences among populations in these values (heart rate at 18 °C, heart rate at 8 °C, and Q_{10}), we performed linear mixed effects modeling using the python package statsmodels v. 0.14.0 (Seabold and Perktold 2010). To test for an effect of population on these responses, we included population as a fixed effect and colony membership as a random effect. We chose to include colony membership as a random effect because there is the potential for differences in heart rate to be driven by colony-level phenomena (genetic differences, blastogenetic stage, etc.) and we were primarily interested in differences among populations. Because we hypothesized that there would be an ordering in these values according to population latitude (SDGO expected to exhibit lowest heart rate at 8 °C, followed by BDGA, then SITK), we encoded population according to an ordinal scale, with SDGO assigned a value of 0, BDGA a value of 1, and SITK a value of 2. To assess how much variation in the response variables can be attributed to colony membership, we calculated the intra-class correlation coefficient (ICC) by dividing the variance explained by colony membership by the sum of itself and the unexplained variance in the model (*scale* value in model output). To perform post-hoc pairwise comparisons among populations, we repeated the linear mixed effect modeling on groupings containing data for population pairs, adjusting p-values using the Bonferroni method to account for multiple comparisons.

RESULTS

Environmental temperature

As expected, environmental temperature varied strongly with latitude (Figure 4-1B). However, at certain times during the summer of 2020, mean daily temperatures overlapped between BDGA and SITK. Similarly, during the experimentation periods in the summer of 2023 we observed overlap between the *in situ* temperatures at BDGA and SITK (Figure 4-1C).

Heat tolerance

In total between the three sites, 1,021 oozooids from 48 clutches were included in the experiment (Table 4-1). Logistic survivorship curves illustrate how heat tolerance varied according to population (Figure 4-2). LT_{50} estimates for SITK, BDGA, and SDGO were 31.54, 31.52, and 32.28 °C, respectively (confidence intervals provided in Table 4-1). A model with separately estimated LT_{50} values for each population explained the data significantly better than a model including a jointly estimated LT_{50} value for all populations ($\chi^2(2) = 132.86$, $p < 0.001$). Post-hoc comparisons between pairs of sites revealed significant differences between SDGO and SITK ($p < 0.001$), and SDGO and BDGA ($p < 0.001$), but not between SITK and BDGA ($p = 1$), as illustrated by Figure 4-2.

To investigate broader patterns of how LT_{50} varies with environmental temperatures, as a result of local adaptation and/or adaptive phenotypic plasticity, we synthesized east coast data from Tobias et al. (2024) with that collected in the present study. We found that population LT_{50} is strongly associated with the mean summer temperature at each site ($r^2 = 0.75$, $p = 0.0034$) (Figure 4-3). This analysis revealed three groups of populations in regard to heat tolerance: SITK and BDGA with the lowest LT_{50}, SAND and Walpole, ME (DARL) with intermediate LT_{50}s, and SDGO, Falmouth, MA (FALM), Chatham, MA (CHAT), and Tuckerton, NJ (RUTG) with the highest LT_{50}s.

Cold tolerance

Among the three sites, we obtained heart rate records for 86 zooids from 31 colonies. Overall, heart rate decreased linearly with decreasing temperatures over the course of the experiment (Figure 4-4A, Figure S4-1). After removing records that did not span the full 8-18 °C temperature range, 81 zooids from 29 colonies remained (9-11 per site). Linear mixed effect modeling revealed a significant effect of population on heart rate at 8 °C ($p < 0.001$), but not heart rate at 18 °C ($p = 0.684$) or Q_{10} ($p = 0.0875$). Colony membership was observed to have a strong effect on interpolated heart rate at 8 °C, with an ICC of 0.809 (Figure S4-2). Post-hoc pairwise comparisons revealed significant differences in interpolated heart rate at 18 °C between SITK and BDGA ($p_{adj} = 0.0262$), but not between SITK and SDGO ($p = 1$) or BDGA and

SDGO (p_{adj} = 0.294) (Figure 4-4B). Interpolated heart rate at 8 °C followed a latitudinal pattern, with significantly higher heart rates in SITK than BDGA (p_{adj} = 0.0114), significantly higher heart rates in BDGA than SDGO (p_{adj} = 0.0196), and correspondingly significantly higher heart rates in SITK than SDGO (p_{adj} < 0.001) (Figure 4-4C). Q_{10} similarly followed a latitudinal pattern but reversed, with higher values at lower latitudes (Figure 4-4D). However, all pairwise comparisons for Q_{10} were non-significant, likely due to high variability in SDGO.

DISCUSSION

Whether and how species will be able to adapt to rapidly changing environmental conditions is a central question in the contemporary study of ecology, evolution, and conservation (Hoffmann and Sgró 2011). Because they experience novel conditions during introduction, NIS present excellent case studies for understanding modalities of adaptive responses, be they genetic through local adaptation or environmental through phenotypic plasticity (Matesanz et al. 2010; Moran and Alexander 2014). In the present study, we assessed phenotypic differentiation of heat and cold tolerance across a latitudinal gradient during range expansion in the invasive golden star tunicate, *B. schlosseri*. We demonstrate that colder, more northern populations exhibit lower heat tolerance than a warmer, southern counterpart. For cold tolerance, we uncovered a pattern of countergradient variation, whereby northern populations progressively maintain higher heart rates in cold temperatures after a common acclimation treatment. Together, our results illustrate how a widespread invasive species has been able to shift its tolerance to match the local thermal environment, providing insights into the ability of species to adapt to changing environmental conditions on contemporary timescales.

Population differentiation of heat tolerance

We found that the more northern populations of SITK and BDGA exhibited lower LT_{50} values than the southern population of SDGO (Figures 4-2 and 4-3), indicating that they possess lower heat tolerances. Given the prevailing environmental temperatures (Figure 4-1A), we expected to observe a gradient in LT_{50} (SITK < BDGA < SDGO). However, we observed no

significant difference between SITK and BDGA. This could be due to a number of factors. First, while the mean annual temperatures between SITK and BDGA differ in the expected direction, temperatures during the summer can be quite similar. For example, in August 2020 local temperatures in BDGA were actually lower than in SITK (Figure 4-1B), despite the large difference in latitude. We observed this from our own temperature records for 2023 as well, where the mean temperature for two weeks in late July in BDGA was lower than for two weeks in late August/early September in SITK (Figure 4-1C). This lack of a strong latitudinal gradient in summer temperature can likely be explained by summer upwelling off of the central California coast, where strong northwesterly winds drive the surfacing of deep, cold water (Huyer 1983). Thus, because *B. schlosseri* are most reproductively active in the summer (Stewart-Savage et al. 2001; Epelbaum et al. 2009), the selective gradient for heat tolerance in early development between these two sites might simply not exist. Alternatively, or perhaps additionally, the similarity in heat tolerance between BDGA and SITK could be driven by phenotypic plasticity. We previously demonstrated that the temperature during development has a strong effect on heat tolerance after settlement, with higher developmental temperatures driving greater heat tolerance (Tobias et al. 2024). Because the thermal environment the oozooids were experiencing as embryos did not differ substantially between SITK and BDGA (Figure 4-1C), the similarity in LT_{50} may have been an effect of similar environments. Despite the lack of a difference between SITK and BDGA, the observation of a higher LT_{50} in SDGO still provides evidence for differentiation of heat tolerance in the direction we anticipated.

Whether this differentiation of heat tolerance is driven by divergence within the invasive range since *B. schlosseri*'s initial introduction is unclear, however. Genomic data for this species indicates that there have been at least two introductions to the west coast of North America, with populations from southern California forming a lineage that is evolutionarily distinct from populations further north (Chapter 2). These separate lineages may differ physiologically due to variation that evolved within the species' native range rather than post-introduction. Thus, the differentiation of heat tolerance that we observe here could be a signature of preadaptation, where a putatively warm-adapted source seeded the introduction to southern California and a more heat-susceptible population was the source of introductions

further north. Because the aforementioned ongoing genomic investigation sampled strictly from North America, it is not able to address the potential origins of *B. schlosseri* to the west coast, precluding a more detailed examination of preadaptation. Nonetheless, this potential scenario draws parallels to the invasion of the east coast of North America by the European green crab, *Carcinus maenas*. Its original introduction to New England in the early 1800s is likely to have been a from a south-central European source (Roman 2006). While northward spread was originally confined to the Gulf of Maine (Carlton and Cohen 2003), further expansion in recent decades coincided with the arrival of a second introduction from a northern European, cold-adapted source. (Roman 2006; Tepolt and Somero 2014).

With the caveat that adaptive differentiation may have arisen prior to introduction, the observation of higher heat tolerance in the southern population of SDGO is consistent with local adaptation to temperature. However, because the individuals used in the LT_{50} experiments were not reared in a common garden, it is not possible to disentangle genetic and environmental effects. As noted above, *B. schlosseri* oozooids possess appreciable levels of developmental plasticity for heat tolerance. Tobias et al. (2024) showed that for every 1 °C increase in developmental temperature, LT_{50} can increase by as much as 0.187 °C, depending on the population. The difference in mean temperature during the experimentation periods between our coldest site (BDGA, 16.03 °C) and our warmest site (SDGO, 20.87 °C) was 4.84 °C. Thus, if we assume an acclimation response ratio (ARR) of 0.187 °C/1 °C developmental temperature, we would expect a maximum difference in LT_{50} of 0.91 °C between BDGA and SDGO. That we observe a difference of 0.76 °C indicates that the differentiation in LT_{50} could be due solely to phenotypic plasticity. This is of course predicated on the assumption that populations on the west coast of North America harbor similar levels of developmental plasticity as the most plastic population investigated on the east coast (Sandwich, MA, USA; SAND). We previously demonstrated that in addition to exhibiting differentiation of heat tolerance, east coast populations differ in their degree of developmental plasticity and that this appears to be related to the extent of short-term temperature variability, with more variable sites exhibiting greater levels of developmental plasticity. Given that the west coast populations experience lower levels of daily variability (Figure 4-1C vs. Figures 1c, S5 of Tobias et al. (2024)),

we might expect them to possess lower ARRs and thus a smaller predicted difference in LT_{50} than was observed, allowing for some role of genetics in shaping this differentiation. Consistent with this, our ongoing genomic study of North American *B. schlosseri* populations revealed strong genetic differentiation and high genetic diversity, increasing the likelihood of local adaptation (Chapter 2). Ultimately, it is likely that both genetics and the environment shape this observed variation. Future studies should employ a multigenerational common garden approach to fully distinguish between the contributions of local adaptation and phenotypic plasticity in shaping the population differentiation (or lack thereof) that we observe between the three populations.

By including the LT_{50} estimates from Tobias et al. (2024), we were able to more closely examine how heat tolerance varies with geography, and, correspondingly, environmental temperatures, across a greater array of populations. We noted a strong relationship between the LT_{50} of a population and its mean summer temperature (Figure 4-3), suggesting that *B. schlosseri* is able to adaptively respond to the local thermal environment. This pattern demonstrates the consistency of the response to environmental temperature across both coasts of North America, spanning ranges where it is invasive (Northeast Pacific) and where its status is unclear (Northwest Atlantic). Of course, if this differentiation is driven solely by developmental plasticity, this is not altogether unsurprising. Conversely, if genetics does play a role, this speaks to how evolutionary adaptation may drive differentiation between populations on contemporary timescales, providing insights into potential species responses to global change.

Heat tolerance is a critical trait for species persistence in a rapidly changing ocean (Somero 2010; Pinsky et al. 2019). Mean sea surface temperature is increasingly rapidly across much of the global ocean (Cheng et al. 2022), and this is compounded by the increasing frequency of more local marine heatwave events (Oliver et al. 2021). Understanding how species may be able to shift their heat tolerance is thus of vital importance for making predictions of range shifts, local extirpations, and even species extinction (Somero 2010; Sunday et al. 2012; Diamond 2017; Morley et al. 2019). By examining geographic variation of thermal tolerance as a lens through which to view temporal responses to climate warming, a

space-for-time approach (Pickett 1989), while controversial (Damgaard 2019; Perret et al. 2024), may indicate which populations may be at risk and whether a species has the capacity to shift its physiology to match future environmental conditions (Sorte et al. 2011; Blois et al. 2013; Lovell et al. 2023). The patterns of heat tolerance observed in the present study and previously (Tobias et al. 2024) illustrate how this critical phenotype varies across space, and how it may be shaped by both genetics and environment. Given *B. schlosseri*'s flexibility and its capacity to match its heat tolerance to the prevailing environmental temperature (Figure 4-3), we may conclude that it is poised to be among the "winners" in an era of climate change (Somero 2010). Other studies have indicated that invasive ascidians may fare comparatively well under a regime of increasing ocean temperatures (Sorte et al. 2010b; Dijkstra et al. 2011; Zhang et al. 2020), demonstrating synergistic interactions between two key axes of global environmental change – species introductions and climate change (Rahel and Olden 2008; Hellmann et al. 2008; Mainka and Howard 2010).

Countergradient variation of cardiac function at cold temperature

We found that sites differed in their cardiac performance at cold temperatures, with more northern populations exhibiting progressively higher heart rates at 8 °C (Figure 4-3C). This observation is consistent with a pattern of countergradient variation. The heart rate of ectotherms is inextricably linked to environmental temperatures, with cooler temperatures driving lower heart rates. In the absence of any genetic effects, one might expect to observe lower heart rates in the colder, more northern populations, a pattern driven solely by the prevailing environmental temperatures. However, that we observe the reverse pattern after a common acclimation treatment demonstrates countergradient variation and suggests that compensatory genetic adaptation to cold temperatures may contribute to this pattern.

In contrast to our heat tolerance results, we demonstrate that the pattern for cold tolerance is clinal along the latitudinal gradient, with SITK possessing the greatest cold tolerance, followed by BDGA, and then by SDGO. It is worth noting that this may be an effect of experimenting on adult animals for cold tolerance in contrast to post-larvae (oozooids) for heat tolerance. As noted above, summer temperatures were overlapping between SITK and BDGA,

potentially driving the lack of differentiation in heat tolerance we observed between these two sites. Because oozooids are only present in the summer and early fall, when temperatures at the sites are similar, there may be no selective pressure for divergence of heat tolerance. For cold tolerance, on the other hand, we experimented on adults, which overwinter. Mean annual temperatures, and especially winter temperatures, do indeed follow a gradient with latitude (Figure 4-1B). Thus, in the case of cold tolerance, there may be differential selection among the populations, driving the pattern of countergradient variation we observed.

While animals were acclimated to a common temperature of 18 °C, it is possible that lingering environmental effects contributed to the observed pattern. We previously demonstrated developmental plasticity of heat tolerance (Tobias et al. 2024), and it is conceivable that increased cold tolerance could be conveyed by colder developmental temperatures as well. Because we could not control the juvenile environment, we were not able to fully separate what might be a plastic response from a presumed genetic adaptation. Further, as phenotypes can be shaped by parental or even grandparental environments through transgenerational plasticity (Shama and Wegner 2014; Donelson et al. 2018), without multigenerational experiments it is impossible to definitively prove that local adaptation contributes to the observed pattern of countergradient variation. However, there are a great number of studies that use field-collected, lab-acclimated organisms to infer the potential for evolutionary divergence of thermal physiology (e.g. Tepolt and Somero 2014; Chiba et al. 2016; Thyrring et al. 2019; Broitman et al. 2021). These approaches are especially important for the most non-model of species that lack tractable methods for intergenerational culture.

While we cannot rule out that phenotypic plasticity may be contributing to countergradient variation of heart rate, other lines of evidence bolster the case for genetic adaptation. We observed that in addition to variation in cold tolerance among populations, there is substantial variation among colonies *within* populations. This likely has a genetic basis, as individuals from the same colony tended to express more similar cardiac responses to cold than individuals from other colonies (Figure S4-2), as indicated by the high intra-class correlation coefficient. This strongly suggests the existence of substantial genetic variation upon which natural selection could act to drive evolutionary divergence of cold tolerance

among populations. Furthermore, as with differentiation of heat tolerance, genomic data can provide additional insights into *B. schlosseri*'s potential for thermal adaptation. Populations from San Francisco Bay and northward to Sitka appear to form a single evolutionary lineage that is distinct from populations in southern California. That we observe differentiation of cold tolerance between SITK and BDGA, which are evidently within the same clade, indicates that this divergence likely arose during range expansion. Given that *B. schlosseri* was first detected in northern California in 1947 (Carlton 1979) and was only found in Sitka in 2001 (Ruiz et al. 2006), this speaks to the rapid pace at which evolutionary adaptation may occur. Lastly, several characteristics of *B. schlosseri* point towards the high potential for local adaptation, including strong population genetic differentiation (Grosberg 1987; Yund and O'Neil 2000), pronounced genetic diversity (Chapter 2), and high mutation rates in ascidians (Tsagkogeorga et al. 2012; Berna and Alvarez-Valin 2014). Similar to heat tolerance, we recommend that future studies utilize a multigenerational approach to fully disentangle the contributions of genetic and environmental effects on the differentiation of cold tolerance.

In contrast to studies of heat tolerance, there have been comparatively fewer studies that examine intraspecific geographic patterns of cold tolerance in marine ectotherms (but see Jansen et al. 2007; Tepolt and Somero 2014; Wallace et al. 2014; Thyrring et al. 2015, 2019; Broitman et al. 2021; Dwane et al. 2023 and others), perhaps due in part to the difficulty of conducting such experiments but also given the relevance of assessing heat tolerance in an era of climate warming. Similar to our findings, Dwane et al. (2023) found that individuals of the intertidal snail *Littorina saxatilis* from northern populations maintained higher heart rates at cold temperatures than their more southern counterparts. Higher physiological rates among populations at higher latitudes or elevations have been observed in a diverse array of organisms (Conover and Present 1990; Schultz and Conover 1997; Milla et al. 2009; Kaluthota et al. 2015; Storz et al. 2019) and is a central component of the metabolic cold adaptation (MCA) hypothesis (Krogh 1916; Scholander et al. 1953; Wohlschlag 1964). MCA is a type of countergradient variation whereby populations from colder environments exhibit higher metabolic rates at a common test temperature than their warmer counterparts. While the MCA hypothesis remains controversial (Pörtner et al. 2006), as investigation has revealed some

confirmatory (Addo-Bediako et al. 2002; White et al. 2012) but largely contradictory evidence (Clarke and Johnston 1999; Harper et al. 2000; Lardies et al. 2004; Messamah et al. 2017), it remains a useful theoretical framework for testing how key physiological parameters vary across thermal gradients. While we did not measure metabolic rate in the present study, heart rate is often closely linked to metabolism in both ectotherms and endotherms (Green et al. 2001; Green 2011; Bruning et al. 2013; Kinoshita et al. 2022), allowing us to interpret our results in the context of MCA. In contrast to many studies investigating MCA in marine ectotherms (Holeton 1974; Clarke and Johnston 1999; Sokolova and Pörtner 2003; but see Tepolt and Somero 2014; Thyrring et al. 2015; Dwane et al. 2023), we found evidence of latitudinal compensation, whereby northern, putatively cold-adapted populations maintained higher heart rates at a common cold reference temperature than populations further south. Again, whether this truly represents evolutionary adaptation or long-term phenotypic plasticity is unresolved. Nonetheless, that we observe this pattern in *B. schlosseri* is in accordance with MCA, reinforcing the notion that while MCA may not be the rule, it should not be discounted entirely (Pörtner et al. 2007).

Thermal limits, species invasions, and responses to novel environments

Temperature is a fundamental abiotic factor shaping ecological niches (Hochachka and Somero 2002), and understanding species' thermal breadths, and to what extent they are flexible, is critical to predicting responses to global climate change (Chown et al. 2010; Sunday et al. 2012). By examining both heat and cold tolerance in the same set of populations, we demonstrate how *B. schlosseri* has been able to shift its thermal physiology in response to prevailing local environmental temperature. Further, by assessing differentiation of thermal tolerance in a NIS, we can make inferences about how local adaptation and/or phenotypic plasticity are able to drive divergence of thermal tolerance on contemporary time scales.

We observed that northern, putatively cold-adapted populations are more sensitive to heat stress and tolerant of cold, whereas southern, putatively warm-adapted populations are more tolerant of heat and sensitive to cold. The negative correlation between heat and cold tolerance indicates that there may be costs associated with maintaining thermal tolerance in

the non-selective environment. Trade-offs between heat and cold tolerance have been observed in other studies (Bennett and Lenski 2007; Rodríguez-Verdugo et al. 2014; Schou et al. 2022; Xiao et al. 2024), suggesting constraint upon the evolution of thermal limits. Because anthropogenic change is not only shifting global mean temperatures upwards, but also increasing the frequency of weather extremes (Rahmstorf and Coumou 2011; Ummenhofer and Meehl 2017), the questions of whether and how species are able to simultaneously adapt to both heat and cold are relevant to their persistence. Several studies have demonstrated how rare, extreme weather events, rather than climatological means, can drive the rapid evolution of species (e.g. Campbell-Staton et al. 2017; reviewed in Grant et al. 2017). While considered by some as a "silver lining" (Coleman and Wernberg 2020), it is unclear whether these evolutionary shifts ultimately increase the likelihood of population persistence, as they may prove maladaptive under future environments (Lyberger et al. 2021). If the evolution of heat tolerance is constrained by cold tolerance, and vice versa, this may be a major limitation to the ability for adaptation to promote persistence in a more volatile future.

Through encountering novel environments during introduction, NIS present valuable case studies for understanding species responses to rapid environmental change (Huey et al. 2005; Sax et al. 2007). It is possible that some evolutionary divergence of the measured thermal tolerance traits, particularly heat tolerance, occurred prior to introduction through preadaptation (see above). However, the high potential for local adaptation in this species and the fact that we observe differentiation of cold tolerance within a single evolutionary lineage suggests that at least some of the differentiation we observe is driven by rapid evolution within the invasive range. Rapid adaptation in invasive species has been demonstrated in terrestrial, aquatic, and marine systems spanning the tree of life (e.g. Maron et al. 2004; Colautti and Barrett 2013; Sultan et al. 2013; Sotka et al. 2018; Mittan and Zamudio 2019). These evolutionary shifts, and indeed, those in response to extreme weather events discussed above, suggest that some species/populations have the capacity to rapidly adapt to novel environmental conditions, whether in the context of species invasions or global change. However, whether or not species contain the adaptive potential necessary to "keep up" with environmental change is an open question (Visser 2008; Munday et al. 2013; Martin et al.

2023). Predicting evolutionary responses to global change is a monumental task, one that requires not only more detailed assessments of adaptive variation in key physiological phenotypes, like that presented here, but also integration across multiple lines of inquiry, from theory to 'omics approaches (Urban et al. 2023). By investigating how a NIS is able to shift its physiology over contemporary time scales, we present valuable information of the pace of adaptive change in response to novel environmental conditions.

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TABLES

Table 4-1. Site information and heat tolerance experiment data, with lower and upper 95% confidence limits around LT_{50}.

Location	Code	Lat	Lon	Oozooids	Clutches	LT_{50} (°C)	Lower	Upper
San Diego, CA	SDGO	57.058	-135.354	535	14	32.28	32.20	32.37
Bodega Bay, CA	BDGA	38.329	-123.057	338	24	31.52	31.39	31.64
Sitka, AK	STIK	32.767	-117.231	148	10	31.54	31.40	31.68

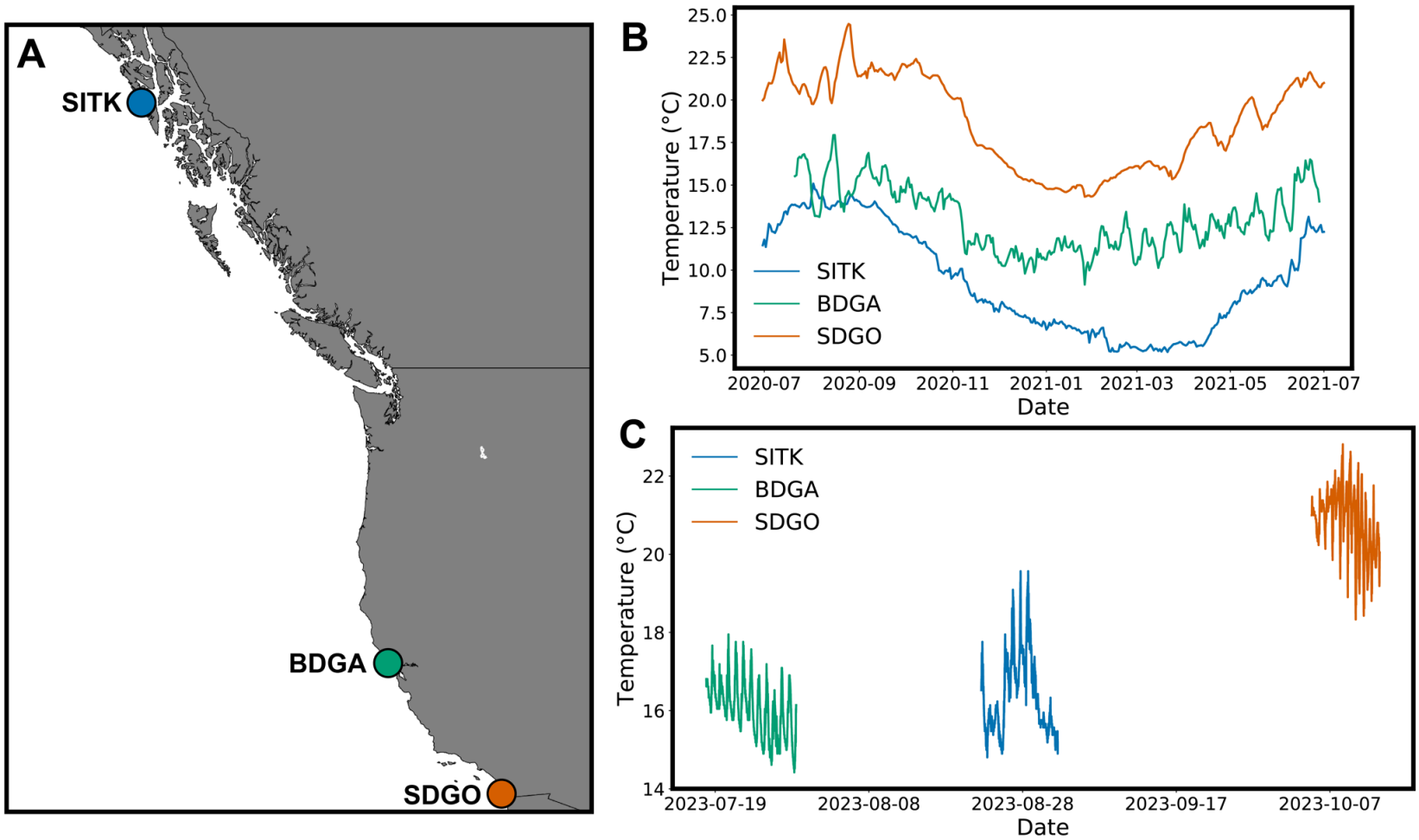

Figure 4-1. A) Map of study sites. SITK = Sitka, AK, USA. BDGA = Bodega Bay, CA, USA. SDGO = San Diego, CA, USA. B) Mean daily environmental temperatures at the three sites from July 2020 to July 2021. C) *In situ* temperatures recorded during experimentation periods in the summer/fall of 2023.

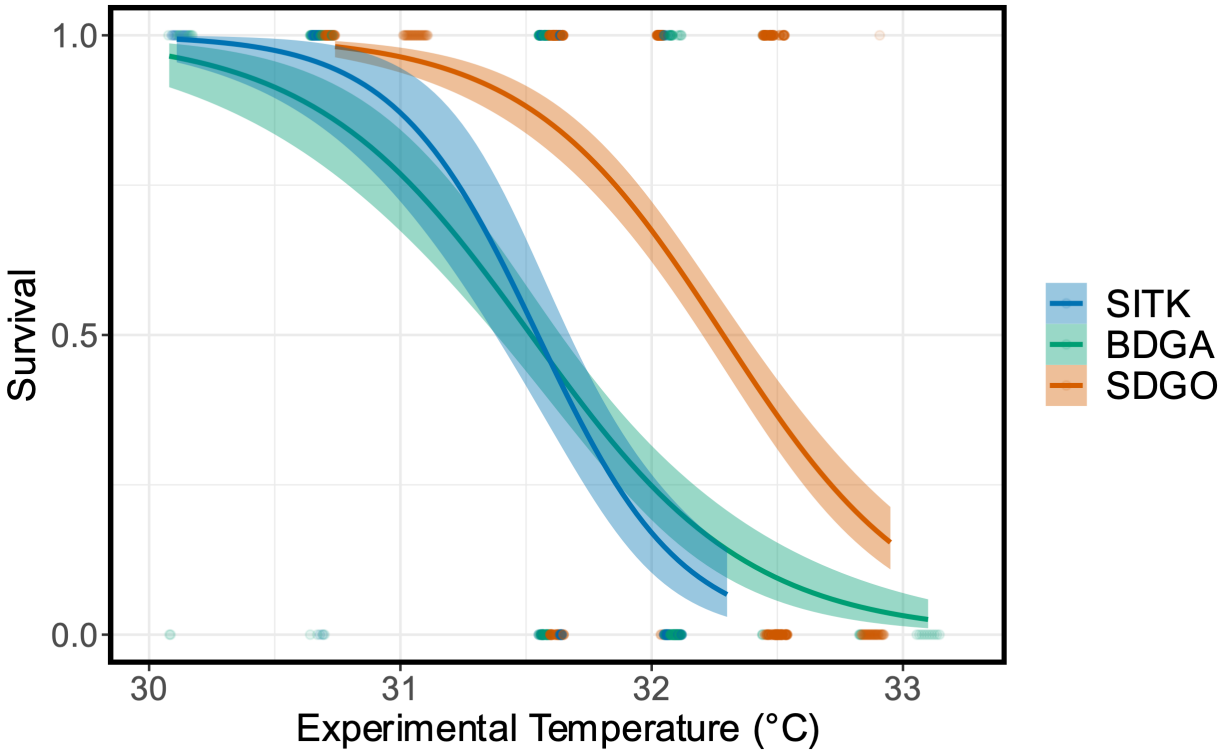

Figure 4-2. Differentiation of heat tolerance, with SDGO exhibiting significantly greater heat tolerance than SITK and BDGA. Points represent individual survivorship data with lines illustrating logistic regressions of these data. Shaded areas around each line represent 95% confidence interval.

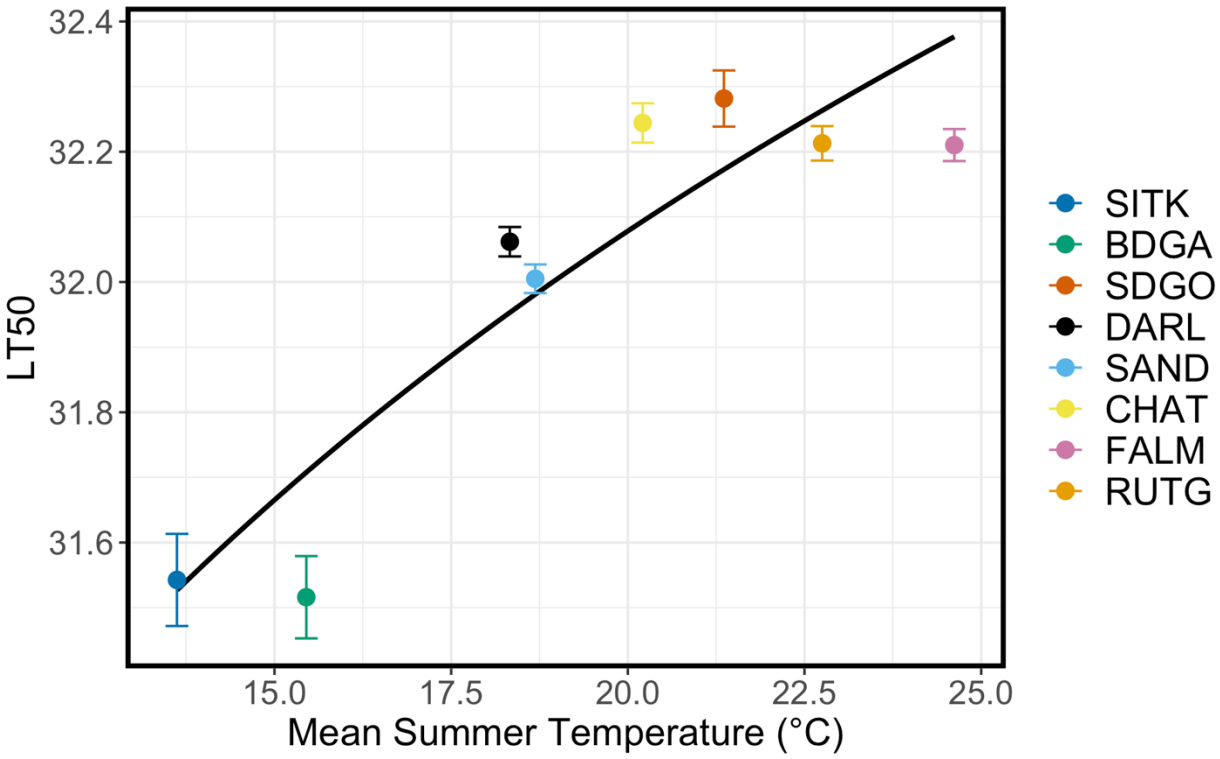

Figure 4-3. Heat tolerance increases with environmental temperature. Points represent LT_{50} estimates from the present study and Tobias et al. (2024). Error bars represent 95% confidence intervals around each LT_{50} estimate. Black line is a linear regression of the natural logarithm of the LT_{50} estimates ($r^2 = 0.75$, $p = 0.0034$).

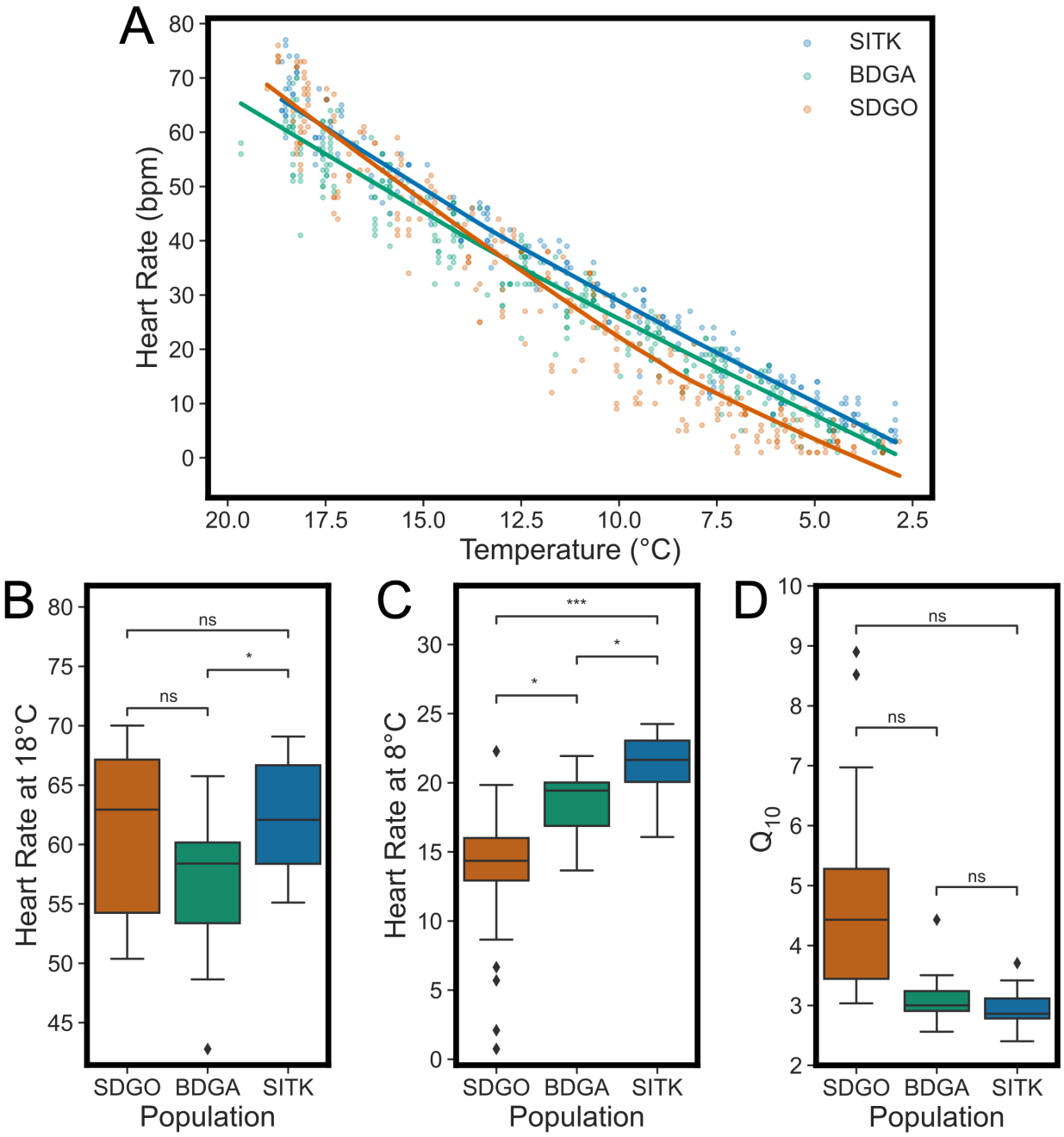

Figure 4-4. Populations differ in cardiac performance during a cold challenge. A) Heart rates during the course of the experiments. Points represent heart rate in a zooid at a given experimental temperature. Lines are locally weighted scatterplot smoothing (LOWESS) fits to each population's heart rate data. X-axis is reversed to reflect the progression of the experiments. B) Boxplot of interpolated heart rates at 18 °C by population. C) Boxplot of interpolated heart rates at 8 °C by population. D) Boxplot of Q_{10} values between at 8 and 18 °C by population. Two outlier values not displayed for SDGO (24.37 and 69.69) due to *y*-axis limits. Significance testing between pairs of populations was carried out by linear mixed effect modeling, with p-values adjusted via the Bonferroni method. * = $p_{adj} < 0.05$, ** = $p_{adj} < 0.01$, *** = $p_{adj} < 0.001$, ns = non-significant.

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SUPPLEMENTARY FIGURES

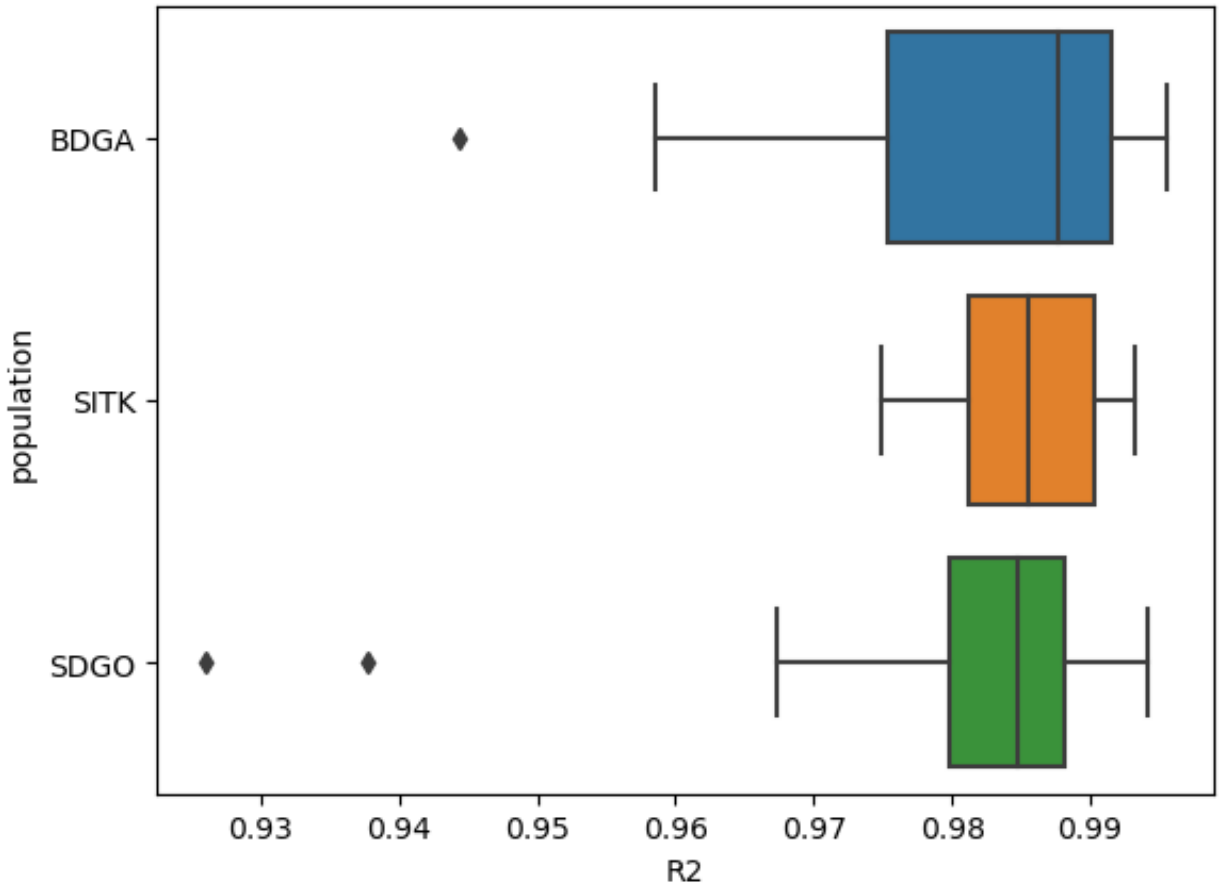

Figure S4-1. Distributions of R^2 values of linear regressions of heart rate to experimental temperature by site.

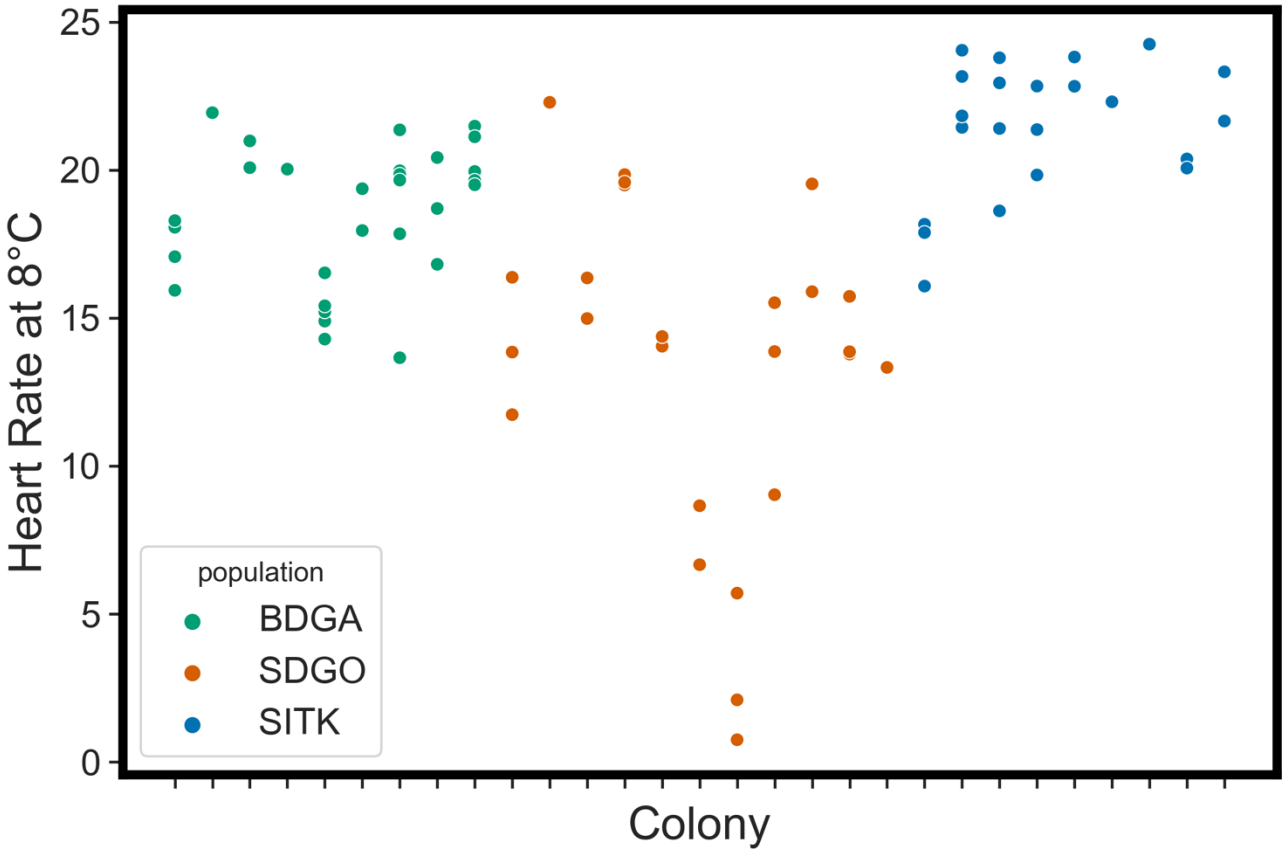

Figure S4-2. Interpolated heart rate at 8 °C of zooids by colony membership.

V

Conclusion

Throughout this thesis I have woven genomic and physiological lines of inquiry to understand how *Botryllus schlosseri*, a marine NIS, copes with variation in environmental temperature across geographic and temporal scales. By describing intraspecific variation of thermal tolerance and its genomic and environmental correlates, I contribute valuable information of how evolutionary adaptation and phenotypic plasticity may shape differentiation of a critical trait on contemporary timescales. Correspondingly, I demonstrate how using a biological invasion as a natural experiment can yield important insights into the pace of adaptive change in wild populations. This work was built on a foundation erected by previous researchers in such diverse fields as invasion biology, ecophysiology, bioinformatics, evolutionary biology, marine biology, and others. In this conclusion, it is my goal to place my doctoral research within the broader context of these fields and draw meaningful conclusions from a selection of my primary findings. Along the way, I inevitably engage in (perhaps more than) a few digressions, as well as point out some important caveats for interpreting my results. Lastly, I offer some parting thoughts on the potential scope of future efforts toward understanding rapid adaptive shifts of critical phenotypes during an era of global change.

Strides towards understanding the pace of adaptive change

A major theme of my dissertation research concerns the rate of adaptive change. By studying a marine NIS with a reasonably well-characterized chronology of introduction, I was able to place upper bounds on the timescales over which differentiation emerged, whether at the genomic or physiological levels. Importantly, the genomic data presented in Chapter 2 allowed me to better interpret the implications of the physiological data presented in later chapters, in particular Chapter 4. Specifically, the knowledge that all populations within San Francisco Bay and northward to Sitka, Alaska comprise one evolutionary lineage allowed me to determine that differentiation of cold tolerance between Sitka and Bodega Bay, California has emerged since *B. schlosseri*'s initial establishment in northern California in 1947 (Carlton 1979). While environmental effects could still contribute to differentiation (see Caveats below), that I observed population-level differences after a common acclimation treatment suggests that this differentiation is at least partly genetically based and that it has emerged within the last eighty

years, potentially much more recently since *B. schlosseri*'s establishment in Sitka by 2001 (Ruiz et al. 2006).

In addition to the potential for rapid evolutionary change, my dissertation research illustrates how *B. schlosseri* is able to respond quickly to environmental conditions through developmental plasticity. In Chapter 3, I demonstrated that short-term temperature history affects the heat tolerance of settled larvae (oozooids), whereby warmer environmental temperatures had a positive effect on oozooid LT_{50}. This response occurred on the order of days, speaking to the rapid pace at which developmental plasticity is able to shape thermal tolerance at later life stages. Taking these results together, putative local adaptation to temperature (most strongly evidenced by Chapters 2 and 4) and developmental plasticity of heat tolerance (demonstrated by Chapter 3) contribute to *B. schlosseri*'s ability to cope with variation in its thermal environment over spatial and temporal scales.

There are open questions as to whether species will be able to adapt to the plethora of changes they face in an era of great environmental upheaval (Visser 2008; Hoffmann and Sgró 2011; Martin et al. 2023; Urban et al. 2024). One of the most apparent and well documented environmental shifts is the increase in global mean temperatures. Both adaptive evolution and phenotypic plasticity have been invoked as potential bulwarks to this rapid change in temperature (Somero 2010), but it is unclear if they will be sufficient to buffer species from the worst of global change (Gunderson and Stillman 2015; Radchuk et al. 2019). By using the invasion and subsequent range expansion of *B. schlosseri* as a natural experiment, I evaluated the potential for this species to shift its thermal tolerance on contemporary time scales. While invasive species may be more physiologically flexible (Davidson et al. 2011) and thus should potentially be treated as an upper bound on the possible rate of adaptive change (see Caveats), my dissertation research serves as a potent example for how species can rapidly shift their physiology through genetic adaptation and phenotypic plasticity.

In the endeavor to predict evolutionary responses to global change, discourse has shifted from whether adaptation is possible (Parmesan 2006; Merilä 2012) to the specific factors that constrain or promote evolutionary adaptation to a changing world (Martin et al. 2023; Urban et al. 2024). Considerations such as the extent of standing genetic diversity

(Barrett and Schluter 2008), rates of gene flow (Aitken and Whitlock 2013), and interactions with phenotypic plasticity (Ghalambor et al. 2007) can all affect the likelihood and rate of adaptive evolution. By providing a pool of potentially adaptive standing genetic variation, high genetic diversity is likely to contribute to the adaptive potential of populations and species (Jump et al. 2009). In the case of *B. schlosseri*, I demonstrated high levels of genetic diversity (Chapter 2), which may contribute to its potential for local adaptation to temperature across its range. Interestingly, a substantial component of this variation was shared between both coasts of North America, but only a very small proportion was responding to thermal selection similarly in both lineages. This illustrates how high levels of standing genetic variation can facilitate local adaptation, but how the molecular mechanisms underlying it can be divergent (see Insights into evolutionary parallelism below).

In contrast with genetic diversity, gene flow has a more complex relationship with local adaptation. On one hand, gene flow can result in an influx of maladapted alleles in to a focal population, stifling local adaptation through gene swamping (Lenormand 2002). Contrastingly, gene flow can redistribute adaptive variation across space, which may be especially important in cases where environmental characteristics are shifting (Aitken and Whitlock 2013). For *B. schlosseri*, the often-extreme levels of genetic differentiation we observed (Chapter 2 Figure 2-3A) indicates severely restricted rates of gene flow among populations. Thus, *B. schlosseri* might be expected to possess a significantly greater likelihood of becoming local adapted to environmental temperature. However, limited gene flow may also mean that individual populations may not be able to rely on standing variation segregating within a larger metapopulation, instead having to rely on existing variation within populations or *de novo* mutations. The detection of clines in allele frequency at loci associated with environmental temperature suggests that a large component of this variation is segregating within populations rather than fixed, indicating that there may be further capacity for adaptive shifts.

The interactions between phenotypic plasticity and adaptive evolution are complex and may have a variety of outcomes on species' abilities to adapt to global change (Ghalambor et al. 2007; Fox et al. 2019). Adaptive phenotypic plasticity historically was assumed to hinder adaptive evolution by shielding genetic variation from natural selection (Huey et al. 2003).

However, the potential for initially environmentally induced phenotypes to become constitutive through genetic assimilation and/or accommodation may quicken adaptive evolution (Kelly 2019). Further, not all phenotypic plasticity is adaptive (Gibert et al. 2019); maladaptive phenotypic plasticity may increase selection coefficients and thus may speed local adaptation (Ruell et al. 2015). Finally, in its ability to cause rapid shifts in critical traits, phenotypic plasticity may be a means of "buying time" for adaptation to occur (Diamond and Martin 2021). *Botryllus schlosseri* clearly possesses phenotypic plasticity for temperature tolerance and is also likely locally adapted to temperature. It is unknown, however, how the two may interact in this species. Given that we observed variation for developmental plasticity among populations on the east coast of North America (Chapter 3), future investigation may focus on these populations and their respective "adaptability," perhaps via an experimental evolution approach.

Insights into evolutionary parallelism

Parallelism at the phenotypic level has long been of interest to naturalists and evolutionary biologists because it presents some of the most convincing examples of adaptation in nature (Bolnick et al. 2018). The observation that similar environments can drive the evolution of similar forms in independent lineages lends itself to a deterministic basis of evolution (Blount et al. 2018). Despite landmark examples of parallel phenotypic evolution being mediated by parallel changes at the molecular level (e.g. Cooper et al. 2003; Mundy et al. 2004; Chan et al. 2010), it is still far from clear if this is the norm, as there also exist many countervailing examples demonstrating parallel adaptation via divergent molecular means (e.g. Westram et al. 2014; Therkildsen et al. 2019; Fischer et al. 2021). By investigating the potential for parallel differentiation of thermal tolerance on the Atlantic and Pacific coasts of North America and examining the genomic basis of putative local adaptation to environmental temperature on both coasts, this dissertation contributes to our understanding of evolutionary parallelism in nature.

In Chapters 2 and 3 I undertook physiological studies examining how populations spanning latitudinal gradients in temperature differ with respect to their thermal physiology. In

both cases, I observed that more northern, putatively cold-adapted populations are more susceptible to heat stress than their southern counterparts, potentially reflective of parallel local adaptation to temperature on either coast. That we observed this among independent evolutionary lineages (as documented in Chapter 2) indicates that differentiation of thermal tolerance likely evolved repeatedly. While I only investigated cold tolerance across the Pacific coast, I would venture that given the high potential for local adaptation in this species and the genomic patterns indicative of local adaptation to temperature, we would likely observe similar patterns across the Atlantic coast. Because this dissertation spans its putative native range (east coast of the United States [*sensu* Yund et al. (2015)]) and its invasive range (west coast of the United States), it appears that this parallelism I observed in temperature tolerance across latitude arose on two vastly different time scales.

In addition to establishing parallel patterns of thermal tolerance on either coast, my genomic investigation shed additional light on the potential for evolutionary parallelism. In two independent evolutionary lineages, one on each coast, I detected thousands of loci whose allele frequencies significantly covaried with environmental temperature, suggestive of their role in local adaptation. Interestingly, despite sharing a substantial proportion of overall genetic variation, only a small subset of loci was implicated in local adaptation on both coasts. This suggests that parallel evolution of thermal physiology on either coast may be mediated by contrasting genetic mechanisms.

It is a useful exercise to speculate as to why this might be. While our understanding of the factors that mediate whether parallel phenotypic change will be accompanied by parallel changes at lower levels of biological organization remains incomplete, there exist some theoretical expectations (Bolnick et al. 2018). First, the type of genetic variation upon which selection acts can influence the degree of observed genetic parallelism. Parallelism at the molecular level is predicted to be much more likely for adaptation from standing genetic variation relative to that from *de novo* mutations (Schlötterer 2023). My results from Chapter 2 indicate a substantial proportion of genetic variation is shared between the two lineages on either coast of North America, potentially allowing natural selection to draw on the same pool of variation and “reuse” the same alleles in parallel adaptation to temperature. Nonetheless, I

observed minimal overlap in the identify of loci implicated in local adaptation to temperature, no more than would be expected due to chance.

Second, divergent population history can affect the probability of parallelism by introducing contingencies. Populations that have been evolving independently for long periods of time have more opportunities to accumulate differences through drift, minimizing the likelihood of parallel adaptation at the genetic level. Indeed, studies have demonstrated that more recently diverged population are more likely to utilize similar genetic mechanisms than those that have been in isolation longer (Bollback and Huelsenbeck 2009; Conte et al. 2015). The degree of divergence between the Pacific and Atlantic lineages of *B. schlosseri*, in terms of F_{ST}, is quite extreme, perhaps shedding light upon why we observed so few shared putatively adaptive loci. It would be informative to explore the potential for adaptive clines in allele frequency along other latitudinal gradients within *B. schlosseri*'s distribution, for example, in Europe, Oceania, or South America. If the lineages occupying these other gradients differ with regard to the level of divergence among one another, it would be a prime opportunity to test whether the extent of parallelism at the genetic level scales with the degree of divergence.

Lastly, the genetic architecture underlying traits under selection is expected to affect the observed degree of parallelism at the molecular level. Polygenic architectures, where many loci of small effect contribute to the emergence of a trait, are thought to result in high levels of potential redundancy, allowing for many evolutionary routes to the same phenotypic outcome (Barghi et al. 2020). Using a conservative approach, I found thousands of loci that are putatively adaptive for temperature (Chapter 2). This suggests that thermal tolerance is a highly polygenic trait in *B. schlosseri*, in accordance with studies in other species (Healy et al. 2018; Rose et al. 2018). Thus, given its polygenic basis, it is perhaps unsurprising that even with a substantial proportion of shared variation, local adaptation to temperature appears to be underlain by contrasting genetic means in the Atlantic and Pacific lineages of *B. schlosseri*.

Potential for local adaptation of phenotypic plasticity

Because genetic variation is required for natural selection to drive adaptation, much emphasis has been placed on the importance of disentangling genetic and environmental

contributions to phenotypic divergence. In this vein, for much of the twentieth century phenotypic plasticity was considered as a nuisance in the study of evolution (Falconer 1952; Pigliucci 2005). More recently, there has been heightened interest in understanding the complex interplay between evolutionary adaptation and phenotypic plasticity, and how it may mediate responses to environmental change (Ghalambor et al. 2007; Reed et al. 2011). Furthermore, there has been a shift towards viewing phenotypic plasticity as a trait in itself, one subject to natural selection (Pigliucci 2005). Studies that investigate variation for phenotypic plasticity, then, are important for documenting its potential to evolve.

Theoretical expectations have established that greater phenotypic plasticity should evolve in variable environments (Bradshaw 1965; Levins 1968; Schlichting 1986). Nonetheless, there remain few empirical examples that demonstrate local adaptation of phenotypic plasticity (Hendry 2016). In Chapter 3 I demonstrated that *B. schlosseri* possesses developmental plasticity of heat tolerance, whereby development at higher temperatures results in greater heat tolerance after settlement. Critically, I established that populations that experience greater short-term temperature variability exhibit greater magnitudes of developmental plasticity. If phenotypic plasticity were to become locally adapted, we would expect greater levels of plasticity in variable environments, as observed. Of course, because I could not fully disentangle genetics from environment (see Caveats), it is possible that plasticity operating over a longer time course (i.e. transgenerational plasticity) affected the degree of developmental plasticity I observed. Nonetheless, that we observed a difference in the magnitude of developmental plasticity that corresponded with differences in temperature variability does provide some evidence of local adaptation of phenotypic plasticity. An interesting follow-up study would be to measure the magnitude of developmental plasticity across a broader gradient of short-term temperature variability, as my investigation strictly compared a low-variability site to two high-variability sites. Clinal variation of developmental plasticity would provide firmer evidence for the potential local adaptation of phenotypic plasticity in this system.

Implications for management of biological invasions

Throughout this thesis I have emphasized the utility of species invasions for gaining insight into the evolutionary process. However, as major agents of global environmental change (Vitousek et al. 1996), NIS are also deserving of study insofar as it can inform appropriate management. Many knowledge gaps remain in our ability to design appropriate policy interventions to prevent and manage biological invasions (Clout and Williams 2009). Among these are understanding the sources and vectors of introduction and the prediction of where incipient NIS may colonize next. In pairing genomic and physiological investigation of *B. schlosseri*, a pernicious marine NIS, I provide some key insights that may benefit its management in North American waters and beyond.

Genetic approaches are often critical for unraveling the often complex invasion histories of NIS (Cristescu 2015). By describing patterns of genomic variation of *B. schlosseri* along both coasts of North America, Chapter 2 brings some clarity in understanding the potential sources of introduction. For example, in Newfoundland I found that there have likely been multiple introductions, one to Conception Bay that may have arisen from southern Maine or a closely related site, and potentially another from central Nova Scotia. Without the knowledge of multiple introductions, management may be targeted towards containing its spread from where it was first detected, treating it as a beachhead invasion. Understanding that recurrent invasions have occurred can help policymakers design more appropriate interventions, perhaps those that focus on interprovincial translocation rather than local spread. A similar case can be made for Southeast Alaska, where I found that populations are more closely related to those further south along the United States coast rather than the nearby populations in northern British Columbia. This knowledge may make the case for focusing on introduction via hull fouling from vessels arriving from further south along the United States coast.

Once a NIS is established, it is often extraordinarily difficult to eradicate, and generally only possible at the earliest stages of invasion (Parkes and Panetta 2009). As such, prevention, early detection, and rapid response are critical to the effective management of species invasions (Reaser et al. 2020). Much of the existing efforts aimed towards predicting where NIS may turn up next depend on species distribution modeling (Gallien et al. 2010). This approach

characterizes the abiotic environment where a NIS is found to make inferences about its environmental tolerances and thus where it may be capable of invading. While a useful approach, by inferring habitat suitability by investigating its current distribution it ignores the potential for niche under-filling: species may be capable of enduring environmental conditions outside their current regime. To more accurately describe the environmental tolerances of potential invaders, empirical studies of NIS physiology are required. In Chapters 3 and 4 I presented new data on the heat and cold tolerance of *B. schlosseri*, providing useful information of the environmental tolerances of this marine NIS that could potentially be used to inform mechanistic modeling that more directly incorporates physiology (Lennox et al. 2015).

Importantly, I describe how thermal tolerance varies across geography. Most species distribution modeling treat species as a monolith, sharing a single set of environmental tolerances. Incorporating intraspecific variation of environmental tolerances can yield substantially different predictions of suitable habitat (e.g. Oney et al. 2013; Hu et al. 2021). For NIS, when there is appreciable genetic structure and lineages may differ in their physiology (e.g. via local adaptation), directly modeling this variation substantially alters predictions of where they may be capable of invading (Compton et al. 2010; Fraimout and Monnet 2018). By demonstrating differentiation of thermal tolerance among populations and lineages of *B. schlosseri*, this dissertation emphasizes the importance of accounting for this variation when making predictions about future spread.

Beyond characterizing the potential for intraspecific variation of environmental tolerances, I have provided clues into how evolutionary adaptation may underlie invasion success in *B. schlosseri*. At a physiological level, perhaps most convincing is the observation that individuals from more northern, cold-exposed populations in the Pacific have apparently adapted their cardiac physiology, maintaining higher heart rates in cold temperatures than their southern counterparts (Chapter 4). At a genomic level, clines in allele frequencies that are coincident with a gradient of environmental temperature suggests there has been widespread genetic adaptation to temperature across *B. schlosseri*'s invasive range in North America (Chapter 2). These results demonstrate the potential for invasive species ecological niches to expand or shift during the course of invasion. Failure to model these evolutionary shifts in the

niche of NIS is an additional shortcoming of species distribution modeling, which by their design assume evolutionary stasis. My results, then, demonstrate the importance of accounting for potential niche shifts/expansion through adaptation in modeling efforts, which has been done for native species (Benito Garzón et al. 2019; DeMarche et al. 2019) but has rarely been applied in studies of NIS (Gallien et al. 2010; but see Chapman et al. 2017).

Caveats

Species invasions can serve as potent examples of rapid evolutionary change, but how representative are these examples of species at large? Indeed, there is much to be learned about the pace of adaptive change from the study of successful NIS (Huey et al. 2005; Sax et al. 2007; Westley 2011), but by focusing on the "winners", conclusions drawn from these examples should perhaps be treated as an upper bound of what is possible (*sensu* Moran and Alexander 2014). Invasive species have been hypothesized to be more phenotypically plastic than their native counterparts (Baker 1965), which has been confirmed in a meta-analysis by Davidson et al. (2011) (but see Palacio-López and Gianoli 2011). These higher levels of phenotypic plasticity may be one significant factor underlying invasion success, but also indicate that caution should be exercised when drawing conclusions from NIS to native species. Given the importance of evolutionary adaptation to the success of species invasions, it is conceivable, too, that NIS harbor elevated rates of evolutionary change compared to natives, though I am not aware of any support for this hypothesis in the literature. Thus, concerning the rate of adaptive change, in particular in response to global change, it is likely that drawing conclusions from NIS will overestimate the adaptive capacity of native species (Moran and Alexander 2014). Nonetheless, lessons from NIS demonstrate the rapid evolutionary change is possible, providing a glint of optimism in an era of otherwise dire circumstances for the world's biota.

By using genomics and ecophysiology to study patterns of variation in thermal tolerance across space and time, my dissertation provides insights into how local adaptation and phenotypic plasticity shape a critical trait in natural populations. However, I have not endeavored to fully disentangle the contributions of these two processes, as is often the goal of evolutionary studies. Therefore, it is possible that environmental effects have contributed to

the patterns which I ascribe to local adaptation. For example, I use the pattern of countergradient variation of heart rate that I observed in Chapter 4 to argue for compensatory genetic adaptation to colder environmental temperatures in northern populations. While I used a common acclimation treatment to minimize the potential effect of acclimatory plasticity, it is still possible that phenotypic plasticity operating over a longer time course (i.e. transgenerational plasticity) contributed to this pattern. Contrastingly, it is also possible that genetic factors contributed to patterns which I ascribe to phenotypic plasticity. For example, in Chapter 3 I use natural temporal variation of environmental temperature as a proxy for developmental temperature. In certain circumstances this spanned several months. In the intervening time between experiments at a particular site, it is possible that temporally varying selection resulted in more heat-resistant genotypes being dominant later in the summer, producing more heat-tolerant larvae. I consider this rather unlikely, as I also observed shifts in oozooid thermal tolerance consistent with variation in temperature across just days. Nonetheless, this speaks to the potential confounding effects of genetic and phenotypic plasticity in shaping the patterns I observed.

While biological study of *B. schlosseri* is certainly more developed than for many marine invertebrates, it is still far from an established model system of the likes of fruit flies and mice. As such, it lacks some of the critical techniques that allow for disentangling genetic and environmental contributions to phenotypes, namely accessible intergenerational culture. In fact, to my knowledge there are only three labs in the world that regularly rear *B. schlosseri* over multiple generations (Ben-Hamo and Rinkevich 2021). I do not view this strictly as a shortcoming, however. By investigating differentiation of thermal tolerance in a more "non-model" species, my dissertation provides valuable information on the potential for this critical trait to shift rapidly, adding taxonomic breadth to the existing literature that may make it more representative of the diversity of evolutionary trajectories in nature.

Future directions

In seeking to explain life's complexities, biological research often generates more questions than answers. While this dissertation comprises a small but valuable contribution

towards our understanding of how genetics and environment shape thermal tolerance across spatial and temporal scales, it has also brought up a number of questions. Below I will attempt to summarize my thoughts on where we might go from here.

By applying low-coverage whole genome sequencing to two dozen populations across North America, Chapter 2 provided the most high-resolution picture of population genetic structure in *B. schlosseri* to date. In doing so it made strides towards resolving the species' complicated invasion history in the region. A logical next step would be to expand the geographic scope of sequencing efforts. Including specimens from Europe and Asia would be particularly helpful for understanding its global invasion history and would shed light on how the North American lineages fit into a global context. In addition to understanding invasion dynamics, sequencing of populations spanning other latitudinal gradients in temperature would provide further opportunities to investigate parallel adaptation to temperature and whether the genomic basis is shared or divergent. If a more global approach uncovered distinct lineages that differ with respect to their degree of divergence, as is likely, it would be a useful system in which to test the prediction that more recent divergence leads to a greater degree of parallelism at the genetic level.

Another potentially exciting avenue that stems from my research is the potential for local adaptation of phenotypic plasticity. In Chapter 3 I demonstrated that populations which experience more short-term temperature variability exhibit greater levels of developmental plasticity. While far from proving this definitively, this observation provides correlative evidence for local adaptation of developmental plasticity in this system. Future efforts should explore this further. For example, one potential experiment could assess variation in the magnitude of developmental plasticity across a broader gradient of short-term temperature variability, with the expectation that the most variable environments would favor the evolution of the most plastic genotypes. Ideally this would be conducted with multigenerational rearing to establish a heritable basis of increased plasticity. When paired with genotyping, this approach could shed light on the genetic architecture of phenotypic plasticity.

Beyond these more specific research directions into *B. schlosseri* itself, my dissertation research highlights additional questions about species invasions and the evolution of species

more broadly. For example, what are the relative contributions of local adaptation and phenotypic plasticity to the success of NIS? Do invasive species exhibit greater adaptive capacity than native species? If so, what characteristics of NIS promote rapid evolutionary adaptation? How might evolutionary adaptation and phenotypic plasticity interact to shape evolutionary responses to rapid environmental change? When do we expect parallel phenotypic evolution to be mediated by parallel changes at lower levels of biological organization? All of these questions are beyond the scope of any one dissertation, and rather encompass the aims of entire research programs, if not entire subfields. Nonetheless, it is my hope that my dissertation has made some small contribution towards resolving some of these thorny questions.

Parting thoughts

Throughout this dissertation, and, indeed, my nearly six years of doctoral study, I have strived to understand how an obscure marine invertebrate inhabiting the bottoms of docks copes with temperature variability across space and through time. By investigating processes that span levels of biological organization, from nucleotides to populations, my dissertation can be viewed as a truly integrative body of work, one that weaves genomic and physiological lines of inquiry towards understanding the modalities of thermal adaptation. In doing so I hope this dissertation has provided some insights into the roles of local adaptation and phenotypic plasticity in shaping thermal tolerance, and how the pace of adaptive change can inform our understanding of species responses to global change. It is up to the reader to determine whether or not I have succeeded in this task. This judgment notwithstanding, I am delighted by the opportunities I have been provided with to interrogate big questions in this tiny sea squirt, *Botryllus schlosseri*.

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