DIFFERENTIAL SUSCEPTIBILITY OF COASTAL MARINE INVERTEBRATES TO INFECTION BY PARASITIC CASTRATORS: INSIGHTS FROM SPATIALLY VARIABLE INFECTION RISK

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(Under the Direction of James E. Byers)

ABSTRACT

Parasites are ubiquitous in natural populations, and can exert strong selection on hosts to defend themselves from infection pathology. Parasites are also often heterogeneously distributed across host habitats, in terms of pathogenicity, diversity and prevalence. The extent to which parasite infection risk constitutes a force of selection for host resource allocation to defense is expected to depend on both the likelihood of encounter and the fitness consequences of infection. Adaptation of hosts to local parasite selection regimes also depends on host gene flow between transmission hotspots and coldspots. I used two different types of marine invertebrate hosts (snails in the genus *Littorina*, and the shore crab *Hemigrapsus sanguineus*) and their common castrating parasites to test 1) whether wild hosts experiencing divergent parasite selection regimes were adapted to local levels of infection risk, and in the shore crab hosts, 2) whether hosts experiencing a reprieve from infection risk in their invasive range showed altered energy allocation to immune defenses and other fitness traits. In *Littorina* snails, I used a common garden experiment to test whether historical exposure to high or low castrating trematode parasite infection risk predicts the ability of snails to avoid or defend against trematode infection, and whether host local adaptation to infection is influenced by host population genetic admixture

as determined by each species' reproductive most. For the two *Littorina* host species with direct development of offspring and thus low population admixture, snails originating from high infection-risk environments were less susceptible to infection than snails from naïve populations. Next, because introduced species often escape from infection risk from their co-evolved native parasites, we hypothesized that invasive host populations may respond to relaxed parasite selection in the invasive range by redistributing resources away from immune defenses and toward other fitness traits. A common garden experiment comparing infection susceptibility in *H. sanguineus* from the native range, where castrating rhizocephalan barnacle parasites are common, to susceptibility in invasive-range crabs where these parasites are absent showed higher susceptibility among invasive-range crabs. An exploration of immune defense traits and energy storage revealed that invasive range crabs have lower self-maintenance metabolic costs and altered relationships between reproductive energy investment and immune trait investment. In invasive species, these results support that invasive populations that experience reduced selection from the parasites with which they co-evolved may re-allocate defense resources to competing fitness traits, but that reduced defense investment comes at the cost of heightened susceptibility to infection. Overall, this dissertation supports that parasites constitute a strong force of selection acting on host resource allocation, and that the loss or intentional removal of parasites from host populations may result in host populations that are vulnerable to future infections.

INDEX WORDS: local adaptation, invasive species, parasite escape, ecological immunology, trade-offs

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DEDICATION

With extreme gratitude for their encouragement and good humor, I dedicate this document to my family. I especially appreciate the patience and support of Chris Goodson during my extended absences from home while I hunted snails and crabs in various states and countries.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Parasites as forces of selection

Contemporary ecological communities are the product of, and players in, dynamic webs of interacting selective forces. Biotic interactions play some of the strongest roles in shaping species' evolutionary paths. Co-evolution with parasites may represent one of the strongest forces of interspecific selection acting on hosts. Parasitism is by definition an antagonistic interaction from the perspective of hosts, as parasites divert host resources for their own growth and reproduction. In extreme cases, parasite infection results in host death or in reduced fecundity or castration.

In addition to the direct negative effects parasites exert on their hosts through depletion of resources or destruction of host cells or tissues, infection pathology may also be driven by the host's response to the invading parasite. When hosts deploy molecular defenses to limit or stop the spread of an invading parasite, these immune defenses come at a cost to the host (Sheldon & Verhulst 1996). Immune costs generally take either the form of a resource cost (Moret & Schmid-Hempel 2000), or the cost of self-harm if destruction of the parasite requires the production and release of cytotoxic compounds into the host's cells or tissues (Sadd & Siva-Jothy 2006). The study of how trade-offs between defense and host fitness contributes to the distribution, persistence, and evolution of both parasites and hosts has garnered enough attention in the past two decades to initiate a new sub-discipline of ecological immunology (Sheldon &

Verhulst 1996; Rolff & Siva-Jothy 2003; Martin *et al.* 2011). Evolutionary trade-offs between resistance to damaging parasites and fitness costs for other life history traits are emerging as an important driver of heterogeneity in resistance/defense in natural populations.

If immune defenses are costly, they should only be favored in environments where the risk of infection by fitness-limiting parasites is consistently high. Thus across a spatial mosaic of parasite selection (Thompson 1999), hosts may be expected to adapt investment in defense across generations to match local infection risk, if gene flow between patches experiencing different parasite selection pressures is not too disruptive (Lenormand 2002). In Chapter 2 of this dissertation, we test whether intertidal snails in the genus *Littorina* from six sites across the Gulf of Maine are locally adapted to different levels of risk of infection by castrating trematode parasites. In particular, we test for local adaptation to infection risk in two species (*L. obtusata* and *L. saxatilis*) that have local recruitment and high population genetic structure, and one species (*L. littorea*) with high gene flow, which we expected to act as an outgroup.

Chapters 3 and 4 extend this question of local adaptation to infection risk to ask whether the trade-offs between immune defense and fitness traits described above may contribute to the demographic success of invasive species.

Consequences of altered parasite selection in invasions

Biological invasions pose increasing challenges to co-evolved native ecosystems as human civilization operates with ever less regard to natural barriers to species movement (Carlton and Geller). Because they often share no recent evolutionary history with their recipient ecological communities, populations of invasive species necessarily experience a similarly dramatic alteration in selective pressures as the change they impose on the invaded community.

"The mixing of tens of thousands of species worldwide is thus a Whitman's Sampler of competition, predation, and disturbance, with every possible positive and negative outcome…" -- *J.T. Carlton*

To the extent that there are predictable changes in selective pressures experienced by invaders, the loss of co-evolved natural enemies such as parasites is a well-established pattern (Torchin *et al.* 2002; Mitchell & Power 2003; Torchin *et al.* 2003; Blakeslee *et al.* 2013).

In their reprieve from infection by parasites, invaders benefit in two ways – the most obvious (though surprisingly rarely quantified) and direct benefit is that they no longer experience parasite-mediated mortality or loss of fecundity. A less obvious but perhaps equally important benefit is that of "compensatory release" (Colautti *et al.* 2004), whereby invaders benefit from the freeing-up of resources no longer needed for the expensive maintenance and deployment of the immune system, and can instead allocate more resources to growth and reproduction (Blossey & Notzold 1995). A hypothesized consequence of this relaxed parasite selection is that invasive host populations may ultimately be rendered more susceptible to infection (Perkins *et al.* 2008), and studies of invasive plants have sometimes found evidence to this effect (Wolfe 2002; Wolfe *et al.* 2004).

Most empirical studies of infection susceptibility in animal invasions have largely been "community studies" comparing the infection rates of native and invasive host species by common parasites in the invasive range (reviewed by Dunn 2009), rather than explicitly testing the effect of relaxed selection. This community approach is informative of the potential benefits invaders may have over native congeners that suffer natural levels of infection, and also provides a backdrop for understanding the reprieve from infection experienced by invaders in the recipient range – are invaders simply "superior genotypes" that are very resistant to infection? However,

to untangle the effects of invader resistance from native-parasite infectivity, studies comparing the susceptibility of native versus invasive populations to native-range parasites, in which "compatibility" filters (Kuris *et al.* 2007) on the part of the parasite should not operate, are more informative.

Results from a handful of laboratory (reviewed by Duncan *et al.* 2011) and theoretical studies have provided support for the hypothesis that relaxation of parasite-mediated selection may result in a loss of infection resistance in the host population. To our knowledge only one experimental removal of parasites has been carried out in the wild. After experimental removal of parasites from Trinidadian guppies, Dargent *et al.* (2014) tested the susceptibility of offspring to infection by a costly ectoparasite and found that the manipulated population showed higher resistance to infection rather than higher susceptibility. This surprising result suggests that wild populations may respond in more complex ways than lab populations, highlighting the need to carry out more experiments against the backdrop of combinations of selective pressures that may not be reproducible in the lab (Pedersen & Babayan 2011).

Despite the attractiveness of the hypothesis that relaxed parasite selection may lead to altered immune defenses and higher infection susceptibility in invasive populations, in animal systems this topic has so far received more attention in reviews than in empirical studies (Perkins *et al.* 2008; Dunn & Perkins 2012; Dunn *et al.* 2012; White & Perkins 2012). Several studies have capitalized on heterogeneity in parasite distribution across the invasive range of range-expanding invasive species to begin to test for differences in immune traits at the (usually) parasite-free expanding edge (Phillips *et al.* 2010), versus the more parasitized invasion epicenter (Llewellyn *et al.* 2012; Brown *et al.* 2015). One of the most direct tests of the consequences of

relaxed parasite-mediated selection on invaders is whether they differ from conspecifics from their native range in their ability to defend against infection by native-range parasites. In Chapter 3, we compare the susceptibility of invasive-range and native-range shore crabs (*Hemigrapsus sanguineus*) to infection with castrating rhizocephalan barnacle parasites from the native range. As in Chapter 2, we use a common-garden approach focusing on the difference in infection levels between invasive- versus native-range crabs as the bottom-line test of the effect of different parasite infection risk on immune defense investment. Chapter 4 extends the exploration of the consequences of relaxed parasite selection on invader immune defenses by comparing two immune traits and three energy traits between invasive- and native-range H. sanguineus before and after exposure to rhizocephalan parasite larvae. We ask whether general immune traits predict likelihood of infection, and whether these traits trade-off with energy allocation to self-maintenance, energy storage, or reproduction.

The work presented here expands previous studies of parasite-mediated selection that have been carried out in largely laboratory-based settings (but see Bryan-Walker *et al.* 2007; Hasu *et al.* 2009) to a more natural field context (Chapter 2), and to an invasive species context (Chapters 3 and 4). Characterizing the response of invasive hosts to differences in selective pressure in their invasive range and native range is of critical importance for understanding drivers of divergence from known natural history patterns in the native range and for predicting responses of invaders to changing selection pressures in their recipient communities.

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CHAPTER 2

LOCAL ADAPTATION TO PARASITE SELECTIVE PRESSURE: COMPARING THREE CONGENERIC CO-OCCURING HOSTS[1]

[1]Carolyn L. Keogh, Martha E. Sanderson, and James E. Byers. 2015. Oecologia 180: 137-147. Reprinted here with permission of the publisher.

ABSTRACT

Local adaptation may optimize an organism's investment in defenses in response to the risk of infection by spatially heterogeneous parasites and other natural enemies. However, local adaptation may be constrained if recruitment is decoupled from selective pressure experienced by the parent generation. We predicted that the ability of three intertidal Littorinid snail species to defend against trematode parasites would depend on prior levels of population exposure to parasites and on larval dispersal mode, a proxy for population openness. In a common garden experiment, for two snail species with direct development and localized recruitment (*Littorina obtusata* and *L. saxatilis*), hosts from sites with high trematode infection risk were less susceptible to infection than hosts from low-risk sites. However, this relationship was not apparent for a third host species with broadcast larvae (*L. littorea*), suggesting that broad larval dispersal can impede local adaptation; alternatively, the lack of response in this species could owe to other factors that limited experimental infection in this host. Our findings support that locally recruiting hosts can adapt their defenses to scale with localized infection risk.

Key words: ecoimmunology, life history, trematodes, *Littorina*, co-evolutionary arms race

INTRODUCTION

All natural populations experience infection by parasites, which vary greatly in the degree to which they stimulate a defensive response from their hosts. Host responses to parasite infection risk are context-dependent, and the relative levels of resistance expressed by individual hosts determine rates of disease spread and persistence at the level of populations and metapopulations (Ardia et al. 2011; Downs et al. 2014). Thus, understanding drivers of variation in susceptibility to parasite infection in populations under natural selective regimes is a key goal for the emerging field of ecoimmunology (Downs et al. 2014; Martin et al. 2011), and a step toward prediction and control of disease outbreaks in natural populations (Hawley and Altizer 2011).

While all wild hosts are expected to have some ability to defend against parasites, anti-parasite defenses are known to come at a cost to hosts, either by diverting energy from other fitness components, or via autoimmune reactivity (Hasu et al. 2009; Sheldon and Verhulst 1996). Higher defense investment is therefore only advantageous when resistance costs are outweighed by the cost of infection by the parasite, and when the risk of infection is consistent both spatially and temporally (Ardia et al. 2011; Barrett et al. 2008; Zuk and Stoehr 2002). Thus the existence of transmission hotspots across a host range that is otherwise low in infection risk can give rise to divergent selection on traits for resistance, resulting in divergent adaptation of host traits to local levels of infection risk (Kawecki and Ebert 2004). For example, amphipods from natural populations that are naïve to infection have higher susceptibility to trematode or acanthocephalan parasites compared to populations where natural infection is common (Bryan-Walker et al. 2007; Hasu et al. 2009).

Although host immune investment may be expected to reflect the spatial mosaic of parasite-mediated selective pressure (ie. local adaptation), the extent and effectiveness of any inherited mechanisms of adaptation may also be highly influenced by the amount of gene flow between host populations under different selection regimes (Gandon et al. 1996; Kawecki and Ebert 2004; Lenormand 2002; Slatkin 1987; Urban 2011). Some gene flow is necessary to maintain the variation on which selection acts, but too much mixing counteracts the effects of local selection and inhibits adaptation to local conditions (Lenormand 2002; Slatkin 1987). Given sufficient standing genetic variation, host populations that have high rates of local recruitment (i.e. with offspring retained in the parents' habitat) may be more likely to evolve phenotypes that reflect local selection pressure without the disruptive effect of gene flow from non-adapted populations. In contrast, hosts with broad propagule dispersal may be selected to maintain generalist phenotypes and greater phenotypic plasticity to ensure their success in a broader range of potential habitats (Warner 1997; Yamada 1989). Finally, because host-parasite interactions are co-evolutionary in nature, the amount of dispersal and admixture of the parasites themselves may also be an important determinant of the hosts' ability to locally adapt (Gandon et al. 1996; Hoeksema and Forde 2008; Lively 1999; Roth et al. 2012).

Marine organisms provide an ideal venue for testing these questions because of the diverse types of larval dispersal strategies (and their associated rates of gene flow) often found among sympatric, closely related species. We used a snail-trematode system to explore the interaction of historical parasite exposure (as a proxy for parasite-mediated selective pressure) and larval dispersal mode on the local adaptation of host defense against parasite infection. In the New England rocky intertidal zone, three congeneric species of *Littorina* snails occur in

sympatry. *Littorina saxatilis* and *L. obtusata* are native to New England and Europe, while *L. littorea* is a naturalized invader from Europe that has inhabited New England rocky shores for over 150 years (Blakeslee et al. 2008). These species differ greatly in their reproductive strategies: *L. saxatilis* brood their young and fully developed juveniles emerge from the mother's brood sac; *L. obtusata* lays benthic egg masses that hatch as crawl-away juveniles; and *L. littorea* releases pelagic larvae which spend an estimated 4-7 weeks in the water column (Reid 1996). The amount of gene flow within each species conforms to expectations based on their life histories: *L. saxatilis* and *L. obtusata* have higher levels of population genetic structure, while *L. littorea* shows high levels of genetic admixture and little differentiation (Berger 1973; Johannesson and Johannesson 1996; Johannesson 2003; Schmidt et al. 2007; Snyder and Gooch 1973). *Littorina saxatilis* in particular has been found to have strong genetic clines over the scale of a few meters, and strong evidence of population isolation on separate but neighboring islands (<15km distant) (reviewed in Johannesson 2003; Johannesson and Tatarenkov 1997).

Among the three snail hosts, spatially heterogeneous patterns of infection by castrating digenean trematode parasites have been well documented (Blakeslee et al. 2012; Blakeslee and Byers 2008; Byers et al. 2008; Byers 2015; Granovitch et al. 2000). In New England, *L. littorea* is most commonly infected by the trematode *Cryptocotyle lingua*, while *L. saxatilis* and *L. obtusata* are more commonly infected with *Microphallus similis*, but there is considerable overlap of the trematode assemblages in all three host snails (Blakeslee and Byers 2008). Trematode parasites utilize sexually mature (adult) *Littorina* snails as first intermediate hosts in their complex lifecycles, reproducing asexually in the region of the snail's digestive gland and gonad, and causing castration of the snail host (Robson and Williams 1970; Rothschild 1942).

Free-swimming cercariae are released from the snail and penetrate a second intermediate host (fish for *C. lingua*; crabs for *M. similis*), which is consumed by a seabird definitive host (e.g. eider ducks, cormorants, and gulls (*Larus marinus*, *L. argentatus*)). As the infected birds forage, loiter or nest on rocky shores, they deliver trematode eggs via their guano to the vicinity of the snail hosts. *Microphallus similis* and *C. lingua* eggs are ingested by grazing snails to complete the life cycle (Stunkard 1930; Stunkard 1957). Thus, gull colonies, which are often found on uninhabited rocky islands, and gull attractors such as fishing piers, emerge as hotspots of trematode transmission to snails (> 50% prevalence in adult snails at some sites), while many other rocky intertidal sites exhibit low to no trematode prevalence (Byers et al. 2008; Hoff 1941; Matthews et al. 1985).

The unique features of this host-parasite system provide a valuable opportunity to test for the relative influence of local selective pressure and dispersal-mediated gene flow on host local adaptation. We carried out a common garden experiment to test for differences in susceptibility to trematode infection in wild-caught snails originating from locations with high or low trematode infection risk history (HR vs LR), hypothesizing that snails from locations with high parasite selective pressure would show lower susceptibility to infection, reflecting local adaptation of host defenses. We further hypothesized that this effect would only hold for the two snail species with local reproduction modes. We also tested the assumption that trematode parasites have high levels of genetic mixing in this system that might reduce their tendency to locally adapt to or specialize on differentiated snail genotypes.

METHODS

Snail collections

To test for an effect of parasite selective pressure on susceptibility to trematode infection, the three species of *Littorina* snails (*L. obtusata, L. saxatilis, and L. littorea*) were collected from multiple intertidal sites along the coast of New England, USA between June 28 and July 1, 2011 for use in a laboratory common garden experiment (Figure 1). Specifically, we collected each snail species from three sites where at least one study over the previous 10 years had identified high risk (HR) of trematode infection (i.e., >10% trematode infection prevalence in at least one of the three species, and high densities of shorebird definitive hosts), and from three sites with known low risk (LR) of trematode infection (<10% infection rates in prior surveys of any species, and/or low density or absence of shorebirds) (Table 1). Not all snail species were present at all sites in sufficient numbers, so the six collection sites are not identical for each species (Figure 1, Table 1). We sourced snails from multiple HR and LR sites to ensure broad characterization of each category and thus minimize the influence of isolated, site-specific effects on snail performance.

To determine the consistency of selective pressure acting on the current generation of snails at our snail collection sites, and thus to validate our categorization of a site as either HR or LR, we examined ~100 adult snails of each species at each site for trematode infections. Because snails live for multiple years and successful infections are usually permanent in the snails, measurements of trematode parasite prevalence in the adult snail population at each site, including larger, older snails, allowed us to infer infection risk integrated over multiple years, giving a fuller picture of the consistency of parasite infection risk at each site (Graham 2003).

For these surveys, snails representing the full range of adult sizes were collected haphazardly at low tide from a broad area (>100m length whenever possible, including high and low intertidal). We measured the collected snails from the apex to the anterior tip of the aperture and then dissected them under a stereomicroscope and examined the gonad and digestive tissues for presence of trematode cercariae, sporocysts or rediae. Trematode species were identified using published keys and descriptions of trematodes infecting *Littorina* sp. (James 1968; Stunkard 1983).

Our estimates of trematode prevalence in each host species at each collection site were evaluated against data from previous studies to confirm or refute each collection site's *a priori* assignment to the HR or LR category (Byers et al. 2008, Blakeslee and Byers 2008, Blakeslee, unpublished data). Consistency of high parasite pressure was affirmed if trematode infection prevalence in at least one snail species exceeded 10% in both a previous report and the current study (Table 1). We attempted to collect snails of each historical infection risk category (LR and HR) over as large a domain as possible; however, due to limitations in available qualifying sites, our range of collections was more geographically constrained for HR snails. Specifically, because of the uncertainty in the consistency of parasite mediated selection pressure at one of our *a priori* designated HR sites (Odiorne, NH), we excluded snails from this site from our analysis. The two sites that showed consistent infection in the snails are both on gull nesting islands, where gulls and other sea birds are known to aggregate annually at high densities (Ellis and Good 2006), further supporting their categorization as HR sites. These two island sites are relatively geographically close with the potential for greater connectivity by dispersal for the host species with pelagically dispersed larvae. For the two species with closed populations (*L.*

obtusata and *L. saxatilis*), these two collection sites are still likely to represent separate demes, given they are on different islands and these two species can exhibit small-scale genetic differentiation (Johannesson and Tatarenkov 1997; Schmidt et al. 2007).

For the focal experimental snails used in the laboratory common garden experiment, we collected ~200 small-sized adult snails of each species at each site. Small adult size ranges were approximately 4.5-8mm max spire height for *L. saxatilis*, 6-9mm for *L. obtusata*, and 7-12mm for *L. littorea*. Among sexually mature (adult) snails, smaller individuals were preferred for use in experiments because they are susceptible to trematode infections, but are less likely to harbor infections than larger, older adults which are more likely to have previously encountered trematode infective stages (Graham 2003). Collected snails were held in sea tables with running ambient seawater at the Shoals Marine Laboratory, Appledore Island, Maine.

Common garden infection experiment

We used a common garden experiment to test whether a history of trematode infection risk (i.e. HR versus LR) affected susceptibility to trematode infection in each host species. A random subset of 50-100 snails of each species in the small adult size class from each site was painted to denote their collection site using BriteMark paint pens. We combined these groups of snails in a single tray of a flowing seawater table, evenly distributing them into one of two identical 1.4m^2 mesh bags (made from 0.33mm^2 black screening material) populated with rocks and *Ascophyllum* seaweed to form a semi-natural habitat. The water was drained from the sea table tray once daily for a period of 2-3 hours to simulate a low tide, and the tray was rinsed regularly to prevent the buildup of snail waste and algae. We exposed all experimental snails to infective trematode eggs by adding seabird guano, which contains trematode eggs from infected

birds, to the common garden habitat. Bird guano was collected from a common seabird loitering area that was separate from any snail collection site (Figure 1). Dissections of snails inhabiting this guano collection area over the 10 past years have consistently shown high levels of trematode prevalence in this area, suggesting that the site is a hotspot for transmission from infected birds to the snail first intermediate hosts (Byers et al. 2008; Byers 2015). During the guano collection period, daily counts (n=11) of seabirds utilizing the intertidal area (~100m x 30m) from which the majority of guano was collected averaged 27.5±2.8 (mean±SD) black back gulls (*Larus marinus*), 2.2±0.7 Double-crested cormorants (*Phalacrocorax auritus*), and 4.0±1.3 herring gulls (*Larus argentatus*).

Fresh guano deposits were scraped off the rocky substrate into a 50mL plastic tube, and diluted in a 1:2 ratio with seawater to form a homogenous slurry. This slurry was either poured evenly across the habitat in both snail containment bags during the simulated low tide each day or used to coat fresh seaweed (usually *Ascophyllum* with a mix of *Ulva* and *Fucus* sp.) which was then distributed in the habitat as a supplemental food source. For a period of 24 days, an average of 12.5± 1.3mL of guano per day was added to each bag (i.e., 37.5mL per bag after seawater dilution), alternating between the two application methods. This volume of guano is substantially higher than would be encountered daily over a similar area in nature where the deposits are discrete and patchy; however using the large diluted volume allowed us to ensure even distribution across the bags to standardize access to infective stages for the experimental snails. After the inoculation period, the snails were maintained in the sea table habitat for an additional four weeks, and in aerated seawater tanks for an additional two weeks to allow any

induced infections to reach patency. After this six-week incubation period, all experimental snails were dissected to assess infection status.

The other half of the collected small adult snails were used to control for baseline levels of background field-acquired infection at each site, and were maintained in a separate sea table from the experimental snails to prevent parasite exposure. These control snails were maintained in Tupperware containers with mesh sides to allow water circulation, and were separated by collection site. The snails were provided with seaweeds collected from a cove on Appledore Island with low bird density and low natural trematode infection in snails to avoid introducing new infections. We subjected these snails to the same simulated tidal cycle and sea table cleaning schedule as the experimental snails. The snails were held for a minimum of four weeks to allow any field-acquired infections to reach patency, and then dissected over the course of four weeks. Though these control snails were necessarily maintained under slightly different conditions to prevent the acquisition of new infections, they should accurately quantify the baseline prevalence of pre-existing infections in wild-caught snails in the experimental size class.

Data analysis

Because induced infection levels were clearly quite different between the three snail species, and the species of trematode infecting one of the sentinel snail species was different, we analyzed each species separately. Each individual snail was considered an independent replicate, with a binomial response of infected or not-infected. Because one *a priori* HR site was dropped due to inconsistency in parasite pressure (Odiorne), our HR category encompasses snails from two collection sites, and the LR category represents snails from three collection sites. To focus our analysis on the impact of parasite-mediated selection in determining each snail's response to

exposure, we statistically accounted for collection site-level impacts by including individual collection site as a random variable in our model, and used the category of infection risk history (HR vs LR) as a fixed effect.

Thus for each species, our analysis consisted of a binomial generalized linear mixed effects model with experimental treatment (control or guano addition), infection risk history (HR or LR), and their interaction as fixed effects, and individual collection sites included as a random effect. The interaction term (experimental treatment x infection risk history) is particularly important because it relates whether experimental trematode exposure differentially affects snails with HR versus LR histories. Also, holding treatment constant (exposed), we calculated the differences in the log-odds of infection for LR relative to HR-sourced snails. Specifically, we exponentiated the sum of the GLMM model parameter estimates for the fixed effects of infection risk history and the interaction of risk history with experimental treatment. Analyses were carried out in RStudio v. 0.98.1056 (RStudio 2012).

Trematode genetic structure

Our infection experiment assumed that the site of origin of the trematode eggs would not influence infectivity in snails from different sites due to relative genetic homogeneity of the trematode populations across the area of our study. To test this assumption, and to evaluate the overall amount of genetic admixture in the parasite population, we assessed population genetic structure of the most common trematode species in our study, *Microphallus similis,* across two sites that span the geographic range of our study. In June 2012, trematode tissue samples were collected from all *M. similis*-infected snails originating at Appledore Ledges and from a site ~170 km away on Damariscove Island in Boothbay Harbor, ME, a geographically distant site

that had a sufficient number of *M. similis* infections to conduct the test (Figure 1). A single trematode DNA sample was collected from each *M. similis*-infected individual from Damariscove Island and from Appledore Island, and saved in 95% ethanol. DNA extraction was carried out using Purgene (Gentra) reagents. We amplified a mitochondrial CO1 gene using primers published for a different Microphallid species (Leung et al. 2009). Sequencing was carried out by Macrogen and alignment of the resulting sequences was carried out in Geneious v. 6.1.2 (Biomatters). We used Hudson's S_{nn} (nearest-neighbor) statistic (Hudson 2000), which is robust to small sample sizes, to test for genetic differentiation between the two collection sites, using 10 sequences from the Damariscove site, and 6 sequences from Appledore Ledges. Values near 1 indicate strong differentiation, while values near 0.5 indicate low heterogeneity or panmixis (Hudson 2000). The statistic and associated p-values were calculated in DNAsp (Librado and Rozas 2009).

RESULTS

Common garden infection experiment

In our common garden infection experiment, naturally collected seabird guano produced trematode infections in all three snail species, especially *L. obtusata* and *L. saxatilis*. Infection prevalence in these two species increased from near 0% in control snails to 54.5% and 82.9%, respectively. Infection levels in *L. littorea* were markedly lower, increasing only by 6.5 percentage points overall (Figure 2).

For the two direct-developers (*L. obtusata, L. saxatilis*), our model indicated a significant interaction of infection risk history (HR or LR) and experimental exposure on infection (Table 2), after accounting for variation due to collection site-level differences. Experimentally exposed

snails from locations with low infection risk history (LR) had 2.13 times greater odds of becoming infected relative to HR history snails for *L. obtusata*, and 3.11 times greater odds of infection for *L. saxatilis*.

On the other hand, the interaction of infection risk history with exposure was not significant for the broadcast spawning species, *L. littorea*. Snails from both HR and LR-history sites increased in infection, with HR snails trending toward higher infection than LR snails. Because the interaction of infection risk history and experimental exposure was not significant, we did not calculate the ratio of odds of infection for this species.

Many of the infections observed during dissections of experimentally infected snails were immature and did not possess mature cercariae with distinguishing morphological characteristics. However, toward the end of the 4-week period that was required to complete dissections of experimental snails, many infections had developed distinguishing characteristics, and the majority of these were identified as *Microphallus similis* infections.

Trematode genetic structure

Samples of *M. similis* separated by 170km represented 11 haplotypes (Supplemental figure 1) across the >800bp mitochondrial CO1 gene region we sequenced. There was no differentiation based on site of origin, suggesting high gene flow at the spatial scale of our study ($S_{nn} = 0.64$, $p = 0.08$).

DISCUSSION

Adaptation of hosts to local parasite-mediated selective pressure is an important co-evolutionary process with strong ramifications on disease dynamics in wild populations. Adaptation in the hosts is predicated on the strength of selection pressure exerted by the parasites

relative to defense costs, the consistency of such pressure across a time frame relevant to the development of a response, and the isolation of the population under selection from disruptive introduction of maladapted genotypes. In our study system, castrating trematode parasites exert strong selective pressure on snail hosts to avoid or defend against infection. In agreement with our expectation, we found that for the two host species with relatively closed populations, snails from sites with consistently high parasite pressure were significantly less susceptible to infection than hosts that were more naïve to infection threats (Figure 2). Our results contribute to a growing body of evidence that anti-parasite defenses involve trade-offs for hosts, such that strong defense is only selected for when the probability of infection is consistently high. The results also suggest that host gene flow plays a role in governing hosts' ability to locally adapt to parasite infection risk. The effect of prior exposure to reduce susceptibility was specific to our two host species with local recruitment. The one host species we examined with open populations, *L. littorea*, did not exhibit evidence of lower susceptibility among snails with higher prior exposure to trematodes, suggesting that they may not adapt to match local parasite risk. However, this result could also stem from the lower overall susceptibility of this host species to the predominant trematode obtained from the field (*M. similis*), limiting the power of our experiment to detect adaptation in this species.

Effect of prior exposure

Consistent use of particular sites, such as our HR island sites, by seabird definitive hosts results in high levels of parasite infection risk for snails across generations (Byers et al. 2008; Fredensborg et al. 2006; Levakin et al. 2013), and this can impose directional selection on snail resistance mechanisms such as immune defenses (Gorbushin and Borisova 2014; Iakovleva et al.

2006). From the host perspective, consistent parasite-mediated selective pressure should enhance host immunity over generations via a combination of changes in frequencies of relevant alleles and epigenetic inheritance (Ardia et al. 2011). Our findings of higher defense among snails from HR-history sites are consistent with other studies indicating that immune defenses are only selected for when parasite infection risk is sufficiently high and is stable across generations (Bryan-Walker et al. 2007; Hasu et al. 2009; Kawecki and Ebert 2004), whereas defenses are selected against when infection risk is low because of costs of immune defense such as resource diversion or self-harm (Sheldon and Verhulst 1996). Among our experimental *L. obtusata* and *L. saxatilis*, we observed higher mortality during the experiment among snails originating from HR sites, which could reflect a cost of higher defense investment.

Non-immunological sources of defense such as behavioral avoidance, self-medication, beneficial symbionts, and fecundity compensation may also constitute an important component of a host's response to parasites (Parker et al. 2011). For example, *L. littorea* are able to detect and behaviorally avoid seabird guano, the delivery medium of the trematode eggs, at exceedingly small concentrations (Davies and Knowles 2001). While this avoidance behavior may operate in habitats where guano is relatively rare, the extensive guano coverage achieved in our common garden experimental enclosures likely eliminated this defense option, instead enhancing reliance on other defense mechanisms, including immune defenses. If infection risk and immune defense costs are high, hosts demes experiencing consistent parasite pressure may be selected to tolerate some level of infection in favor of maximizing reproductive effort under the threat of castration. In White Sea populations of *L. saxatilis*, uninfected snails from HR locations compensate for the high likelihood of castration by having higher age-specific fecundity than uninfected females

from LR areas (Granovitch et al. 2009). Different versions of this "fecundity compensation" have been observed in several other snail hosts that experience high trematode infection risk (Fredensborg and Poulin 2006; Ibikounle et al. 2012; Jokela and Lively 1995; Lafferty 1993). Whether this tolerance response operates in addition to higher defense responses in our host populations is another area for future study.

Effect of larval dispersal mode

High larval retention in the parent habitat that results from the direct development life history of *L. obtusata* and *L. saxatilis* promotes the adaptation of resistance in these two hosts to local levels of parasite risk. In some cases limited mixing can be a detriment, but only if genetic diversity is so limited that it minimizes the chance of adaptive alleles being present. Our data suggest that the restricted gene flow of the two direct developing snail species and the resulting minimal disruptive immigration of non-adapted immigrant genotypes gives them an advantage in adapting to their more broadly dispersed trematode parasites. It also implies costliness to maintaining resistance when it is not needed (Sheldon and Verhulst 1996). Our test for gene flow within the focal parasite species, *M. similis*, in agreement with prior studies of trematodes, shows high admixture of parasite genotypes at the spatial scale of our study, reflecting its relatively broad dispersal by seabird definitive hosts (Dybdahl and Lively 1996; Keeney et al. 2008; Keeney et al. 2009; Prugnolle et al. 2005).

The relatively high amount of genetic mixing in *M. similis*, in addition to its generalist tendency to infect multiple snail species, suggests that the collection site of the parasite should not strongly influence its infectivity in hosts from different locations. Indeed, in our study, if the relative proximity of the two HR sites to the guano collection site had positively influenced

infectivity of the parasites coming from nearer sites (ie. parasites performing better in sympatric hosts), we would expect the snails from these sites to have shown a greater increase in infection than snails from more distant sites. Instead, the fact that *L. obtusata* and *L. saxatilis* coming from sites closer to the parasite collection site had lower odds of experimental infection than more distant LR-site snails supports our overall hypothesis that the hosts have the advantage in the host-parasite arms race thanks to their local recruitment promoting adaptation to local parasite-mediated selection. Future experiments that reciprocally test the susceptibility of multiple HR-site snails to sympatric versus allopatric parasite populations would provide a more formal test of the co-evolutionary arms race (Hoeksema and Forde 2008; Kawecki and Ebert 2004), building on our more general approach comparing host performance based on high versus low infection risk history.

From the parasite's perspective, a high level of mixing relative to their hosts does not necessarily beget a co-evolutionary disadvantage (Gandon et al. 1996; Lively 1999), and could even be advantageous if other aspects of the parasites' biology allowed positive new alleles to be quickly spread through the parasite population (ex. fast generation times, direct transmission). However, in our particular host-parasite system there are several factors at play that may limit adaptation of the parasite to local host demes. First, the amplification of a subset of parasite genotypes that occurs when the trematode reproduces clonally within the first intermediate snail host may serve to reduce the effective population size of the parasite, reducing its evolutionary potential (Jarne and Theron 2001; Mulvey et al. 1991). Second, the complex three-host life cycle of the parasite may considerably lengthen the parasite's generation time, restricting its evolutionary pace relative to other types of parasites (Barrett et al. 2008). Third, because the

primary trematode found infecting our experimental snails, *M. similis*, is thought to be a generalist species that infects multiple first intermediate host species, specialization by the parasite may be actively selected against in our system (Barrett et al. 2008; Gandon 2002; Roth et al. 2012). This final point may also be a key difference from other trematode-snail systems in which parasites appear to have the co-evolutionary upper hand (Lively and Dybdahl 2000; Lively et al. 2004), or in which neither host nor parasite demonstrates local adaptation (Prugnolle et al. 2006).

Because *L. littorea* has pelagic larvae and thus higher genetic admixture within the metapopulation, we expected this species to act as an outgroup in terms of capacity for local adaptation when compared to the two direct-developing species. Indeed, although we did see a significant effect of treatment and a strong trend of influence of infection risk history (HR vs LR) on infection in *L. littorea*, we did not see an interaction effect indicative of local adaptation, perhaps because field-acquired infections contributed a relatively large proportion of ultimate infections in the HR snails. It is possible that the snail's likelihood of locally adapting to its trematode parasites could also be influenced by a disruption in their coevolutionary dynamics resulting from *L. littorea*'s invasion history. We think this explanation is unlikely to contribute heavily to the observed pattern of minimal local adaptation because 1) *L. littorea* is still heavily infected by trematodes at sites within northeastern North America, including the two HR sites in the current study (Table 1, Byers et al. 2008), and 2) the associations between *L. littorea* and its trematodes are not novel since all five of its trematode parasites in North America are found in the snail's native range in Europe (Blakeslee and Byers 2008). Overall levels of induced infection in *L. littorea* were much lower than the levels induced in the other two host species.

While the relatively low amount of infection achieved in *L. littorea* may seem to suggest a high amount of defense by this host, the levels of infection experimentally induced in our three target species are not readily comparable because of the difference in trematode species that preferentially utilize these hosts. The majority of identifiable infections that were induced experimentally in *L. obtusata* and *L. saxatilis* were identified as *M. similis*, a species which usually occurs at very low levels in *L. littorea* in the field (Blakeslee and Byers 2008). The species of trematode that constitutes the majority of infections in *L. littorea* in the field (*Cryptocotyle lingua;* Byers et al. 2008) did not seem to be amenable to our fecal manipulation. Thus a reduced chance of experimental infection may have reduced the power of our experiment to detect differences in adaptation between *L. littorea* from LR and HR sites. Further experiments that are successful in inducing *C. lingua* infection in *L. littorea* would allow for greater comparison of the defense abilities across the three snail host species.

Conclusion

Co-evolutionary relationships between parasites and hosts are antagonistic and dynamic, providing a fertile testing ground for the ecological and evolutionary importance of local adaptation. The ability of individual hosts to resist or limit parasite attack has implications for disease emergence and persistence at population and meta-population levels. Our experimental approach demonstrates that, for two species with local recruitment, historical parasite exposure can alter hosts' susceptibility to infection. The expansion of this approach to include increased replication at the level of dispersal mode (i.e. host species identity) would provide a more robust test of the effect of dispersal on host local adaptation. Future studies may build on these findings to uncover potential feedbacks of host local adaptation to disease emergence and spread.

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Table 2.1 Prevalence of trematode infection in each species of *Littorina* snails from each site of origin in 2011 and in prior studies. Not all species were present at sufficient densities to be sampled at each site, so more than six total sites are included.

Site lat/lon	**Species collected**	**Adult % Prevalence (N)**	**Mean size (mm) ±SD**	**Prior % Prevalence (N) and Source**
Appledore, ME*	*L. littorea*	27.43 (113)	15.51 ±2.56	29.0 (279) †
42º 59.09 N	*L. obtusata*	15.94 (69)	10.22 ±1.08	
70º 36.79 W	*L. saxatilis*	15.94 (69)	10.32 ±1.42	
Smuttynose, ME*	*L. littorea*	23.47 (98)	15.58 ±3.21	30.7 (205) †
42°59.01 N	*L. obtusata*	10.81 (111)	10.58 ±1.42	
70°36.13 W	*L. saxatilis*	4.67 (107)	9.30 ±1.60	
Boothbay, ME	*L. littorea*	4.85 (103)	16.42 ±4.15	9.21 (76) ‡
43°50.55 N	*L. obtusata*	1.06 (94)	8.55 ±0.94	0.00 (87) ‡
69°37.55 W	*L. saxatilis*	3.85 (52)	9.34 ±1.29	3.85 (156) §[1]
Hilton Park, NH	*L. littorea*	0.00 (78)	15.31±3.55	1.9 (157) †
43º 07.21 N				
70º 49.63 W				
Merepoint, ME	*L. littorea*	1.06 (94)	15.77±3.72	
43°49.41 N	*L. obtusata*	5.71 (105)	10.07 ±1.02	
70°00.59 W				0.00 (90 *L. saxatilis*) §[2]
N. Hampton, NH	*L. obtusata*	1.01 (99)	10.57 ±1.10	NA
42°57.54 N	*L. saxatilis*	0.00 (81)	9.88 ±1.35	
70°46.13 W				
Rye Harbor, NH	*L. saxatilis*	0.00 (120)	9.63 ±1.41	4.96 (121)^
43º 00.22 N				5.9 (169 *L. littorea*) †
70º 44.99 W				
Odiorne Pt., NH ¶	*L. littorea*	3.03 (99)	16.96 ±3.80	11.8 (178) †, 13.25 (317)‡
43º 02.59 N	*L. obtusata*	0.00 (105)	9.96 ±1.42	8.08 (198) ‡
70º 42.65 W	*L. saxatilis*	7.69 (78)	10.17 ±1.82	26.21 (145) ‡

* denotes sites designated *a priori* as high-prevalence sites based on data from prior year(s)

† (Byers et al. 2008) – sampling carried out summer 2002

‡ (Blakeslee and Byers 2008) – sampling carried out summers 2002-2005

§ Blakeslee, unpublished data – sampling carried out 2003 and 2008[1] or 2010[2]

¶ denotes site designated *a priori* as high prevalence but dropped due to inconsistency

Table 2.2 Results of binomial generalized linear mixed models for the outcome of infection for each species. Each model includes fixed effects of infection risk history (HR or LR) and experimental treatment (Control or Exposed), along with their interaction, and also includes collection Site ID as a random effect. The reference categories are HR (risk history) and Control (treatment).

	Parameter estimate	**Std. Error**	**z-Value**	**Pr(>|z|)**
L. obtusata				
Intercept	-3.46	0.41	-8.35	<0.001
Treatment (Exp)	3.14	0.46	6.83	<0.001
Category (LR)	-1.85	1.08	-1.71	0.088
Treat. x Category	2.61	1.11	2.35	0.019
	Variance	**Var. St. Dev.**		
Site ID	<0.001	<0.001		
L. saxatilis				
Intercept	-3.11	0.41	-7.68	<0.001
Treatment (Exp)	4.17	0.49	8.56	<0.001
Category (LR)	-1.96	0.82	-2.37	0.018
Treat. x Category	3.09	0.92	3.36	<0.001
	Variance	**Var. Std. Dev.**		
Site ID	0.026	0.16		
L. littorea				
Intercept	-3.10	1.17	-2.64	<0.01
Treatment (Exp)	1.18	0.34	3.47	<0.001
Category (LR)	-2.80	1.88	-1.49	0.14
Treat. x Category	-0.44	0.77	-0.57	0.57
	Variance	**Var. Std. Dev.**		
Site ID	2.55	1.60		

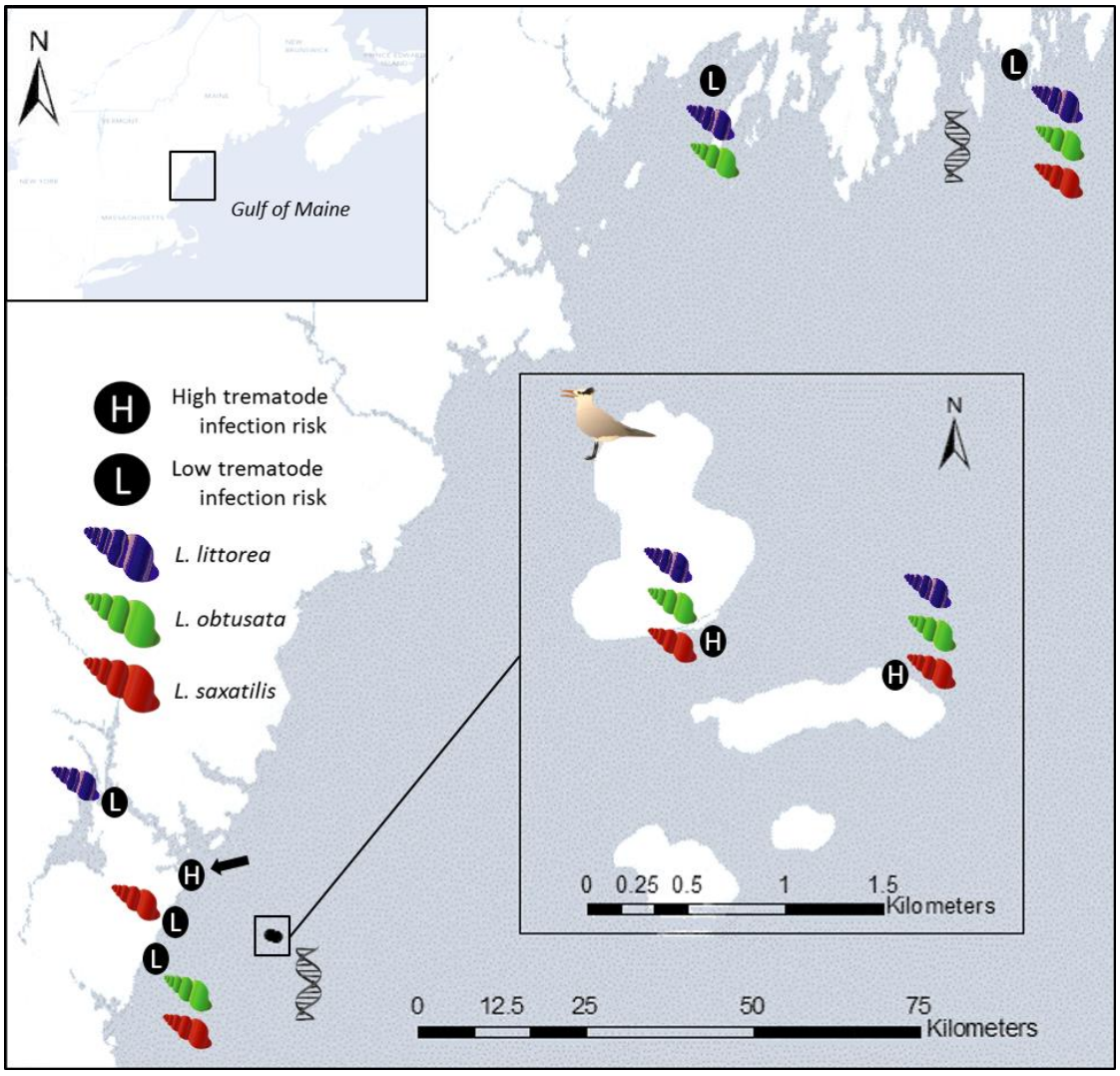

Figure 2.1 Spatial distribution of snail collection sites on the coast of the northeastern USA (New England). Top left inset indicates location of study area within New England. Lower right inset shows two high trematode infection risk sites. DNA helixes denote collection sites for *Microphallus similis* DNA samples. Bird icon marks site of experimental guano collection. Black arrow points to proposed high trematode infection risk site that was ultimately excluded due to inconsistency in parasite pressure

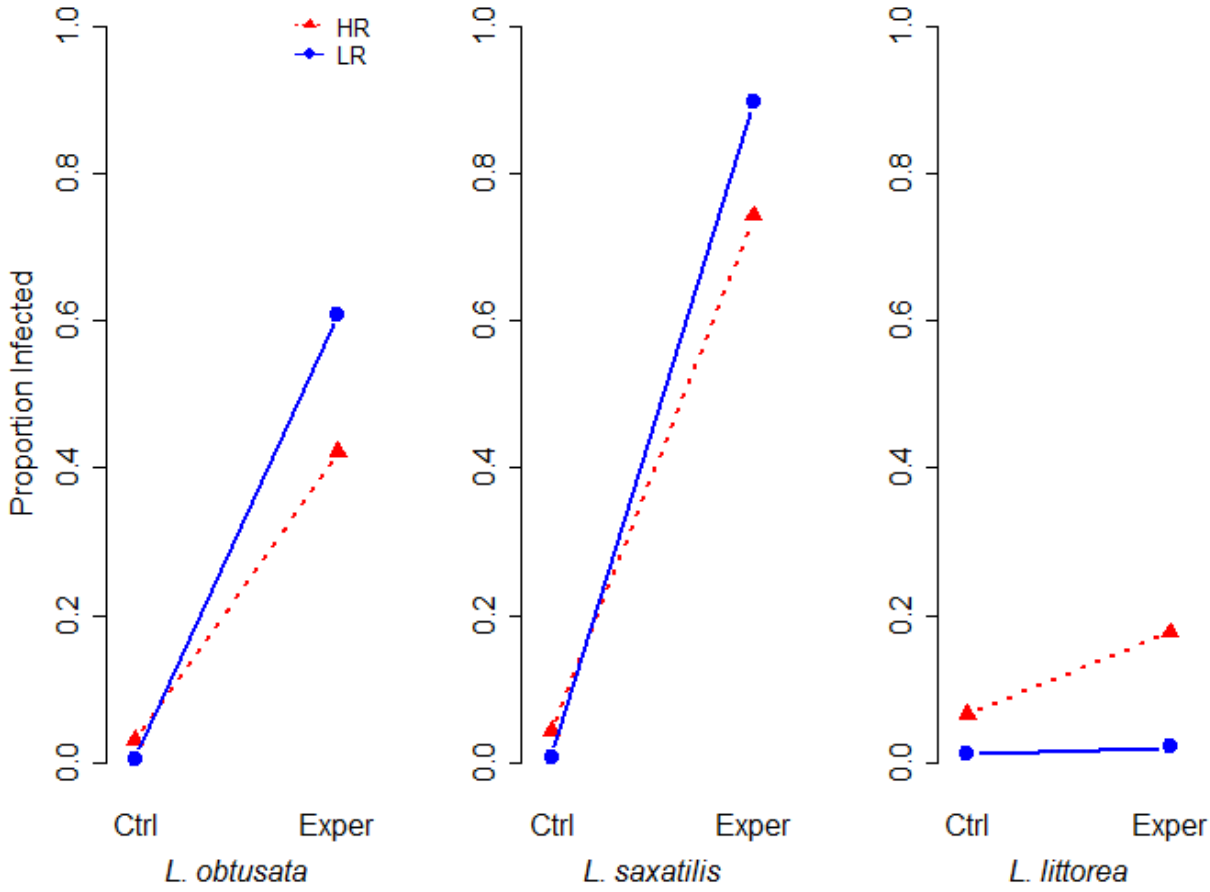

Figure 2.2 Change in proportion of snails infected after experimental exposure in common garden (Exper) for each species relative to baseline infection prevalence (Ctrl), showing difference for each species in response among snails with high infection risk history (HR; red triangles) and low infection risk history (LR; blue circles)

CHAPTER 3

THE DOUBLE EDGE TO PARASITE ESCAPE: INCREASED INFECTION SUSCEPTIBILITY IN AN INVASIVE CRAB[2]

[2]Carolyn L. Keogh, Osamu Miura, Tomohiro Nishimura, and James E. Byers. 2016. To be submitted to *Ecology Letters*.

ABSTRACT

Invasive species that escape from their native range parasites may benefit not only from reduced infection pathology, but also from relaxed selection on costly immune defenses, promoting reallocation of resources towards growth or reproduction. Ultimately however, this relaxed selection could come at the cost of increased infection susceptibility. We conducted laboratory studies of the shore crab *Hemigrapsus sanguineus* from highly-parasitized native (Japan) populations and less parasitized invasive (USA) populations to test for differences in susceptibility to infection by native-range rhizocephalan parasites, and to explore differences in host resource allocation. Invasive individuals showed at least 1.8 times greater susceptibility to infection than their native counterparts, and had reduced standing metabolic rates, signifying lower physiological self-maintenance costs. Our results support an indirect advantage to parasite escape via the relaxation of costly physiological defenses. However, this advantage comes at the cost of heightened susceptibility, a tradeoff of parasite escape that is seldom considered.

Keywords: ecological immunology, enemy release hypothesis, Polyascus, common garden, standing metabolic rate

INTRODUCTION

Biological invasions continue to pose challenges to native ecosystems as humans increasingly transport species across natural barriers to their movement (Carlton & Geller 1993; Lodge *et al.* 2006). The mechanisms by which invaders achieve success in novel habitats, the evolutionary trajectories of invaded communities, and traits upon which to base predictions of new invasions and impacts (Simberloff *et al.* 2013; White *et al.* 2013; Bock *et al.* 2015) are all pressing issues in the field of invasion biology. One of the commonly cited explanations for the often higher performance of invasive populations is that they have escaped from co-evolved natural enemies that may limit populations and shape life-history trade-offs in the native range (Elton 1958; Keane & Crawley 2002). Dramatic reductions in both the prevalence and intensity of parasite infection (i.e. parasite escape) have been documented for numerous invasive populations of both plants and animals, across terrestrial, freshwater, and marine ecosystems (Mitchell & Power 2003; Torchin *et al.* 2003; Torchin & Mitchell 2004; Blakeslee *et al.* 2012; Blakeslee *et al.* 2013).

A reprieve from parasite infection could benefit invasive populations in two key ways. First, and perhaps most obviously, escape from parasites alleviates the negative impacts of parasite infection on host survivorship or fecundity (Anderson & May 1978; Dobson & Hudson 1992). This direct, regulatory benefit has often been cited in studies comparing the performance of parasite-free invaders against other members of the recipient community that harbor natural parasite loads (Dunn et al 2009), though rigorous comparisons of parasite impacts on host fitness between the invasive and native range are still largely lacking. Second, a less obvious, but perhaps equally important benefit of parasite escape is that of "compensatory release" (sensu

Colautti *et al.* 2004), whereby invasive organisms in the absence of parasites may free-up resources no longer needed for the expensive maintenance and deployment of the immune system and potentially re-allocate them towards growth and reproduction (Blossey & Notzold 1995; White & Perkins 2012).

The expected compensatory benefit to invaders assumes that hosts pay costs (either evolutionarily or physiologically, or both) to develop, maintain and deploy immune defenses (Sheldon & Verhulst 1996; Zuk & Stoehr 2002; Graham *et al.* 2011). These immune defense costs may be constitutively expressed, as demonstrated by selection experiments that have revealed, for example, lower fecundity (Vijendravarma *et al.* 2009) or competitive ability (Kraaijeveld & Godfray 1997) in the absence of parasites in populations selected for increased infection resistance (additional examples reviewed in Duncan *et al.* 2011). Alternatively, costs may be induced upon immune system activation as reflected by reduced survivorship of immune-stimulated bumblebees (Moret & Schmid-Hempel 2000), or by increased metabolic energy expenditure in a range of immune-challenged vertebrates (Martin *et al.* 2008) and insects (Ardia *et al.* 2012).

Due to these trade-offs between immune defense investment and other fitness traits, the relaxation of parasite infection risk in populations of invaders experiencing parasite escape is expected to result in directional selection away from defense and toward other traits that increase fitness in the absence of parasites (Blossey & Notzold 1995). However, the possible energetic benefit that invasive populations may gain by reducing immune defense investment may pose long-term risks. Specifically, a lack of immune defense maintenance or a constitutive reallocation of resources to other traits could result in increased susceptibility of invasive

populations to both newly-introduced natural enemies from their native ranges, and fast-evolving generalist parasites in their novel range (Perkins *et al.* 2008), as has been observed in invasive plants (Wolfe 2002; Wolfe *et al.* 2004).

Whether invaders differ from native-range populations in their ability to defend against infection by native-range parasites provides a critical test of the proximate and ultimate consequences of relaxed parasite-mediated selection among invasive populations. Not only does the measurement of infection susceptibility provide information about whether invaders are indeed selected to reduce immune defense investment in favor or other fitness traits, as ecological immunology theory would predict, but it also provides insight into the long-term consequences of the novel selective regime in the invasive range on the future capacity of hosts to respond to infection risk. However, to our knowledge only one study comparing the susceptibility to native-range parasite infection of invasive versus native populations has been carried out in an invasive animal (Fromme & Dybdahl 2006), and the clonal reproduction of the snail hosts in this study probably limited their likelihood of adapting rapidly to the change in parasite infection risk. More recent studies of immune traits in invasion-front cane toads that have escaped some of their parasites in Australia have provided insight to changes in immunity that may result from the altered selection pressures at the expanding edge of their invasive range, but the susceptibility of toads under these varying selection pressures has not been studied (Llewellyn *et al.* 2012; Brown *et al.* 2015).

Here we present an experiment using a common garden approach to compare the susceptibility of individuals from native and invasive populations of a marine invertebrate to a castrating rhizocephalan barnacle parasite from which it has fully escaped infection in its

invasive range. We find that individuals from the invasive population are more susceptible to infection than individuals from native populations. Further, we measured standing metabolic rate as an indicator of self-maintenance costs in uninfected native and invasive-range individuals, and found that invasive range individuals had lower standing metabolic rates compared to native-range individuals, supporting that invaders may be selected to spend less of their available energy on physiological upkeep of immune defenses. This study contributes to our understanding of parasite-mediated selection in the wild by underscoring the cost of maintaining parasite resistance and revealing impacts of enemy escape that are important for understanding invasive species success.

Study system

The shore crab *Hemigrapsus sanguineus* is native to Asia, ranging from the southern coast of Russia to Hong Kong, including Taiwan and the Japanese archipelago. The crab was introduced to the northeastern coast of the United States likely as larvae in ballast water and has been established since at least 1988 (McDermott 1991). The crab reaches averages densities of $>200/m^2$ in central parts of its invaded range in the Northwest Atlantic (O'Connor 2014), and has replaced *Carcinus maenas* as the dominant intertidal crab in this system (Lohrer & Whitlatch 2002; O'Connor 2014). Invasive populations have more recently (1999) become established in Europe (Dauvin 2009; Micu *et al.* 2010).

Three factors likely contribute to the parasite escape experienced by *Hemigrapsus* in the invaded US range (Blakeslee *et al.* 2013). First, because ballast water was the likely vector of introduction, the crab was ostensibly introduced as larvae, which are typically uninfected. Second, the *Hemigrapsus* introduction is relatively new, and parasites of invasive species tend to

accrue slowly over time (Torchin & Mitchell 2004). Finally, the *Hemigrapsus* genus is phylogenetically unique to the Atlantic ocean basin, decreasing the likelihood that host-specific parasites in the Atlantic can readily use this new host. In its native range, *H. sanguineus* is host to five species of Digenean trematodes and one unidentified microsporidian parasite, along with three species of castrating Rhizocephalan barnacles in the genera *Polyascus* and *Sacculina* (Takahashi & Matsuura 1994; Yamaguchi *et al.* 1994; Yamaguchi *et al.* 1999; Blakeslee 2009; McDermott 2011). In contrast, invasive populations on the Northeast coast of North America experience a near complete reprieve from parasite infection risk, having been found to harbor only one type of rare adult nematode (Blakeslee 2009) and a species of larval acanthocephalan at a single site (Christiansen *et al.* 2009; McDermott 2011).

Rhizocephalan barnacle parasites in particular may strongly and negatively affect the host in its native range, as these directly transmitted parasites castrate their crab hosts and have been observed to influence host populations in other systems (Torchin 2001). In the native range in Japan, Rhizocephalan infections are widespread throughout the crab's range, and can reach prevalences exceeding 80% (Yamaguchi *et al.* 1994). Because rhizocephalan barnacle parasites should exert strong selective pressure on their crab hosts and are prevalent in native-range populations of *H. sanguineus* while being fully absent in the invasive range, we focused our infection experiment on this group of natural enemies. In the invasive range of *H. sanguineus* in the Northwest Atlantic, none of the three species of native-range rhizocephalan parasites have invaded, and we have never seen a rhizocephalan infection in any of the >1000 *H. sanguineus* we have collected in the invasive range between 2009 - 2015 (Keogh, pers. Obs). Resistance to rhizocephalan infection is not fully understood in this host-parasite system, but likely involves

the deployment of the host's encapsulation response (i.e. walling off the invading infective stage with layers of host cells) as has been observed in other rhizocephalan-decapod interactions (Kuris *et al.* 2007). The encapsulation response is known to be energetically costly in other invertebrate hosts (Kraaijeveld *et al.* 2001; Rantala & Roff 2005; Ardia *et al.* 2012).

METHODS

Collections and husbandry

We collected *H. sanguineus* from three spatially distant sites in their native range in Japan (Figure 1A), incorporating both the Pacific Ocean and Sea of Japan coasts in order to represent maximal population genetic diversity (Yoon *et al.* 2011). Live specimens were collected from three sites spanning the invasive range on the US East Coast (Figure 1B), and shipped to a common garden laboratory site at Kochi University, Kochi, Japan. The prevalence of reproductively mature (i.e. externally visible) rhizocephalan infections was 0% in crabs from the invasive USA sites (consistent with the continued absence of rhizocephalan parasites throughout the invasive range), and ranged from 0-14% in the three native-range sites (Figure 1). Because rhizocephalan infections are not externally detectable before they reach maturity, and internal phases of early-stage infections are difficult to distinguish microscopically within the crab, we developed an 18s rDNA PCR assay to detect the presence of latent pre-reproductive infections within host tissues (Appendix 1). We used this assay to quantify the prevalence of pre-reproductive infections in a subsample of 20-30 crabs per collection site from the native range, and found rates of internal infection ranging from 32-45% (Figure 1A, Supplemental Table 1), suggesting parasite selection pressure was operating at all native-range sites. Background

internal infection was not assayed in invasive-range crabs because rhizocephalan parasites do not infect *H. sanguineus* in the invasive range.

All crabs collected in both source regions were held during transport to the lab in coolers with ice packs and moistened cloths to prevent desiccation. Despite the obviously greater geographic distance travelled by the crabs collected in the invasive range (US), transit times between collection and arrival in the laboratory were approximately equal between US and Japan sites (48 -72 hours). Upon arrival in the laboratory, crabs were transferred to aerated locally-collected seawater in closed aquaria (i.e. with no possible outflow of invasive range crabs or larvae to the environment). All crabs were starved for one week to standardize nutrition status. After the starvation period, all crabs were provided *ad libitum* access to the seaweed *Undaria* spp. 2-3 times per week.

Common garden susceptibility experiment

To test for differences in susceptibility to rhizocephalan parasite infection between invasive- and native-range crabs, we carried out a common garden experiment using between 20 - 30 individuals per site for two invasive and two native-range sites, as well as some additional (≤ 10) individuals from a third invasive and native site, for a total of 66 invasive- and 54 native-range sentinel crabs (Supplemental Table 1). We excluded a small percentage of native-range crabs that showed external signs of rhizocephalan infection. Thus, the sentinel crabs showed no external signs of infection prior to the start of the experiment, and ranged in size from 12 to 26 mm carapace width (measured at the widest point of the carapace just anterior to the third spine). All crabs were held in a common 60 L tank filled with approximately 45 L of aerated seawater from a nearby coastal area in which both the host crab and parasite were not observed (thus the

seawater should not contain parasite infective stages). This common holding tank was partially submerged in a basin of chilled water to maintain a constant temperature between 18 and 22°C (mean= 18.29 ± 0.04 SE). Inside the common tank the sentinel crabs were maintained in 14.5 x 6.5 x 9cm plastic mesh boxes (0.1 x .5 cm mesh size) in groups of 2-4 to reduce cannibalism, and were given access to *Undaria* spp. every 2-4 days. Approximately 30% of the tank water was changed weekly.

To achieve semi-natural exposure to rhizocephalan infective stages, we added to the common tank ~45 infected native-range crabs with reproductively mature rhizocephalan infections. To reduce the potential bias on the experimental infection rates by local adaptation between sympatric host parasite populations, the infected crabs that were the source of infective rhizocephalan parasite larvae were collected at a site that was distinct from any of the three native-range sentinel crab collection sites (Figure 1A). The infected crabs were contained individually in 5 x 6 cm compartments within two 35 x 22 x 5 cm plastic storage boxes with 0.5 cm diameter holes that allowed released parasite larvae to move freely out of the boxes and into the water column. The boxes with sentinel crabs floated near the top of the water column, while the infected crabs were at the bottom of the tank. Such vertical stratification of the infected and sentinel crabs allowed us to capitalize on the positive phototaxic behavior of the parasite larvae (Hoeg 1984) to ensure a high contact rate of infective larvae with the sentinel crabs. Each of three water samples taken from the tank at 2-week intervals after the addition of the sentinel crabs confirmed the frequent presence of parasite larvae in the common garden tank.

To ascertain the effect of exposure time on infection as a function of source region, we staggered the introduction of sentinel crabs into the common garden tank in three exposure

blocks containing an even mix of native and invasive-range individuals, thus creating three different exposure lengths (4, 3, or 2 weeks). After all crabs had been exposed for at least 2 weeks, the infected source crabs were removed from the tank, and the sentinel crabs were maintained for an additional 4 weeks to allow any successful infections to establish and reach a detectable level. No infections were externally detectable after the 4-week incubation period, so all crabs were dissected and target tissues harvested for PCR- detection of infection (Appendix 1).

We used generalized linear mixed models (GLMM) with a binomial distribution to determine the likelihood of experimental infection as a function of native or invasive source region, exposure block, crab size (carapace width) and sex as fixed effects, and collection site within the two source regions as a random effect in the initial full model. GLMM models were implemented via the R package lme4 (Bates 2014). From the full model, we used backward elimination based on conditional AIC values to select the best fitting model. As an measure of model fit, the proportion of variance explained by fixed effects alone (marginal R^2) and by both fixed and random effects (conditional R^2)(Nakagawa & Schielzeth 2013) were extracted using the rsquared.GLMM function in the MuMIn package. All analyses were carried out in R v3.1.2 (R Core Team 2015).

Standing metabolic rate

As an indicator of the energetic cost of self-maintenance, we measured resting oxygen consumption as a proxy for standing metabolic rate (SMR) in a separate group of crabs from the same three native-range and invasive-range sites (6 individuals per site) using an intermittent-flow respirometry system (Q-Box AQUA, Qubit Systems). These crabs were collected and

maintained in a separate tank under the same conditions as the common garden experimental crabs. All crabs used in this assay were acclimated for approximately 10 weeks prior to measurement.

To conduct measurements, we placed each focal crab into a 140mL cylindrical holding chamber. To reduce crab movement during the measurement period, we fitted the chamber with a 3.5 x 13.5cm piece of rigid plastic mesh to provide a stable substrate for the crabs and covered the chamber with a dark cloth. Oxygen depletion by each crab in the closed 0.213L system was measured with a Clark-type electrode for four replicate 3-minute intervals. Each crab was acclimated to the holding chamber for 4 - 8 minutes prior to the beginning of measurement, and each replicate measurement phase was followed by a 1-minute flushing phase with aerated seawater from the sump to replenish oxygen in the chamber. Trials were run across three consecutive days, and measurement of invasive and native-range individuals was alternated to reduce any effect of temperature fluctuation or machine calibration across the data collection period. After the assay was completed, all individuals were dissected to assess rhizocephalan parasite infection status using PCR (Appendix 1), and only data from individuals that were negative for infection were included in the analysis (n=10 native-range and n=13 invasive-range individuals).

We averaged across each crab's four replicate runs and corrected the mean rate of oxygen depletion for each crab by the mean rate of background oxygen consumption (i. e. with the chamber empty) during each date of data collection to standardize measurements across collection date. Mass-specific oxygen consumption for each crab was calculated as the rate of oxygen consumption per kg per hour. We used a linear mixed effects model to test for an effect

of source region on resting oxygen consumption rate, with crab size (carapace width) as a covariate. Sex is also accounted for in the model, as sex-based differences in SMR have been reported in some decapods (Cerezo Valverde *et al*. 2009) though not all (Laird & Haefner 1976). Individual collection site was included as a random effect.

RESULTS

Common garden susceptibility experiment

A model including Source region as a fixed effect and Site as a random effect best described crab infection prevalence (Table 1). The susceptibility of invasive and native-range individuals to parasite infection was significantly higher among invasive-range individuals compared to native range crabs (Table 2, Figure 2). If all infections in native range crabs were new, the infection susceptibility in invasive range crabs was 1.8 times greater. This value is likely to represent a highly conservative estimate of the difference in the rate of experimentally induced infection between native and invasive range crabs. That is, given our finding of an estimated mean 38% prevalence of latent (pre-reproductive) infection in native range crabs, pre-existing latent infection may account for a large proportion of the infection detected in our experimental native-range sentinel crabs (represented by dashed line in Figure 2). Thus, infection susceptibility in invasive range crabs could be as high as 6 times greater.

Standing metabolic rate

Across body sizes, native range crabs had significantly higher resting oxygen consumption rates than invasive-range crabs (Table 3), indicating higher standing metabolic rate (SMR). Mass-specific oxygen consumption rates decreased significantly with increasing crab size, and the relationship between body size and respiration did not differ by source region (Table 3, Figure

3). Male crabs had significantly higher SMR than females. Temperature varied by ≤ 1.5°C across collection dates.

DISCUSSION

Populations of invasive species provide a unique opportunity to test hypotheses about the strength of various forces of selection and the potential for rapid evolution in wild populations (Sax *et al.* 2007). We quantified a heightened susceptibility of invasive-range *H. sanguineus* to infection by castrating rhizocephalan parasites from the host's original native range, signifying that invasive populations that have escaped the threat of parasite infection may be selected to reduce investment in defense. Specifically, after exposure to infective larvae in a laboratory common garden experiment, invasive-range crabs conservatively harbored ~1.8 times more infections than native-range crabs. Although infected invasive-range crabs must have acquired all of their rhizocephalan infections during the brief (2-4 week) exposure period in the lab, the number of internal infections detected in the native-range individuals represents not only lab-acquired infection but also infections acquired through natural exposure in the field prior to collection (i.e., background internal infection prevalence; dashed line in Figure 2). If we assume that roughly 38% of native-range crabs already harbored internal rhizocephalan infections at the beginning of the experiment, then invasive-range crabs were closer to 6 times more susceptibile to infection than the remaining uninfected native-range crabs. This finding of higher experimental infection in the invasive-range crabs highlights that increased susceptibility to infection by native-range parasites may be an under-studied consequence of relaxed parasite-mediated selection on invaders.

The few previous studies of the effect of relaxed parasite-mediated selection on host susceptibility have been carried out largely with laboratory-reared organisms (Duncan *et al.* 2011; Dargent *et al.* 2013). Here we capitalize on the broad natural experiment resulting from species invasion to test hypotheses about the consequences of relaxed parasite-mediated selection in a somewhat more complex ecological context. To our knowledge, only one other study in an animal system so far has compared the susceptibility of native and invasive-range individuals to infection by native range parasites. Fromme and Dybdhal (2006) tested the relative susceptibility of invasive populations of the clonally reproducing snail *Potamopyrgus antipodarum* to native range trematodes (*Microphallus* spp) that were sympatric or allopatric to the source populations of the snails, and found no evidence of a loss of resistance in introduced snail populations. However, evolutionary potential in clonal populations is much lower than in sexual reproduction systems (Lively & Dybdahl 2000).

In contrast, *H. sanguineus* may exhibit high evolutionary potential due to sexual reproduction, increasing the likelihood that invasive populations have responded adaptively to their new rhizocephalan-free environment. Our finding of an increase in susceptibility to infection among sexually reproducing invaders may reflect a response to selection favoring a decreased investment in costly immune defense in the invaded range where rhizocephalan parasites do not occur. This is the scenario expected if selection against resistance is mediated by trade-offs between resistance and other fitness-related traits such as growth or reproduction. Our findings of higher susceptibility to infection among invaders released from parasite selection and lower self-maintenance costs in these same crabs (indicated as lower SMR) support that immune defense against rhizocephalans is costly.

The primary form of immune defense that is likely to be involved in resistance against rhizocephalan parasites is encapsulation (Kuris *et al.* 2007). The encapsulation response expends host hemocytes in capsule formation, and the subsequent melanization of the capsule may incur additional costs by releasing reactive oxygen species and other cytotoxic molecules that aid in killing the parasite, but which may also cause self-harm to the host (Cerenius *et al.* 2008). Evidence supporting energetic and potential auto-immune costs of the encapsulation response comes from studies reporting lower encapsulation response in the absence of parasite selection in both laboratory selection experiments (Kraaijeveld *et al.* 2001) and natural populations (Bryan-Walker *et al.* 2007), and reduced encapsulation under resource limitation (Konig & Schmidhempel 1995). The formation of melanized capsules around putative rhizocephalan vermigon (internal infective stage) has been observed in other crab hosts (Kuris *et al.* 2007), and was also found in the present system (Appendix 1). Native-range crabs that experience consistent selection to defend against rhizocephalans are likely selected to expend more of their available energy on the production of hemocytes, proteins (e.g. phenoloxidase) and other macromolecules needed to mount a successful encapsulation response. Increased enzyme activity has been linked to higher standing metabolic rate across nine species of Drosophila (Berrigan & Hoang 1999), and a positive correlation between an induced encapsulation response and metabolic rate has been found among multiple species of insects (Ardia *et al.* 2012). Thus higher SMR among native crabs may be a reflection of the physiological cost of maintaining constitutive supplies of molecules and enzymes needed for recognizing pathogens and mounting a successful encapsulation response.

If invasive-range individuals spend less energy on immune defense, they may in turn allocate a higher proportion of their assimilated energy to other fitness related traits such as growth and reproduction. In marine environments in particular, invasive-range individuals are often characterized by achieving large sizes and higher fecundity relative to native-range individuals (Grosholz & Ruiz 2003) and see (Torchin 2001; Darling *et al.* 2011; McGaw *et al.* 2011; Gribben *et al.* 2013; Parker *et al.* 2013). Though a direct comparison of demographic performance of *H. sanguineus* in the native versus invasive range has not yet been undertaken, one previous comparison of native and invasive-range densities (Lohrer *et al.* 2000), along with our own observations in the field and recent reports of extremely high invasive-range densities (O'Connor 2014), support that *H. sanguineus* reaches higher biomass in the invaded range than in the native range (Fukui 1988). In another well-known invasive shore crab (*Carcinus maenas*), individual body size is higher and population biomass is greater in rhizocephalan-free invasive-range sites compared to native-range sites where rhizocephalans prevalence is negatively associated with biomass and individual size (Torchin *et al.* 2001).

Increased susceptibility to infection coupled with lowered SMR in invasive-range crabs is most likely driven by divergent selection on invaders causing rapid adaptation to reduced parasitism; however, other factors may be at play. Specifically, in addition to the probable role of divergent selection, three other mechanisms may play a role in generating the difference we found in susceptibility to infection by common parasites, including 1) protective effects of previously induced responses to rhizocephalan threats among wild-caught adult native-range crabs, 2) differences in standing genetic variation in genes related to immunity (Hawley *et al.* 2006), and 3) heritable epigenetic differences (Palacios *et al.* 2011). For example, a loss of

diversity in genes related to parasite resistance could be a consequence of a genetic bottleneck or founder effect that may have resulted from a small initial population size in the invaded range, as has been observed in house finches afflicted with Mycoplasmal conjunctivitis in their invaded range in the US (Hawley *et al.* 2006). A future study using lab-reared crabs from both within-region (native x native) and cross-region (native x invasive) matings (Brown *et al.* 2015) would provide further insight into the relative role of induced versus inherited differences in resistance. A population genetic study comparing native and invasive populations of *H. sanguineus* is currently underway and will help to shed light on the potential contribution of differences in standing genetic variation to changes in host susceptibility to infection.

Conclusion

Heterogeneity in susceptibility to infection is common in wild populations, potentially as a result of balancing the costs and benefits of investing resources in immune defenses (Sheldon & Verhulst 1996). Populations of invaders that have "escaped" from parasites provide an opportunity to study the strength of parasite mediated selection by observing the extent to which resistance is retained when selective pressure is relaxed. An important proximate consequence of this response to selection against defense is the greater availability of resources to allocate to other fitness gains such as faster growth or higher reproduction. Our exploration of resting energy expenditure supports that the hosts in our system may have more energy available to invest in fitness due to relaxed immune defense investment invaded range. This mechanism of invader success may work in concert with regulatory release and other novel adaptations to allow invasive populations to dominate invaded ecosystems. However, parasite escape has an important double-edge. A consequence of a lack of directional selection favoring immune

defenses in invasive populations may be a reduction the diversity of immune defense potential and result in a loss of evolutionary potential to respond to parasite stressors (Perkins 2008). While invaders may retain defense against some ubiquitous generalist parasites (Wendling & Wegner 2015), our experimental results highlight that invaders may be highly vulnerable to infection by specialist parasites from the native range that have the potential to regulate host populations.

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Table 3.1 Model selection results for full and reduced generalized linear mixed models of infection outcomes in common garden exposure, selected by conditional AIC (AICc). Marginal (m) and conditional (c) R^2 values are included as measures of model fit. Source refers to source region (native or invasive), Time refers to exposure length (2, 4, or 6 weeks), Size is measured as carapace width (mm), and Site is the specific collection site within each source regions, which is treated as a random effect.

Parameters	$R^2_{GLMM}(m)$	$R^2_{GLMM}(c)$	**AICc**	**ΔAICc**
Source, Site(random)	**0.12**	**0.22**	**145.9**	**0.0**
Source, Sex, Site(random)	0.14	0.24	146.6	0.7
Source, Sex, Time, Site(random)	0.15	0.25	150.0	4.1
Source, Sex, Time, Size, Site(random)	0.15	0.25	152.3	6.4

Table 3.2 Results of reduced generalized linear mixed model of infection (binomial) outcomes from common garden exposure experiment. Native source region is the reference category, with site as a random effect.

Variable	**estimate**	**Std Error**	**z-value**	**p-value**
Intercept	-0.1	0.50	-0.21	0.84
Source Region	1.45	0.72	2.02	0.04
		Variance	**Var. Std. Dev.**	
Site (random)		0.40	0.63	

Table 3.3 Results of linear mixed effects model of the contribution of source region, size, and sex to rates of resting oxygen consumption. Native range is the reference category for source region, and female is the reference category for sex. Individual collection site within region is included as a random effect.

Variable	**estimate**	**Std Error**	**z-value**	**p-value**
Intercept	-24.14	221.31	-0.11	0.91
Source (Invasive)	-72.96	25.29	-2.88	0.009
Size	-18.67	4.92	-3.80	<0.001
Sex(Male)	119.07	24.15	4.93	<0.001
		Variance	**Var. Std. Dev.**	
Site (random)		0.0	0.0	

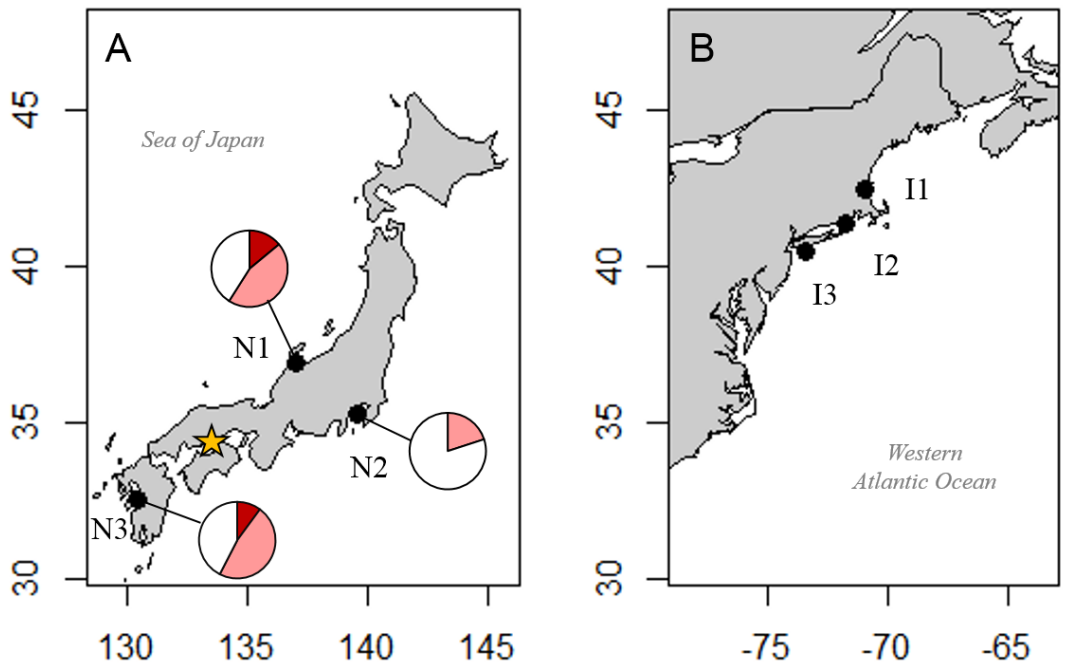

Figure 3.1 *Hemigrapsus sanguineus* sampling locations in A) Native range collection sites in Japan (N1-N3) and B) Invasive-range collection sites in Eastern United States (I1-I3). The gold star in panel A shows the collection site for rhizocephalan-infected crabs used as a source of infective larvae in the common garden experiment. Pie charts in panel A show the percent of crabs at each native-range sample site with visible, reproductively mature *Polyascus* spp. infections (red), internal infections detected by PCR (pink), or without infection (white). In the invasive range in the Northwest Atlantic, *Polyascus* spp. infections (or Rhizocephalan infections of any species) are absent in *H. sanguineus*.

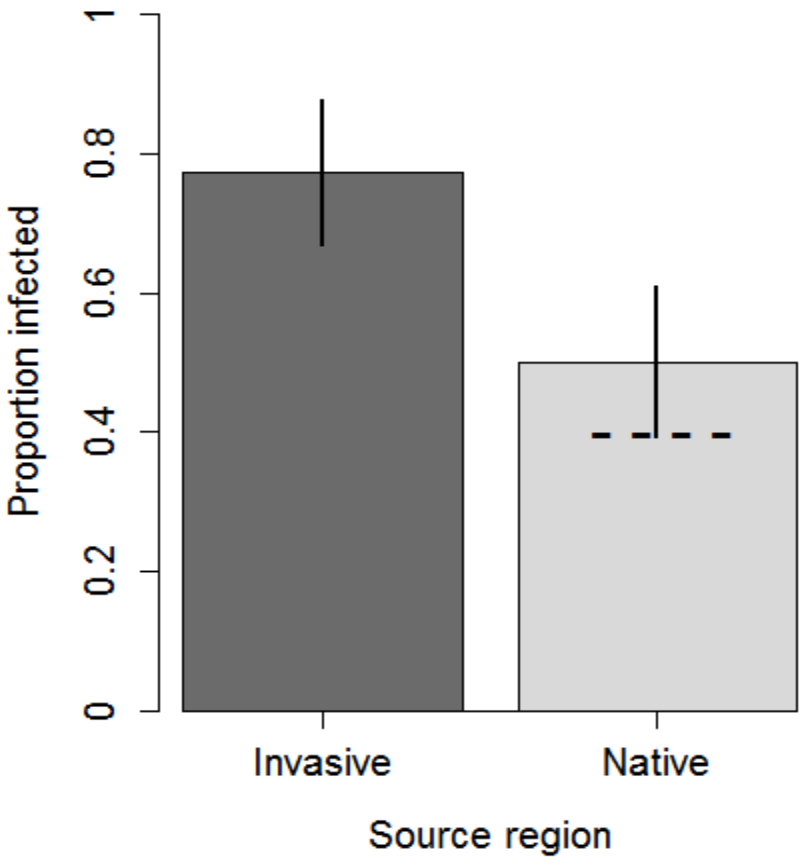

Figure 3.2 Proportion of individuals infected after common garden exposure, expressed as the weighted mean of the three sites within each source region. Error bars represent standard error of the proportions. Dashed line represents our estimate of latent ambient rhizocephalan prevalence averaged across the native range sites (as detected by PCR), indicating that a substantial amount of the prevalence detected in the native-range sentinel crabs which were obtained from this same source pool may represent pre-existing natural infections acquired in the field.

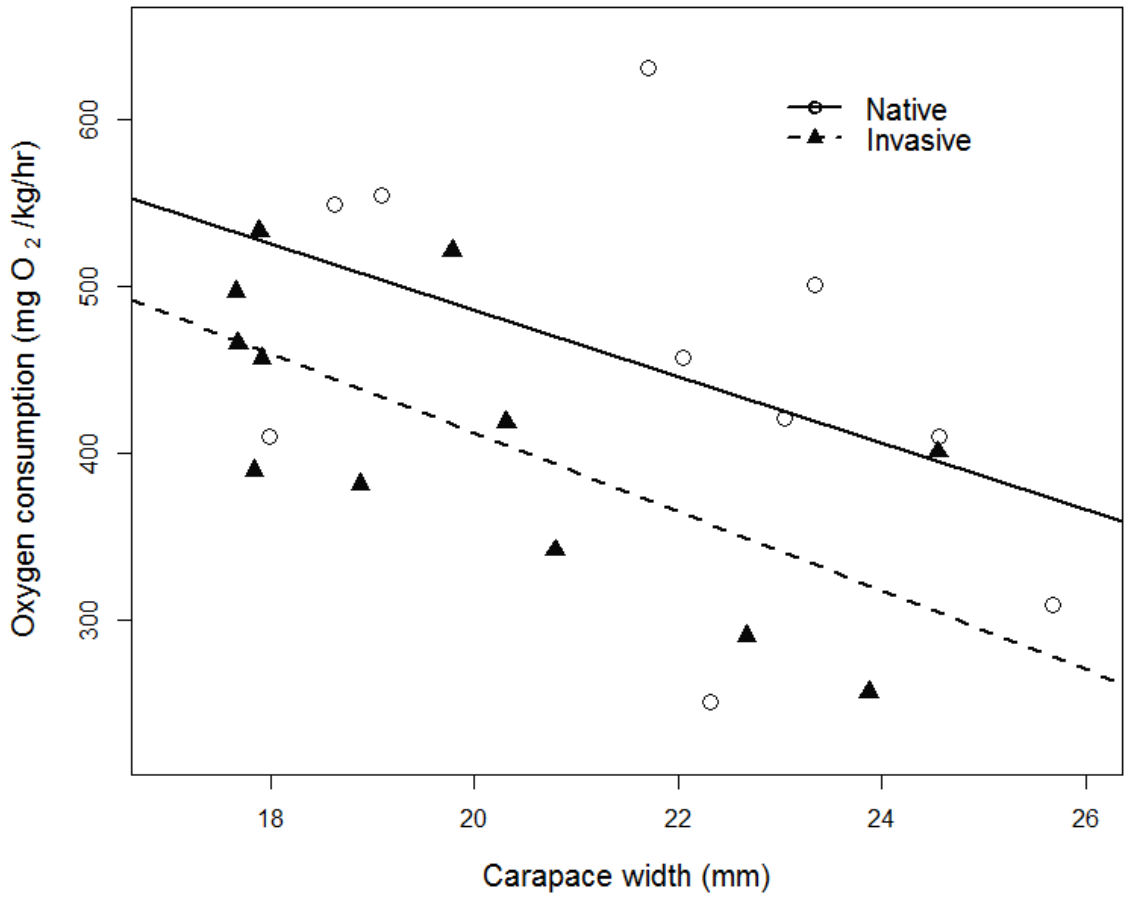

Figure 3.3 Resting oxygen consumption rates for uninfected *H. sanguineus* from native (open circles, solid line) and invasive (filled triangles, dashed line) source regions, across carapace width (body size).

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CHAPTER 4

ECOIMMUNOLOGY OF AN INVADER: IMMUNE TRAITS AND ENERGY ALLOCATION IN NATIVE VERSUS INVASIVE POPULATIONS OF THE SHORE CRAB *HEMIGRAPSUS SANGUINEUS* [3]

[3]Carolyn L. Keogh, Osamu Miura, Tomohiro Nishimura, and James E. Byers. 2016. To be submitted to *Functional Ecology* following substantial revision.

INTRODUCTION

Most natural populations contend with the threat of infection by parasites. Across the animal kingdom, hosts have evolved diverse traits for limiting damage from parasite attack, ranging from infection avoidance behaviors (Parker *et al.* 2011) to tolerance of infection by less-virulent parasites, to resisting infections via the sophisticated molecular tools of the immune system (Raberg *et al.* 2009). Despite the sometimes severe consequences of infection for individual fitness (i.e., pre-reproduction mortality or castration), heterogeneity in resistance to parasite infection is a common characteristic within and among populations of natural hosts (Hawley & Altizer 2011). If parasites exert strong selection on host immune defenses, as evidenced by the array and specificity of immune defense strategies that have evolved and been highly conserved across evolutionary timescales for most branches of the animal phylogenetic tree, why does heterogeneity in resistance persist?

While some amount of variation in host susceptibility to infection may be accounted for by evolution on the part of parasites themselves, differing degrees of host susceptibility to infections may alternatively reflect underlying variation among hosts in immune defense adaptations or physiological investment. Evolution of immune defenses may be constrained if the threat of infection by multiple types of parasites results in multivariate selection pressures (Jokela *et al.* 2000), or if antagonistic pleiotropy with genes involved in other fitness-related physiological traits reduces the fitness benefits of novel immune adaptations (Schmid-Hempel 2011). Over putatively shorter timescales, hosts immune responses may be constrained by resource-based costs associated with maintaining and deploying immune defenses. One of the primary explanations for variation in the strength of immune defenses is that immunity comes at

a resource cost to the host, and therefore is only adaptive when parasites are present (Sheldon & Verhulst 1996; Zuk & Stoehr 2002; Martin *et al.* 2011). Even a small cost of immune defense that lowers the fitness of the host relative to less defended hosts in the absence of parasites should be sufficient to maintain variation in the strength of immune defenses (Sheldon & Verhulst 1996; Martin *et al.* 2011).

Invasive species often experience a dramatic reduction in the prevalence and diversity of parasite infections when they are introduced to a novel environment (Mitchell & Power 2003; Torchin *et al.* 2003). Recent reviews from the growing field of ecological immunology have highlighted potential insights to be gained from using invasive populations as experimental tests for the relative importance of immune-defense trade-offs in the wild (Lee & Klasing 2004; Dunn *et al.* 2012; White & Perkins 2012). For example, coastal marine invertebrates are often introduced to novel environments as larvae travelling in ballast water, separated from parasite-carrying adult hosts in the native range (Carlton 1996). While this "enemy escape" may confer a fitness advantage due to the absence of parasite infection, an additional "compensatory" (Colautti *et al.* 2004) mechanism of fitness improvement may occur due to the possible relaxation of selection (Lahti *et al.* 2009) for investment in costly immune defenses (Martin *et al.* 2011; White & Perkins 2012).

Most evidence for resource-based costs of immune defense comes from a range of laboratory selection experiments and from comparative studies of both plants and animals (Duncan *et al.* 2011). Invertebrate immune systems, like their better-studied vertebrate counter-parts, can be classified as either constitutive (always present) or induced (in response to an identified infection), and are based on cellular and humoral components (Loker *et al.* 2004;

Schmid-Hempel 2011). Among invertebrate hosts in particular, numerous types of costs of immunity have been revealed in laboratory studies (Rolff & Siva-Jothy 2003). Findings include decreased larval competitive ability in *Drosophila* artificially selected for parasitoid-resistance (Kraaijeveld & Godfray 1997), increased metabolic rate in four different species of insects fighting acute infections (Ardia *et al.* 2012), reduced starvation tolerance of immune-stimulated *Bombus terrestris* (Moret & Schmid-Hempel 2000), and lower fecundity for microsporidian-resistant lines of *D. melanogaster* (Vijendravarma *et al.* 2009). Additional evidence for immune defense costs comes from studies of wild meta-populations that show heightened resistance only in subpopulations that experience consistent parasite selection (Bryan-Walker *et al.* 2007; Hasu *et al.* 2009; Keogh *et al.* 2015).

Rhizocephalan barnacles are crustacean parasites that castrate their decapod hosts, thus likely exerting strong selection on hosts to resist infection (Hoeg 1995; Kuris *et al.* 2007). Rhizocephalans are directly transmitted from crab to crab and may be quite common in their host's native habitat, but are usually absent from invasive populations of host crabs (Torchin *et al.* 2003; Blakeslee *et al.* 2013). For example, while native European populations of the green crab *Carcinus maenas* harbor rhizocephalan infections that can reach 78% prevalence (Torchin 2001), none of their several invasive populations around the globe have so far been found to harbor rhizocephalan infections (Torchin 2001; Zetlmeisl *et al.* 2011). Further, release from rhizocephalan infection has been implicated as a driver of the increased individual size and vigor of *C. maenas* in its invasive range (Torchin 2001), including the US east coast rocky intertidal.

In more recent decades, a new species of shore crab has invaded the US East coast, replacing *C. maenas* as the numerically dominant intertidal crab (Lohrer & Whitlatch 2002;

O'Connor 2014). *Hemigrapsus sanguineus* is native to East Asia, including southern Russia, China, Korea, and the Japanese archipelago. Like *C. maenas*, *H. sanguieneus* has also escaped from a suite of parasites that may reduce fitness in its native range (Blakeslee 2009). In the crab's native range in Japan, *H. sanguieneus* is infected by three species of castrating rhizocephalan parasites: *Polyascus (Sacculina) polygenea*, *Polyascus (Sacculina) nigra*, and *Sacculina senta* (Nagasawa *et al.* 1996; Glenner *et al.* 2003). Infection prevalence may reach greater than 80% in some enclosed bay populations (Yamaguchi *et al.* 1994). No rhizocephalan infections have been reported in its invasive range in the Atlantic Ocean basin (Blakeslee 2009; McDermott 2011). Because rhizocephalans commonly exert strong selection for host defense against castration in the native range, induce a defense response (encapsulation) that is known to be costly (Ardia *et al.* 2012), and are absent from the invasive range of *H. sanguineus*, rhizocephalans are an ideal parasite with which to test the effect of parasite escape on host immune defense investment.

An earlier study in this system used a common garden experiment to measure the relative susceptibility of invasive versus native-range crabs to infection by rhizocephalan parasites from the native range, and revealed that *H. sanguineus* from the invasive population are significantly more susceptible to experimental infection than their native range counterparts (Chapter 2/Keogh et al. in prep). This differential susceptibility suggests alteration of immune defenses in invasive range crabs, which could reflect selection or plasticity favoring resource investment in other fitness-related traits over investment in immune defenses (White & Perkins 2012). Here we present a follow-up study to explore whether general immune system traits such as the constitutive density of circulating hemocytes, the responsiveness of hemocyte density to parasite

challenge, and the degree of encapsulation of an implanted fiber predict the hosts' probability of rhizocephalan infection. We explored whether the relationships between these immune traits and infection status differ for invasive versus native-range crabs.

Further, if immune defense is energetically costly, then individuals that invest more in immune defense may alter resource allocation (i.e., higher self-maintenance costs, lower reproductive investment) compared to individuals that are less well-defended (Sheldon & Verhulst 1996). We explored whether three immune metrics (constitutive density of circulating hemocytes, induced change in hemocytes, and degree of implant encapsulation) predict energy allocation to storage (relative hepatopancreas mass, HSI) or reproductive effort (relative gonad mass, GSI). Finally, we tested whether energy allocation trades off between storage (HSI) and reproduction (GSI), and whether relationships between these variables differ for native versus invasive and infected versus uninfected crabs. Our results demonstrate that individuals that harbored infections at the end of our experiment show weaker induced immune traits, and that relationships between immune traits and energy expenditure differ in invasive versus native-range crabs.

METHODS

Sample collection and maintenance

Adult *H. sanguineus* (>16mm carapace width) were collected haphazardly by hand from under rocks at low tide at three sites in the US invaded range and three sites in the Japanese native range in early April 2014 (Figure 1). All crabs were maintained in coolers with ice packs and moistened cloths for approximately 72 hours during transit to the the laboratory facility at Kochi University, Kochi, Japan. In the lab, crabs were divided into three 60L holding tanks filled with

approximately 45L of aerated locally-collected seawater, with partial water changes carried out once per week. Native-range crabs were checked for external evidence of rhizocephalan infection, so that only crabs that were putatively free of mature infections were held in the common tanks. Water recirculated between the three tanks via interconnected drains leading to a common sump in which water was filtered before being pumped back into circulation. Individual crabs were separated into 14.5 x 6.5 x 9cm plastic mesh boxes (0.1 x .5 cm mesh size) suspended within the common tanks to prevent cannibalism. All crabs were starved for one week upon arrival to the lab to standardize nutrition status, and then were maintained on an algal diet (*Undaria* spp.) offered 2-3 times per week. This algal diet was intended to subject the crabs to moderate nutrient limitation to improve detection of resource-based trade-offs.

Overview of methods

Rhizocephalan parasites are transmitted between crab hosts when an infected crab releases a brood of free-swimming larvae into the water column, which mature for several days, and then settle on a naïve host. Initially, rhizocephalan parasites form internal infections in hosts that are only detectable through destructive sampling; only when rhizocephalans reach reproductive maturity does an external infection appear. Therefore, some proportion of native-range crabs may have harbored internal rhizocephalan infections upon arrival to the lab. We dissected between 20-30 individuals per native-range collection site in order to determine background rates of internal infection using a PCR-detection method described in Chapter 2 (Keogh et al, in prep), and rates of internal (site-weighted average = 38%) and external infection (site-weighted mean = 9%) are shown for individual collection sites in Figure 1.

While we attempted to exclude native-range crabs that showed external evidence of infection from the experiment, the interconnected common holding tanks housing the sentinel crab hosts were exposed to periodic bouts of a low level of rhizocephalans from 2-3 infected native-range crabs that were unintentionally included in the holding tanks. This background exposure in the holding tank was not quantified, and accidental exposure was expected to contribute a small proportion of infections. To intensify parasite exposure and stimulate immune system induction at controlled time points, we applied two doses of rhizocephalan cyprids (the infective larval stage; 0, 4, 10 or 40 cyprids per dose) collected from broods released by infected native range crabs by exposing crabs individually in ~100 mL plastic food storage containers. These controlled cyprid exposures were carried out for 8 groups (blocks) of crabs over the course of four weeks. Each exposure block consisted of one crab per collection site (3 native and 3 invasive) for each of the four dose treatments (n = 24 crabs per block). Crabs were returned to the common holding tanks after dose exposures and sample collection described below, so that all crabs spent an approximately equal amount of total time in the holding tank. Newly infected individuals did not have time to develop mature, transmissible infections during the course of the experiment, so would not have contributed infective larvae to the holding tank.

Circulating hemocyte density and standing metabolic rate were measured prior to application of the cyprid dose treatments, to provide an estimate of constitutive or background levels of immune defense readiness, and the physiological cost of self-maintenance. Although the timing of these measurements prior to controlled exposures was intended to quantify baseline values of constitutive traits, the unknown timing of unintentional cyprid exposures that happened in the holding tank may have added background variation in these traits since some crabs were

likely exposed to some limited level of parasites prior to constitutive trait measurements. However, these two metrics should nonetheless represent a baseline against which responsiveness to controlled exposures can be compared.

Seventy-two hours following the first controlled exposure for each block, we measured hemocyte density again, along with the strength of host encapsulation of a nylon implant, mimicking a macroparasite threat. Between 4-6 weeks after the conclusion of the controlled exposures for a given block, all crabs in the block were dissected to collect the hepatopancreas (a key energy storage organ in decapods) and gonad tissue for calculation of hepatosomatic and gonadosomatic indices described below. Midgut tissues were also collected for PCR-based detection of rhizocephalan infection status. The PCR-detection technique allowed us to detect early-stage infections (not yet visible) including those resulting from controlled cyprid exposures and random exposures in the holding tanks, as well those that may have already been present as in wild-caught native-range crabs (i.e., were acquired in the field).

Methods for specific immune trait assays

Circulating hemocyte density

Hemocytes (blood cells) carry out a range of physiological functions within the crab, including forming the basis of immune defense against both micro and macroparasites. We measured the density of total circulating hemocytes in each crab both 6-12 hours before and 72 hours after controlled cyprid exposures. Undiluted hemolymph was withdrawn via pipette from a sterile puncture wound (23g needle) in an unsclerotinized walking leg membrane. For hemocyte counts, 10ul of hemolymph was immediately pipette-mixed with a bead of 20uL of modified anticoagulant saline (MAS, per Dittmer et al. 2010). Approximately 10uL of this dilute

hemolymph solution was loaded into an individual gridded well of a KOVA hemocytomer slide and pictures of the four corners of the grid were captured on a compound microscope camera at 200x. Total hemocytes were enumerated with a digital imaging analysis program (imageJ) for at least 3 pictures per individual, and the mean number of cells per uL of dilute hemolymph was calculated. To determine the responsiveness of hemocyte density to experimental cyprid exposure, we calculated the proportional change in circulating hemocyte density for each crab by subtracting initial counts from post-exposure hemocyte counts, and dividing by the initial count. Both pre-exposure (constitutive) hemocyte density and hemocyte responsiveness were log-transformed for analyses described below.

Encapsulation

A key mechanism of invertebrate defense against macroparasites is the encapsulation of foreign bodies in layers of hemocytes. A successful encapsulation response results in the death of the parasite through the melanization of a capsule of hemocytes that is laid down to physically wall off the parasite. The parasite is killed either by smothering, or by the release during capsule formation of cytotoxic molecules and reactive oxygen species (Cerenius & Soderhall 2004). To estimate the "strength" of the general encapsulation response of each crab, a sterilized 0.25cm length of nylon monofilament was inserted into one of the walking leg sinuses of the crab through a puncture in the walking leg membrane (using the same puncture through which post-exposure hemolymph was withdrawn), and retrieved 24 hours later. Each filament was photographed in three contiguous subsections under 40X magnification. Three separate lab personnel estimated the percent surface area of each subsection of the implant that was covered

by hemocytes, and these three triplicate estimates per fiber were averaged to estimate the percent encapsulation of each fiber.

Methods for specific energy assays

Standing metabolic rate

As a measure of the physiological cost of self-maintenance, standing metabolic rate (SMR) of a subset of experimental crabs were assayed via oxygen consumption both prior to exposure to the experimental cyprid dose, and 72hours after exposure. Specifically, the resting oxygen consumption rate of 6 individual crabs per collection site was measured in crabs from the final two exposure blocks mentioned in the description of the cyprid exposure treatments (n=18 total per source region, comprised of an equal number of males and females within each region). We quantified SMR using an intermittent-flow respirometry system with a Clarke-type oxygen electrode (Qubit systems). After allowing the focal crab to acclimate to the holding chamber for 4-8 minutes, oxygen consumption was measured for four replicate 3-minute intervals in a closed 140 mL chamber. Preceding each measurement phase, the chamber was flushed for 1-minute with aerated seawater from the sump to ensure that oxygen availability did not become depleted in the holding chamber. We alternated between measuring invasive-range and native-range crabs during each of three consecutive days, and we corrected for differences in electrode calibration by standardizing values for each date by replicate measurements of oxygen consumption with an empty chamber. Temperature was consistent to within 1°C within each measurement date. Constitutive SMR (SMRc) was calculated as the mass-specific oxygen consumption rate (mg of oxygen consumed per kg per hour) of crabs prior to exposure, and we calculated the proportional change in SMR after exposure (deltaSMR; specifically, final

measurement minus initial (SMRc) measurement divided by initial measurement) as a metric of SMR responsiveness to immune induction. SMRc and deltaSMR were log-tranformed for analysis.

Hepatosomatic index (energy storage)

The mass of the lipid-rich hepatopancreas organ relative to body weight (i. e., hepatosomatic index) is a reliable predictor of energy storage in decapods (Kennish 1997; Griffen *et al.* 2015). Persistent effects of infection or exposure on energy resources were assayed through calculation of the crabs' hepatosomatic index (HSI) approximately 4-6 weeks after their experimental treatment, at the same time as dissection for tissue collection for PCR assays. We measured the relative contribution of hepatopancreas dry mass (the crabs' main energy storage organ) to total body dry mass by separating the hepatopancreas from the rest of the body and drying both the hepatopancreas and body at 70°C for 72-144hours. The dried hepatopancreas and body were massed separately, and HSI was calculated as the ratio of the dry hepatopancreas mass to the remainder of the crab's dry body mass. HSI was log-transformed for analysis.

Gonadosomatic index (reproductive investment)

Similar to HSI, we measured the relative contribution of the crab's reproductive organs (including gametes or eggs in any stage of development) to total body dry mass (i. e., gonadosomatic index; (Kennish 1997)) by separating the reproductive tissues from the rest of the body, drying the tissues and body at 70°C for 72-144 hours until mass no longer changed, and massing them separately. GSI is calculate as the ratio of dry gonad mass to the remaining dry body mass. We log-transformed GSI before using it as a response variable in the analyses described below.

Analysis of relationships between variables

Although there was considerable infection in crabs at the end of our experiment, our generalized linear mixed model (GLMM) of infection status as a function of exposure treatment indicated infection status was not significantly affected by our exposure dosage treatments designed to generate varying levels of infection (Table 1, Supplemental figure 1). This suggests that our controlled cyprid exposures were less successful in producing infection than random exposures that occurred in the holding tank. In fact, this analysis showed equal likelihood of infection between exposed and invasive-range control crabs, which were known to not harbor pre-existing infections, and which must have acquired all infections in the holding tank. Therefore, we focused our analyses on 1) whether exposure was associated with proportional change in hemocyte density or SMR, 2) whether immune traits that signal defense investment (constitutive hemocyte density, change in hemocytes after controlled exposure augmentation, and encapsulation strength) predict the infection status of sentinel crabs at the end of their potential exposure period, 3) whether constitutive SMR reflects differing levels of investment in immune traits, 4) the effect of immune defense investment on host energy traits (standing metabolic rate, hepatosomatic and gonadosomatic indices), and 5) possible trade-offs between energy storage and reproduction. Below we describe the original full statistical models for each trait. For each of the models described below, we used AICc values implemented via the R package lmerTest (Kuznetsova 2016) to select between full models and reduced models from which non-significant interaction terms had been dropped. We report only results from the final models. All analyses used linear mixed effects (LME) regression and GLMM models and were carried out in R v3.1.2 (Team 2015) using the lme4 package for mixed-effects regression analyses (Bates 2014). Plots

of model outputs were generated in the package visreg (Breheny 2015) and tables of model results were compiled using the package sjPlot (Lüdecke 2016).

Effect of exposure on immune induction

To examine the effect of exposure on the proportional change in hemocyte density, we collapsed the three levels of exposure treatment (including low, medium and high treatments) to a binary category (exposed versus control) because the sample size of crabs from which both pre-and post- exposure counts of hemocyte density were counted was limited. Exposure was included as a predictor in a linear mixed effect model of hemocyte responsiveness that included source region as an interaction term, and exposure block and collection site as random factors. Encapsulation was also modeled as a function of exposure treatment, this time including all four possible treatment levels. Exposure block and collection site were treated as random effects.

The effect of exposure on deltaSMR was also analyzed as a LMER model with exposure, source region, and their interaction as predictors. Size (carapace width) was included as a covariate in the regression, and collection site was included as a random effect. This model also used the collapsed exposure category as a predictor because SMR was similarly restricted in sample size.

Immune traits as predictors of infection status

To determine whether circulating hemocyte densities, hemocyte response to challenge, or encapsulation strength predicted PCR-detected rhizocephalan infection status, we analyzed each trait separately in GLMM models of infection that included source region as an interaction term. Experimental treatment (exposed versus not-exposed) was included as an interaction term in the

original full models of the two traits measured after exposure. Exposure block and collection site were included as random effects.

Constitutive SMR as a predictor of immune trait investment

Because SMR gives an indication of physiological self-maintenance costs, which could include metabolic costs of immune system investment, we analyzed whether constitutive SMR predicts constitutive hemocyte density, induced change in hemocyte density, or encapsulation strength using LMER models. We included infection status, size (carapace width), and sex as fixed covariates, and collection site as a random factor.

Relationships between immune traits and energy metrics in invasive and native-range crabs

We also used linear mixed effects regression (LMER) to analyze whether each immune trait predicted energy allocation to storage or reproduction, and whether this differed by source region. Both for models of HSI and for models of GSI, infection status and size were also included as fixed covariates, and block (which accounts for differences in timing of exposure and dissection) and collection site were included as random effects. Because of extreme differences in the magnitude of relative gonad size between males and females, we analyzed GSI separately for males and females.

Though preliminary data exploration did not reveal strong correlations between any of our measured energy traits, we tested for a relationship between GSI and HSI in male and female crabs by source region and infection status. Specifically, we analyzed GSI as a function of HSI, source region, infection status, and their interaction. Size was accounted for as a fixed covariate, and exposure block and collection site were random factors.

RESULTS

Exposure as a predictor of immune induction

With exposure as a binary predictor, we found no significant response of circulating hemocyte density to experimental exposure (Table 2). However, encapsulation response strength was responsive to experimental treatment level, with significantly higher encapsulation in crabs exposed to a low dose of cyprids (Figure 2, Table 3). A low experimental cyprid exposure was also associated with an increase in delta SMR relative to SMRc, though the other treatment levels did not induce a significant change in SMR (Figure 2, Table 4).

Immune traits as predictors of infection status

Constitutive hemocyte density was not a significant predictor of infection status (Table 5). However, the proportional change in hemocyte density after exposure was significantly negatively related to infection probability, and this effect depended on exposure status (Table 5). For crabs that were exposed to a dose of cyprids, hosts with a greater proportional change in hemocyte density were less likely to harbor infection at the end of the experiment. The relationship between infection and hemocyte response to induction was not significantly different for invasive versus native-range crabs.

The analysis of encapsulation strength as a predictor of infection revealed a significant negative relationship between encapsulation strength and the probability of infection; for each one-unit increase in encapsulation strength, the odds of infection were reduced by a factor of 0.55 ($p=0.04$), and this relationship did not depend on source region (Table 5, Figure 3).

Standing metabolic rate as predictor of constitutive or induced immune defense levels

Constitutive SMR (SMRc) was positively related to constitutive circulating hemocyte density in invasive-range crabs, but not in native-range crabs (Table 6). In contrast, for invaders, SMRc was negatively related to the proportional change in hemocytes upon induction (Table 6, Figure 4). SMRc was significantly positively associated with encapsulation strength, and this relationship did not vary by source region (Table 6, Figure 4). Instead, invasive-range crabs had significantly higher encapsulation responses across SMRc. Experimental exposure and crab size were also positively related to SMRc in the encapsulation model (Table 6).

Immune traits as predictors of energy allocation

Energy storage (indicated by relative hepatopancreas mass, HSI) was significantly positively related to constitutive hemocyte density in invasive-range crabs but not native-range crabs (Table 7). Hemocyte responsiveness (change in hemocytes after exposure) after parasite challenge was associated with a significant decrease in HSI (Table 7, Figure 5), and this relationship was somewhat more strongly negative for invasive-range than for native-range crabs ($p=0.06$). The relationship between change in hemoyctes and HSI also differed based on infection status. In infected crabs, higher hemocyte responsiveness was associated with higher HSI (Table 7).

We analyzed the effect of each immune trait on reproductive effort (GSI) separately for male and female crabs. Female crabs from the invasive range showed a negative relationship between constitutive hemocyte density and GSI (measured at the end of the experiment) (Table 8, Figure 6). The change in hemocyte concentration after parasite challenge was negatively associated with GSI in infected females, but not un-infected individuals (Table 8). The relationship between hemocyte responsiveness and GSI did not differ based on source region, but

invasive-range crabs had overall lower GSI than native-range females. Encapsulation strength was negatively related to GSI in invasive-range females, but positively related to GSI in native-range females (Table 8). Among male crabs from both source regions, constitutive hemocyte density was positively associated with GSI, while hemocyte responsiveness was negatively associated with GSI (Table 9, Figure 6). Infection was positively associated with GSI in both of these models. Encapsulation was not overall a significant predictor of male GSI (Table 9). Crab size was significantly negatively related to HSI and significantly positively related to GSI for both males and females across all three immune trait models.

Our analysis of the relationship of HSI to GSI by source region revealed a trend toward a positive relationship between these two energy metrics for invasive range female crabs and a weakly negative relationship for native females, however the result was not statistically significant (Table 10, Figure 8). Males showed a positive relationship between HSI and GSI that depended on infection status, but not on source region (Table 10, Figure 8).

DISCUSSION

Invasive populations that escape from native-range parasites may benefit not only from the reduction in the pathology of infection, but also from the ability to reallocate energetic resources away from immune defense and toward other fitness traits. A previous study of the effect of relaxed parasite selection on susceptibility of invasive-range *H. sanguineus* to rhizocephalan infection revealed a decrease in defense among invaders relative to native-range crabs (Keogh et al. in prep/Chapter 2). While invasive-and native-range *H. sanguineus* ended the experiment with nearly equal amounts of rhizocephalan infection in the present study, the fact that native-range crabs may have already harbored close to 40% (of a total final 44.9%)

internal infection prevalence prior to their introduction to the lab, while invaders necessarily acquired all 42.5% prevalence during their time in the holding tank and exposure treatments supports that invasive-range crabs are less resistant to infection. The present study builds on our earlier finding of poorer defense in invasive-range crabs by exposing differences in resource-based costs of immune traits to native-range crabs.

Exposure as a predictor of immune induction

Though experimental cyprid exposures did not significantly account for the majority of infections acquired during the experiment, the controlled exposures did induce changes in host immune traits and SMR. A low dose of cyprid exposure was positively associated with both increased encapsulation strength among both native and invasive crabs, and with an increase in SMR relative to constitutive SMR. That encapsulation was not increased in the medium- and high-dose treatments could potentially be due to hosts in these treatments needing to divert more of their resources to defense against the higher number of attacking parasites instead of strongly responding to the generic challenge of the nylon implant. The response of hosts to the nylon fiber implant was necessarily very general, likely utilizing similar resources to a wound-repair response. Therefore a strong encapsulation among invaders may reflect the maintenance of this general response by its utility in dealing with a range of common issues such as wound repair or response to novel generalist macroparasites that may attack invasive-range populations of the crab (Blakeslee 2009).

While the encapsulation response of a non-parasite implant may be considered quite general, we did find a possible protective effect of encapsulation against infection by rhizocephalans. For both invasive- and native-range crabs, a more robust encapsulation response

was associated with a lower probability of rhizocephalan infection, regardless of whether crabs had been exposed to a controlled dose of cyprids. Thus encapsulation strength, a measure of defense against macroparasites, appeared to be an effective constitutive and conserved immune trait. Interestingly, constitutive encapsulation ability seems to come at a physiological maintenance cost to hosts, as hosts with stronger encapsulation responses also had higher constitutive SMR regardless of source region. When SMR was accounted for as a predictor of encapsulation, invasive-range crabs are revealed to have higher encapsulation scores than natives across constitutive SMR, suggesting that physiological maintenance costs of encapsulation may be relatively lower in invasive-range crabs than in natives.

Our results also suggest a protective effect of hemocyte responsiveness among crabs. Exposed crabs that exhibited a more positive proportional change in circulating hemocyte density had a significantly lower probability of rhizocephalan infection. This protective effect was restricted to crabs that had been experimentally exposed to cyprids, supporting that the increase in circulating hemocyte density among these crabs was an effective induced response to the threat of infection. Consitutive hemocyte density was not a significant predictor of the likelihood of infection, suggesting that observed hemocyte-mediated protection may rely more heavily on the production or mobilization into circulation of certain hemocyte types upon detection of parasite cues. Alternatively, the negative relationship between hemocyte responsiveness or encapsulation and infection could potentially be attributed to crabs having been already exposed to (and in the process of infection by) infective larvae in the holding tank prior to immune trait measurement. Native-range crabs also could have harbored pre-reproductive internal (and therefore invisible prior to PCR) field-acquired rhizocephalan

infections upon arrival to the lab, which could interfere with both hemocyte responsiveness and encapsulation if the parasite modulates host immunity, and would have also resulted in a negative relationship between hemocyte responsiveness and infection status. Because source region was not a significant predictor, either by itself or as an interaction with hemocyte induction, of infection status, we don't expect existing infections among natives to have strongly influenced our findings of a protective effect of hemocyte increases or encapsulation.

Interestingly, hemocyte responsiveness was associated with higher constitutive SMR only among native-range crabs. This may indicate that heightened responsiveness among native-range crabs reflects a higher level of physiological investment in signaling molecules, receptors, or cell types that are more costly and more strongly related to defense against infection. Among invasive-range crabs, a higher responsiveness of hemocyte density to exposure was not associated with higher constitutive SMR, and may instead have represented a mobilization of hemocytes that were not particularly costly to have maintained (in terms of physiological maintenance). Consititutive SMR was in fact positively associated with constitutive circulating hemocyte density only among invaders, though constitutive hemocyte densities did not differ by source region. Thus invaders incurred higher costs for higher constitutive maintenance of circulating hemocytes, while native-range crabs did not pay increasing costs for constitutive hemocyte densities but instead incurred costs of maintaining hemocytes "at the ready" for mobilization upon immune induction.

A seemingly higher cost of encapsulation among natives, and a positive relationship between hemocyte responsiveness and constitutive SMR found in this group may indicate that, while we measured the same general phenotypic traits (total circulating hemocytes, general

implant encapsulation) in all crabs, measuring such general traits may mask important differences in the characteristics of these traits between natives and invaders (White & Perkins 2012). For example, while only total hemocytes were enumerated, the composition of hemocytes (ex. granulocytes versus leukocytes) or hemolymph (ex. concentration of signaling molecules, antimicrobial peptides, etc) may have varied substantially between source regions, and could account for differences in the costs of these traits between regions. That exposure to cyprid cues would induce a more expensive response, likely consisting of more highly specific defense molecules, in native-range crabs for whom rhizocephalan selection is consistent may provide evidence of trait canalization, which is expected from stabilizing selection (Le Rouzic *et al.* 2013). Stabilizing selection is predicted if there are indeed trade-offs between immune defense and other fitness traits (also reviewed in (Seppala 2015), and such trade-offs are supported in a subset of our results.

Immune traits and energy allocation – costs/benefits for invaders and natives

The relationships between immune traits and energy allocation in the crabs to physiological self-maintenance, energy storage (HSI), and reproductive investment (GSI) differed between source regions for female crabs. In invasive-range crabs, for whom constitutive hemocyte density and encapsulation were associated with increased self-maintenance costs (SMR), these two putatively constitutive traits were positively related to energy storage but negatively related to GSI in females. In contrast, higher hemocyte responsiveness in invaders was not related to constitutively higher self-maintenance costs (SMR), but invaders that more strongly increased circulating hemocyte density did trend toward greater reductions than natives in energy storage, though this results was not significant at the 0.05 alpha level ($p=0.06$). Thus

for invasive-range females, higher constitutive investment in defense-associated traits trades off with reproductive investment but not with energy storage, whereas a strong induced response (change in hemocytes) does incur costs to energy storage. The positive relationship between the two traits that were associated with higher self-maintenance costs (encapsulation response and constitutive hemocyte densities) and energy storage in invaders may suggest that invaders are better than native-range crabs at assimilating or storing energy, or have higher tolerance to nutrient limitation. For example, invasive-range individuals may be better adapted to store energy derived from their laboratory algal diet if their high invasive-range densities result in regular depletion of animal food sources (Griffen *et al.* 2015). If energy budget theory is invoked, the finding of a positive relationship between constitutive physiological traits and energy storage in invaders may be partially explained by our finding (reported in Ch2) of overall lower self-maintenance costs (SMR) in uninfected invasive-range crabs, which should leave more energy available for storage (Artacho & Nespolo 2009). Constitutive defenses that were positively associated with HSI in invaders may be particularly inexpensive to maintain if the hemocytes perform other physiological tasks, as opposed to potentially more costly physiological upkeep or deployment of specific defenses.

The relationships between immune traits and energy allocation were significantly different in native females compared invasive-range females. In direct contrast to invaders, native range females exhibited either no relationship or a weakly negative one between constitutive hemocyte density or encapsulation and HSI, and a positive relationship between these traits and female GSI. Additionally, GSI was higher overall in native-range females. A potential explanation for the difference in female GSI between the two source regions is that

native-range crabs may respond to the threat of infection by increasing reproductive effort, either plastically or as constitutive strategy in native-range sites where the threat of castrating infection selects for earlier and/or higher reproductive effort. Different versions of this "fecundity compensation" strategy have been documented across several species of invertebrates in sites where castrating parasites are prevalent (Granovitch *et al.* 2009; Parker *et al.* 2011; Vale & Little 2012). Alternatively, the seasonal timing of reproduction may be different between the two regions. Invasive-range populations in the Western Atlantic reproduce for 5 months between April to September (McDermott 1998), while reproduction in the native range occurs for 3 months in northern latitudes in Hokkaido (Takahashi *et al.* 1985) and for up to 8 months from March to October or November in the more southern part of the Japanese range (Fukui 1988; Epifanio 2013). A follow-up analysis of GSI by individual collection site and infection status revealed higher GSI in the northern native-range collection sites, where breeding season should more closely reflect season timing of invasive-range crabs (Figure 1, Supplemental figure 3), thus refuting the likely role of seasonality in driving changes.

If differences in breeding season phenology did play a role in differences in resource allocation between natives and invsives, the influence could extend beyond the relationships between immune traits and GSI if seasonally-based differences in resource allocation to GSI trade off directly with energy storage in the hepatopancreas. Because native range females were allocating more resources to reproduction, they are more likely to exhibit such a resource-based trade-off. Our analysis of the relationship between HSI and GSI by source region was suggestive of such a trade-off in native-range females, however the result was not statistically significant. A future study that measured the relationships between HSI, GSI and immune traits at multiple

intervals spanning the breeding season in the invasive and native ranges would be needed to confirm or refute the role of breeding phenology in the differences in relationships between energy and immunity detected between invasive and native-range females. If breeding phenology is playing a role in the differences observed, then our results provide evidence that reproductive effort imposes energetic constraints on immune defense in females of this species, as has been observed in other invertebrate species (Webster & Woolhouse 1999; Vijendravarma *et al.* 2009; Gershman *et al.* 2010).

Relationships between immune traits and energy allocation differed less between source regions for male crabs. Regardless of source region, males demonstrated a trade-off between GSI and hemocyte responsiveness, suggesting a cost of immune induction to future reproductive effort (Gershman *et al.* 2010). As infection is positively associated with GSI for males in two of the three models exploring GSI as a function of immune traits (Table 9), our results suggest that males may respond to infection and the prospect of castration by increasing reproductive effort, similar to the fecundity compensation described for females above.

For both females and males, the relationships between induced immune responsiveness and energy traits differed based on infection status. In infected individuals, hemocyte responsiveness was positively correlated with HSI, and for females, negatively correlated to GSI, whereas hemocyte responsiveness across uninfected individuals was weakly negatively related to HSI, and in uninfected females was positively correlated with GSI. One possible explanation for this flip in energy allocation between storage and reproduction in infected crabs could be manipulation on the part of the infecting rhizocephalan parasites. Mature rhizocephalan infections sterilize hosts either through active take-over of the host's endocrine system and often

degeneration of the gonopores, or by diverting all of the host's non-vital energy to their own reproduction, result in nearly complete cessation of spermatogenesis and vitellogenesis (Hoeg 1995). As they grow inside the host, rhizocephalans form a web of root-like tissue that pervades the host's hepatopancreas, where the parasite acquires energy from the host (Hoeg 1995). Thus a diversion of resource use away from the storage organ and toward gonad energy may represent an adaptive strategy of the parasites to maximize retention of energy for their own growth.

Conclusion

Invasive species that experience a reprieve from parasite infection in their invaded range provide a unique opportunity to test hypotheses about the importance of parasite-selection for maintaining immunity, and the fitness trade-offs that may be imposed by costly defenses (Sax *et al.* 2007). We found that constitutive defense levels in invasive-range *H.sanguineus* females traded-off with reproductive effort rather than energy storage, the opposite of the relationship shown by native-range females. Further, invaders that responded more strongly (hemocyte responsiveness) to experimental exposure did not exhibit higher physiological self-maintenance costs, as was found in native-range crabs. The seemingly lower energy cost of responsiveness in invasive-range crabs may point to a difference in the quality of defensive hemocytes with which invaders responded. Focusing on more specific immune traits may help reveal important evolutionary mechanisms that may be driving these costs in natives, but which are not apparent in invaders. That background defense levels were not associated with a cost in natives, but were costly in invaders suggests that invaders may not exhibit greater general robustness than native-range individuals, as is implied in the "pre-selection" explanation of invader success. However, taken together with our previous finding of higher susceptibility among natives (Keogh et al. in

prep/Chapter 2), the differences in immune trait-energy allocation relationships between invaders and natives revealed in the present study point to a rapid response of invasive populations to changes in parasite-mediated selection, possibly representing a shift toward growth and survival. Our findings represent a step toward addressing the question of whether relaxed parasite selection may help account for the higher fitness achieved by invasive populations (Blossey & Notzold 1995; White & Perkins 2012), and points to the need for further studies of wild populations to help disentangle the complex relationships between immune defense and host life history (Pedersen & Babayan 2011).

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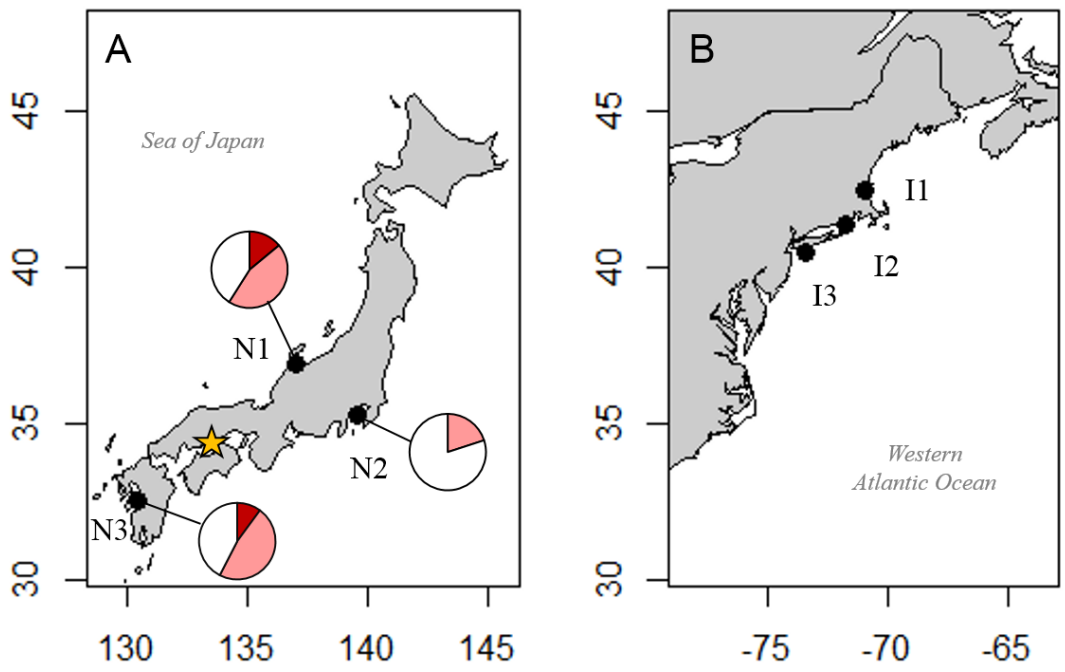

Figure 4.1 Map showing *Hemigrapsus sanguineus* sampling locations in A) Native range collection sites in Japan (N1-N3) and B) Invasive-range collection sites in Eastern United States (I1-I3). Pie charts in panel A show the percent of crabs at each sample site with visible, reproductively mature *Polyascus* spp. infections (in red), internal infections detected by PCR (salmon), or without infection (white). Gold star indicates collection site of native-range crabs from which infective cyprids used in dose treatments were derived. In the invasive range in the Northwest Atlantic, *Polyascus* spp. infections (or Rhizocephalan infections of any species) are absent.

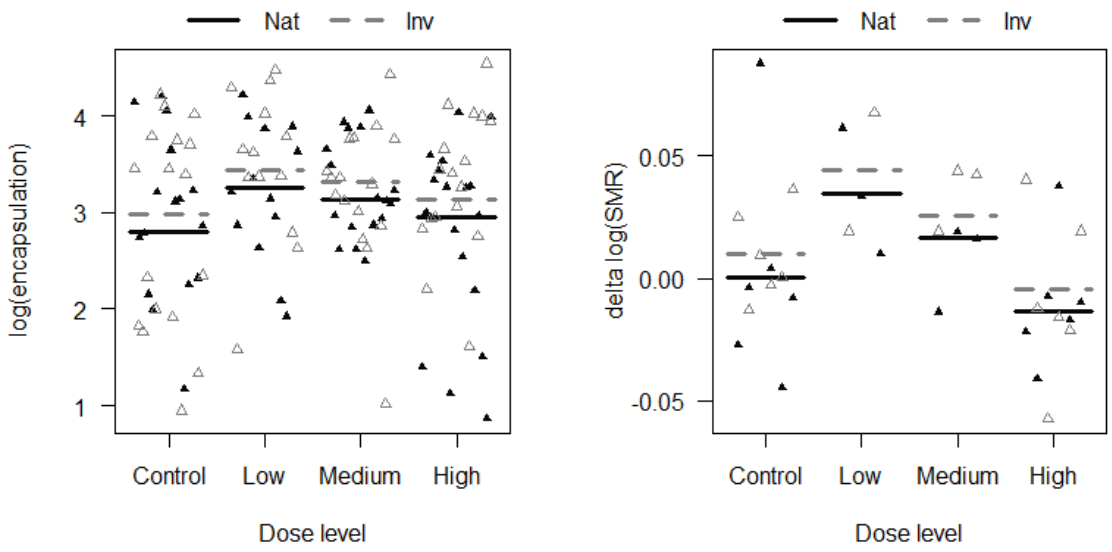

Figure 4.2 (A) Encapsulation strength or (B) delta SMR as a function of controlled cyprid exposure dose level by source region.

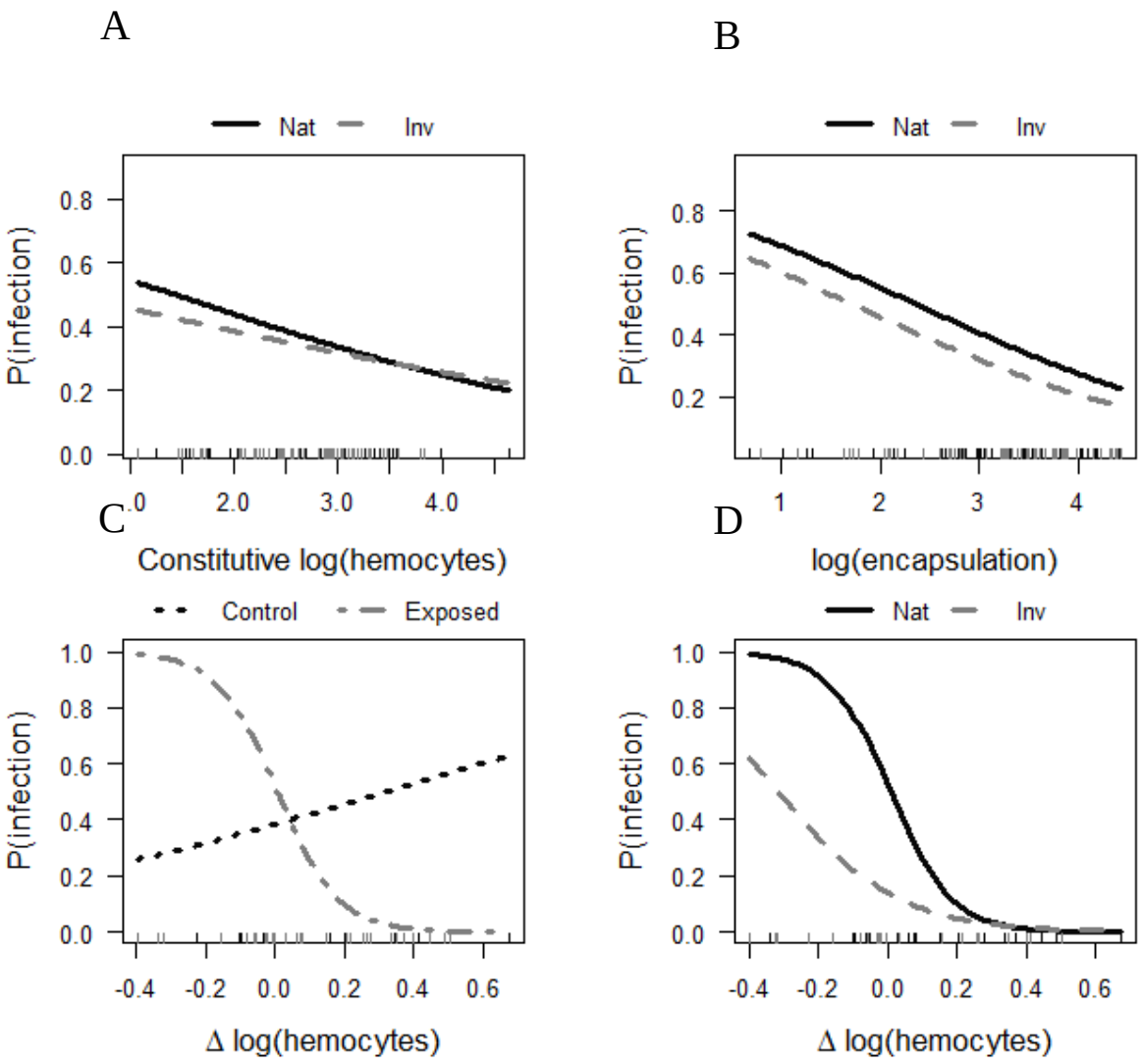

Figure 4.3 Probability of infection as a function of (A) constitutive circulating hemocytes by region, (B) encapsulation strength by region, (C) proportional change in circulating hemocyte density in control (grey) versus exposed (grey dashed) crabs, or in (D) native (solid) versus invasive (dashed) crabs.

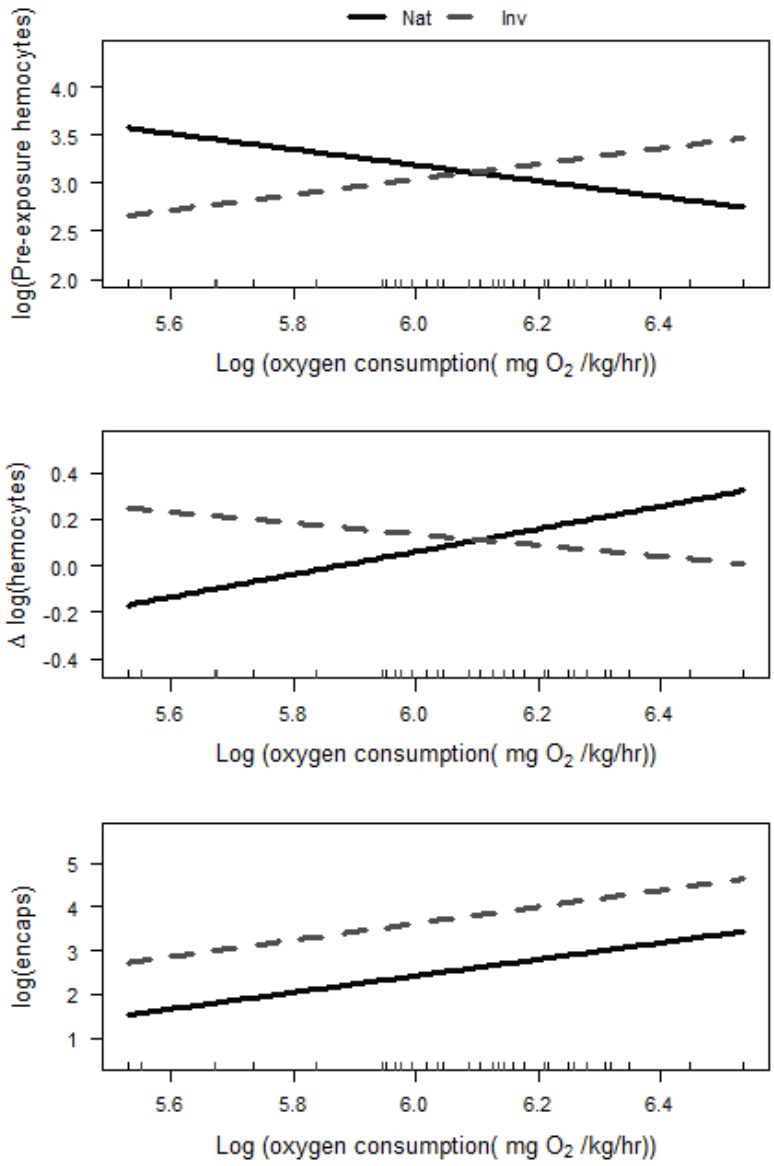

Figure 4.4 SMRc as a predictor of (A) constitutive hemocyte density, (B) change in hemocyte density after cyprid augmentation, or (C) encapsulation.

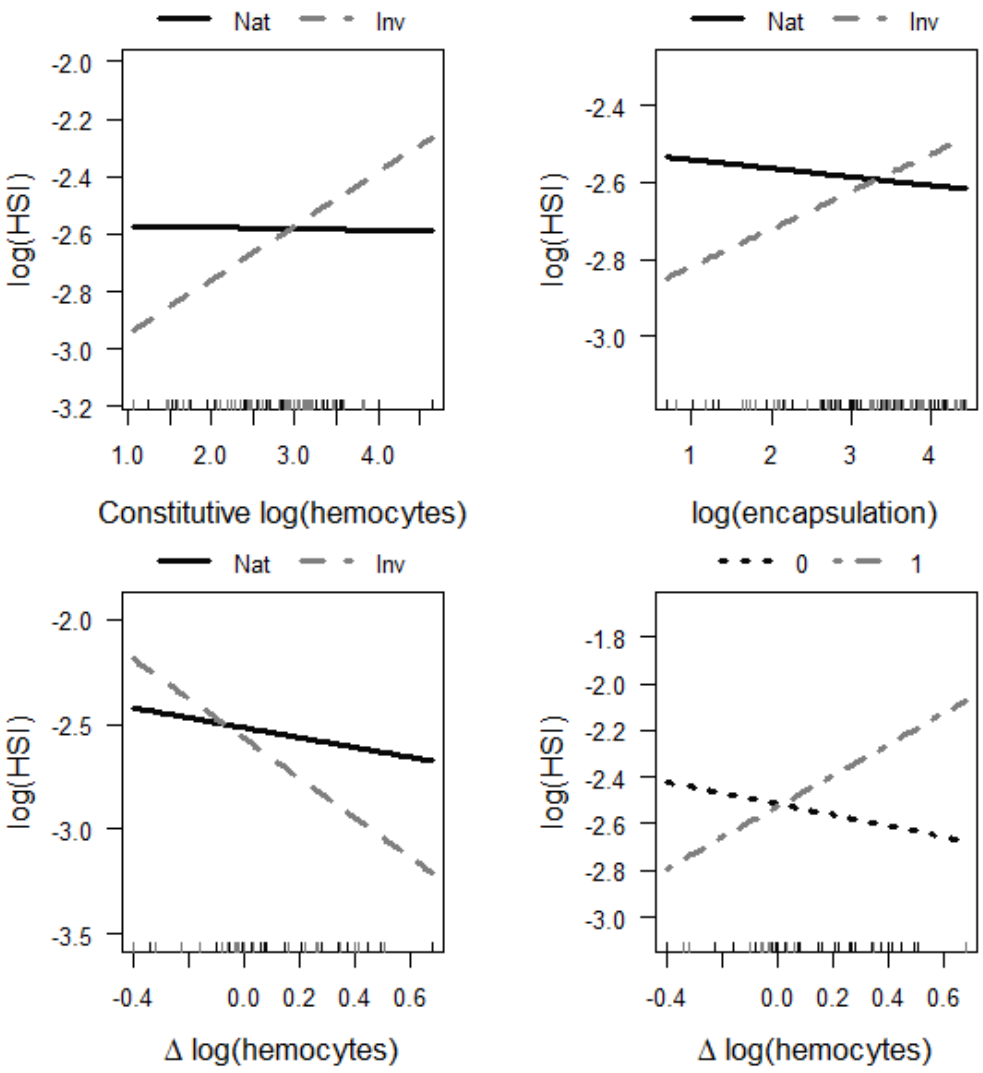

Figure 4.5 Log(HSI) plotted by source region (A-C) as a function of (A) constitutive hemocyte density, (B) encapsulation strength, or (C) proportional change in hemocytes after induction. Panel D shows the change in hemocytes by infection status (1= infected).

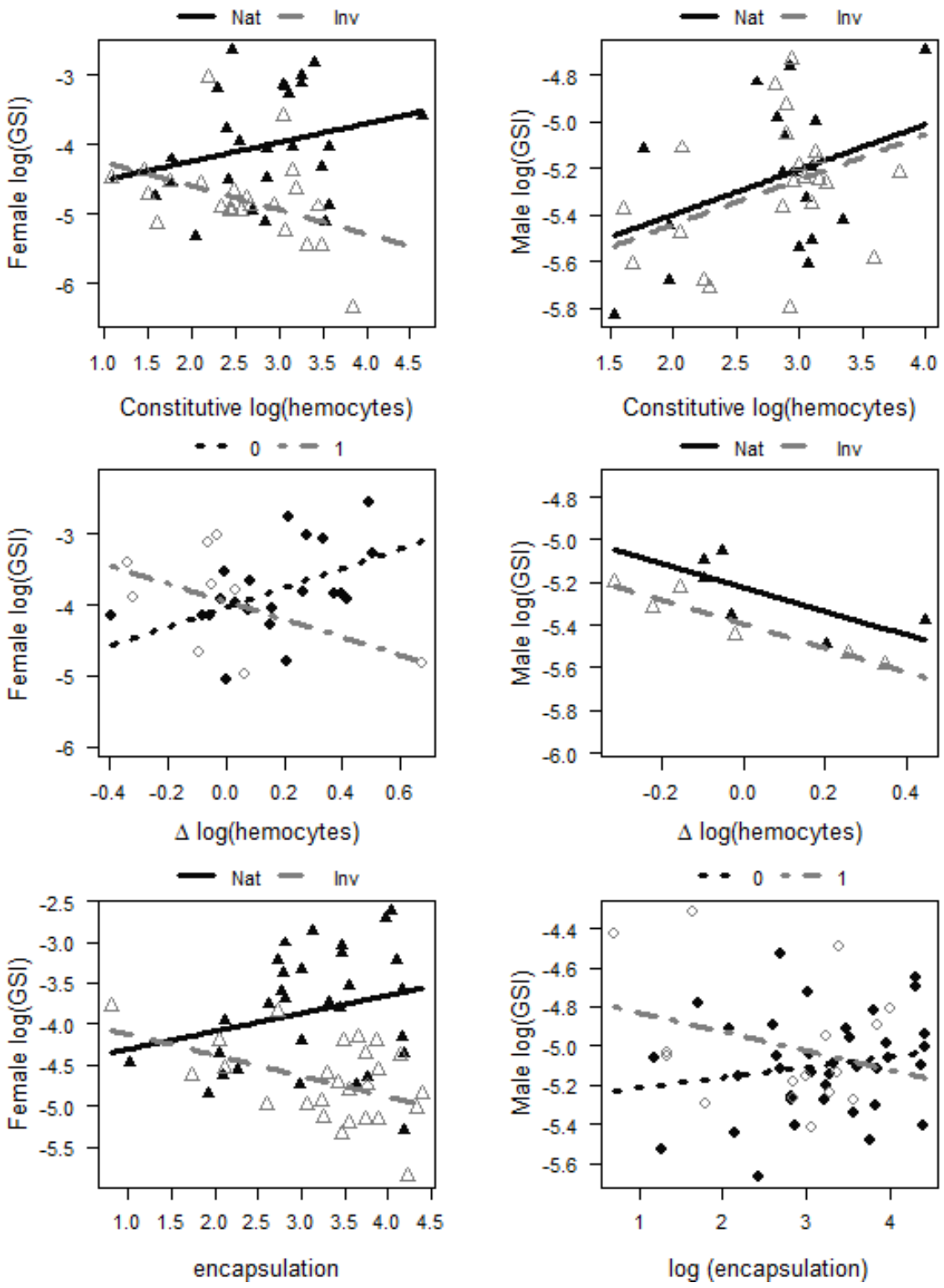

Figure 4.6 Female (A-C) and male (D-F) GSI as a function of immune traits.

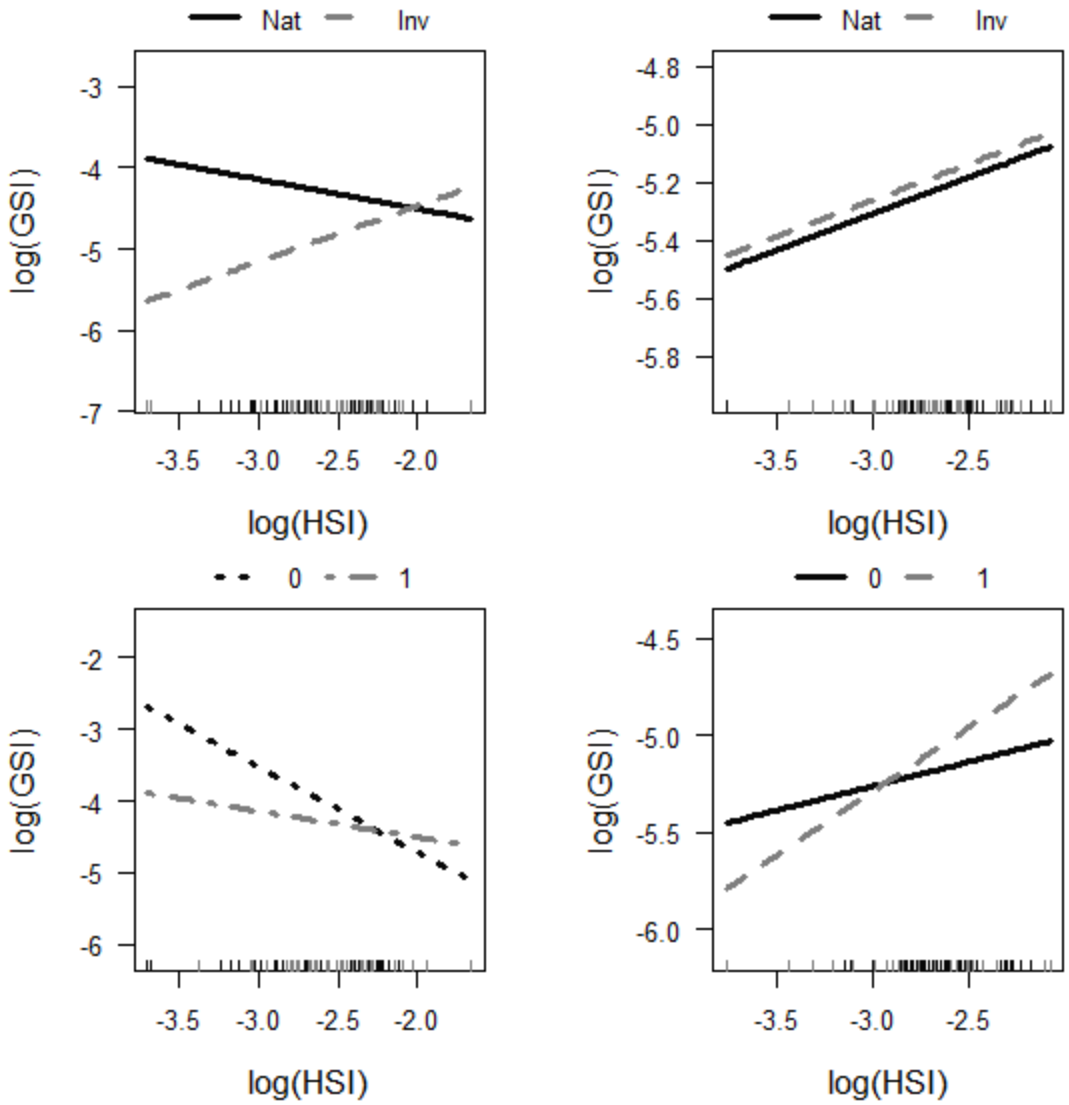

Figure 4.7 GSI as a function of HSI by region (A,B) or infection status (C,D) for female crabs (A,C) or male crabs (B,D).

Table 4.1 Infection status as a function of cyprid dose treatment level and source region

	Full model			Reduced model		
	Odds Ratio	*Conf. Int.*	*p-value*	*Odds Ratio*	*Conf. Int.*	*p-value*
Fixed Effects						
(Intercept)	0.94	0.21 – 4.32	.938	1.04	0.26 – 4.16	.959
treatLow	1.33	0.28 – 6.23	.721	1.18	0.39 – 3.58	.776
treatMedium	0.92	0.18 – 4.61	.923	0.79	0.25 – 2.46	.682
treatHigh	0.92	0.22 – 3.90	.915	0.81	0.29 – 2.30	.697
regionInv	1.00	0.23 – 4.32	1.00	0.82	0.38 – 1.76	.614
treatLow:regionInv	0.78	0.09 – 6.97	.827			
treatMedium:regionInv	0.73	0.08 – 6.81	.782			
treatHigh:regionInv	0.77	0.10 – 5.80	.801			
Random Effects						
N_{block}		8			8	
N_{site}		6			6	
ICC_{block}		0.426			0.425	
ICC_{site}		0.000			0.000	
Observations		158			158	

Table 4.2 Proportional change in hemocyte density (as an indicator of immune induction) as a function of experimental exposure and source region.

	Full model			Reduced model		
	Estimate	*Conf. Int.*	*p-value*	*Estimate*	*Conf. Int.*	*p-value*
Fixed Effects						
(Intercept)	0.10	-0.10 – 0.30	.317	0.09	-0.07 – 0.25	.270
Trt(exposed)	-0.02	-0.24 – 0.21	.890	0.00	-0.16 – 0.16	.997
Region(Invasive)	-0.05	-0.32 – 0.23	.748	-0.02	-0.16 – 0.12	.764
Trt*Region	0.03	-0.29 – 0.35	.842			
Random Effects						
N_{site}		6			6	
N_{block}		3			3	
ICC_{site}		0.000			0.000	
ICC_{block}		0.004			0.004	
Observations		49			49	

Table 4.3 LMER model results of encapsulation as a function of cyprid exposure treatment, with source region as a fixed factor and collection site and block as random factors.

	Est.	*Conf. Int.*	*p-value*
Fixed Effects			
(Intercept)	2.79	2.44 – 3.15	**<.001**
Trt(low)	0.46	0.04 – 0.89	**.035**
Trt(med)	0.34	-0.05 – 0.74	.092
Trt(high)	0.16	-0.23 – 0.55	.433
Region(Invasive)	0.18	-0.10 – 0.46	.218
Random Effects			
N_{block}		6	
N_{site}		6	
ICC_{block}		0.057	
ICC_{site}		0.000	
Observations		127	

Table 4.4 LMER model results of delta SMR as a function of exposure treatment, with source and size, and sex as fixed covariates and collection site as a random effect.

	Est.	*Conf. Int.*	*p-value*
Fixed Effects			
(Intercept)	-0.07	-0.29 – 0.15	.535
Size	0.02	-0.05 – 0.10	.528
Sex(F)	-0.01	-0.03 – 0.01	.238
Trt(low)	0.03	0.00 – 0.06	**.035**
Trt(med)	0.02	-0.01 – 0.04	.287
Trt(high)	-0.01	-0.04 – 0.01	.208
Region(Invasive)	0.01	-0.01 – 0.03	.320
Random Effects			
N_{site}		6	
ICC_{site}		0.000	
Observations		35	

Table 4.5 Output for GLMER models of infection status as a function of constitutive hemocytes, proportional chance in hemocytes after cyprid augmentation (delta Hem), or encapsulation strength. For the two traits measured post-cyprid augmentation (delta Hem, encapsulation), original full models included interactions of each trait with source region and exposure, and reduced models were selected by removing non-significant interactions. Experimental treatment is included as a covariate, and block and collection site are included as random factors.

	Constit. Hem			delta Hem.			Encapsulation		
	Odds Ratio	*Conf. Int.*	*p-value*	*Odds Ratio*	*Conf. Int.*	*p-value*	*Odds Ratio*	*Conf. Int.*	*p-value*
Fixed Effects									
(Intercept)	1.85	0.08 – 41.54	.698	0.62	0.12 – 3.28	.577	4.03	0.46 – 35.18	.208
HemocytesPre	0.65	0.22 – 1.90	.430						
Region(Inv.)	0.61	0.01 – 28.24	.801	0.15	0.02 – 1.01	.052	0.69	0.27 – 1.78	.439
HemPre*Region	1.15	0.29 – 4.60	.846						
delta Hem				4.36	0.01 – 1341.12	.614			
Trt(exposed)				1.77	0.23 – 13.42	.581			
delta H*Trt				0.00	0.00 – 0.06	**.012**			
delta H*Region				279.44	$0.05 – 1.4x10^6$	.199			
Encaps							0.55	0.31 – 0.98	**.042**
Random Effects									

N_{block} / ICC_{block}	6 / 0.229	6 / 0.000	6 / 0.429
N_{site} / ICC_{site}	6 / 0.000	3 / 0.000	6 / 0.000
Observations	98	43	113

Table 4.6 Results of LMER models of SMRc as a predictor of constitutive hemocyte densities, change in hemocyte density after cyprid augmentation, and encapsulation strength.

	Constit. Hem.			Delta Hem.			Encapsulation		
	Est.	*Conf. Int.*	*p-value*	*Est.*	*Conf. Int.*	*p-value*	*Est.*	*Conf. Int.*	*p-value*
Fixed Effects									
(Intercept)	3.80	-6.26 – 13.86	.464	-3.68	-7.79 – 0.44	.089	-20.68	-36.89 – -4.47	**.018**
Const.SMR	-0.82	-1.89 – 0.25	.143	0.49	0.06 – 0.92	**.033**	1.90	0.30 – 3.50	**.026**
Region(Invasive)	-9.91	-19.22 – -0.61	**.045**	4.43	0.65 – 8.22	**.028**	1.20	0.58 – 1.82	**.001**
Size	1.43	-0.35 – 3.22	.125	0.27	-0.46 – 1.01	.476	3.69	0.82 – 6.56	**.017**
Sex	0.13	-0.27 – 0.53	.530	-0.12	-0.29 – 0.05	.179	-0.37	-1.02 – 0.28	.274
Const.SMR*Region	1.63	0.08 – 3.17	**.047**	-0.73	-1.36 – -0.10	**.031**			
Trt(exp)				-0.00	-0.14 – 0.14	.966	0.65	0.07 – 1.23	**.034**
Random Effects									
N_{site}		6			6			6	
ICC_{site}		0.000			0.000			0.000	
Observations		33			32			32	

Table 4.7 LMER model output for HSI as a function of immune traits, with region, infection status, size and treatment as covariates.

	Constit. Hem.			Delta H			Encapsulation		
	Est.	*Conf. Int.*	*p-value*	*Est.*	*Conf. Int.*	*p-value*	*Est.*	*Conf. Int.*	*p-value*
Fixed Effects									
(Intercept)	1.19	-0.34 – 2.73	.130	2.10	-0.10 – 4.29	.069	1.53	0.49 – 2.57	**.005**
Const.Hem	-0.01	-0.12 – 0.11	.930						
Region(Invasive)	-0.57	-1.04 – -0.10	**.021**	-0.05	-0.29 – 0.18	.668	-0.40	-0.80 – 0.00	.055
Infection(Inf)	0.05	-0.06 – 0.17	.364	-0.02	-0.21 – 0.18	.879	0.03	-0.07 – 0.13	.563
Size	-1.25	-1.74 – -0.75	**<.001**	-1.54	-2.25 – -0.83	**<.001**	-1.24	-1.56 – -0.92	**<.001**
Const.Hem*Reg.	0.19	0.04 – 0.35	**.018**						
delH				-0.29	-0.91 – 0.34	.379			
Trt(exposed)				0.05	-0.14 – 0.23	.616	-0.30	-0.69 – 0.10	.142
delH*Region				-0.68	-1.37 – 0.01	.062			
delH*Infect.				0.99	0.20 – 1.79	**.019**			
Encapsulation							-0.12	-0.24 – -0.00	.052
Encaps*Reg.							0.12	0.00 – 0.24	.051
Encaps*Trt							0.10	-0.03 – 0.22	.127
Random Effects									

N_{site} /ICC_{site}	6 / 0.165	6 / 0.146	6 / 0.000
N_{gr}/ICC_{gr}	5 / 0.000	3 / 0.000	6 / 0.052
Observations	91	42	111

Table 4.8 LMER model output for female GSI as a function of constitutive hemocytes, delta hemocytes, or encapsulation. The random effect of block is excluded from the delta Hem model because only two blocks were represented in these data, and the term was not significant when included as a fixed effect.

	Constit. Hemocytes			Delta Hem.			Encapsulation		
	Est.	*Conf. Int.*	*p-value*	*Est.*	*Conf. Int.*	*p-value*	*Est.*	*Conf. Int.*	*p-value*
Fixed Effects									
(Intercept)	-19.78	-25.83 – -13.72	**<.001**	-19.42	-26.08 – -12.76	**<.001**	-15.83	-20.11 – -11.56	**<.001**
HemocytesPre	0.27	-0.15 – 0.69	.210						
Region(Invasive)	0.87	-0.80 – 2.55	.312	-1.06	-1.88 – -0.23	**.045**	0.65	-0.78 – 2.08	.377
Infection(Inf)	0.06	-0.40 – 0.51	.801	0.08	-0.53 – 0.68	.804	-0.21	-0.54 – 0.13	.229
Size	4.95	3.03 – 6.88	**<.001**	5.08	2.96 – 7.21	**<.001**	3.74	2.37 – 5.10	**<.001**
HemPre*Region	-0.62	-1.20 – -0.03	**.045**						
delta Hem				1.38	0.19 – 2.57	**.033**			
deltaH*Infect				-2.63	-4.47 – -0.79	**.010**			
Encaps.							0.22	-0.05 – 0.49	.123

Encaps*Reg.			-0.47	-0.86 – -0.08	**.021**
Random Effects					
N_{site} / ICC_{site}	6 / 0.097	6 / 0.339		6 / 0.308	
N_{gr} / ICC_{gr}	4 / 0.000			5 / 0.000	
Observations	49	30		57	

Table 4.9 Male GSI as a function of constitutive hemocytes, delta hemocytes, or encapsulation. The random effect of block is excluded from the delta Hem model because only one blocks was represented in these data, and the term was not significant when included as a fixed effect.

	Constit. Hemocytes			Delta Hem.			Encapsulation		
	Est.	*Conf. Int.*	*p-value*	*Est.*	*Conf. Int.*	*p-value*	*Est.*	*Conf. Int.*	*p-value*
Fixed Effects									
(Intercept)	-9.05	-11.69 – -6.41	**<.001**	-12.27	-16.05 – -8.50	**<.001**	-8.36	-9.61 – -7.10	**<.001**
HemocytesPre	0.19	0.02 – 0.36	**.034**						
Region(Invasive)	-0.04	-0.26 – 0.17	.722	-0.17	-0.57 – 0.22	.431	0.01	-0.14 – 0.16	.900
Infection(Inf)	0.31	0.09 – 0.53	**.010**	0.55	0.36 – 0.73	**.001**	0.10	-0.08 – 0.29	.276
Size	1.09	0.24 – 1.95	**.020**	2.36	1.13 – 3.60	**.003**	1.06	0.64 – 1.48	**<.001**
delta Hem				-0.56	-0.91 – -0.20	**.022**			
Encaps							-0.00	-0.09 – 0.09	.998
Random Effects									
N_{site} / ICC_{site}		6 / 0.066			6 / 0.793			6 / 0.000	
N_{gr} / ICC_{gr}		4 / 0.000						5 / 0.000	
Observations		40			12			53	

Table 4.10 LMER model output of log(GSI) as a function of the interaction of HSI with source region or infection status for female or male crabs, with size as a covariate.

	Females			Males		
	Est.	*Conf. Int.*	*p-value*	*Est.*	*Conf. Int.*	*p-value*
Fixed Parts						
(Intercept)	-18.78	-23.65 – -13.91	**<.001**	-9.66	-10.95 – -8.36	**<.001**
HSI	-1.19	-2.17 – -0.20	**.021**	0.25	-0.10 – 0.60	.168
Region(Invasive)	1.85	-0.62 – 4.31	.146	1.19	0.13 – 2.26	**.031**
Infection(Inf)	2.13	-0.64 – 4.90	.138	0.05	-0.14 – 0.24	.658
Size	3.87	2.15 – 5.58	**<.001**	1.69	1.22 – 2.16	**<.001**
HSI*Infect.	0.82	-0.11 – 1.76	.090	0.41	0.02 – 0.80	**.046**
HSI*Region	1.05	0.00 – 2.09	.054			
Random Parts						
N_{gr}		6			6	
N_{site}		6			6	
ICC_{gr}		0.000			0.000	
ICC_{site}		0.201			0.111	
Observations		75			72	

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CHAPTER 5

CONCLUSIONS

Macroparasites can exert strong selection on host defenses, but the magnitude and direction of selection may vary greatly over time and space. Because immune defenses incur resource costs, hosts may be selected against investing resources in immunity when the risk of infection is low. Thus heterogeneity in infection risk, combined with energetic constraints faced by hosts, may contribute to the maintenance of differences in susceptibility to infection across wild populations.

This dissertation has explored whether spatial variability in the risk of infection by castrating parasites may drive differences in susceptibility among marine invertebrate hosts. In both a regional-scale study (Chapter 2), and a study of an invader spanning two ocean basins (Chapter 3), we found that consistent differences in selection pressure from castrating parasites predicted host susceptibility to infection, with naïve hosts gaining relatively more experimental infection than their non-naïve counterparts. We also found altered relationships between immune traits and energetic resources in invasive populations that have escaped parasites (Chapter 4), supporting that relaxed parasite selection may benefit invaders by allowing potentially adaptive redistribution of immune defense resources to other fitness traits.

Particularly in the chapters focused on an invasive species experiencing parasite escape, this dissertation represents a step toward filling a critical knowledge gap of the ultimate consequences of relaxed parasite selection for the short term energetic gains and longer-term infection susceptibility that may simultaneously propel invasive populations toward the

disruptively high densities commonly observed while rendering these large populations highly susceptible to damaging infections.

The experiments described in this dissertation used common garden approaches and focused on wild-caught adult snails and crabs that provided a snapshot of the immune defense status of individuals whose development, growth, and immune system had already been shaped by the parent environment from which they were collected. Our results provide a glimpse of the integrative defense outcome of multiple potential mechanisms that may contribute individually or in concert to a host's immune phenotype. Future studies aimed at disentangling the contributions of plastic, epigenetic, and genetic processes to phenotypic divergence in invasive populations in particular will constitute an important next step toward understanding the flexibility of adaptive (maladapted) traits under altered future selective regimes, as well as their dependence on past and future gene flow.

The loss of some species and introductions of others from distant places obfuscate the natural state and functioning of present day ecological communities (Carlton 2009), leaving would-be conservationists sometimes scratching their heads as to which "baseline" should be the target of restoration efforts. A poignant recent example of the difficulty posed by these sliding baselines of ecological history was the recent revelation that an entire iconic (and presumed native) type of coastal ecosystem in South America may be the non-natural product of accidental introduction of the saltmarsh cordgrass *Spartina alterniflora* (Bortolus *et al.* 2015). Compounded with the selective curveballs contributed by rapid human-induced climate change, the evolutionary trajectories of these altered ecological communities are increasingly difficult to predict and preserve - just when our technological capacities and ecological-academic

momentum are at the cusp of enabling us to robustly characterize the diversity and ecology of the natural world.

Though this dissertation focuses on changes in parasite selection that happen in the context of species invasion, other forms of anthropogenic disturbance are resulting in losses of macroparasites from native communities as well, with unknown consequences for hosts and communities. Recent work in reef environments has identified overfishing as a driver of macroparasite diversity loss (Wood *et al.* 2014). Most human activities that dramatically reduce host population size or selectively remove certain host age classes have the potential to drive parasite populations to extinction (Lloyd-Smith *et al.* 2005). Given the important role parasites play as co-evolved agents of selection, and also the recently recognized disproportionate contribution of macroparasites to both biomass and connectivity of food webs (Lafferty *et al.* 2006; Kuris *et al.* 2008), the loss of some types of parasites from natural systems could have important negative consequences for hosts species and the broader ecological community. A lack of baseline knowledge of the identity and distribution of parasites in "healthy" ecosystems is a barrier to answering foundational questions about host-parasite relationships, including accurately characterizing host specificity, co-infection, and the role parasites play as forces of selection structuring host life history traits. Larger and more coordinated efforts at cataloging the diversity, distribution, and ecological roles of parasites are needed before parasites are lost from natural populations.

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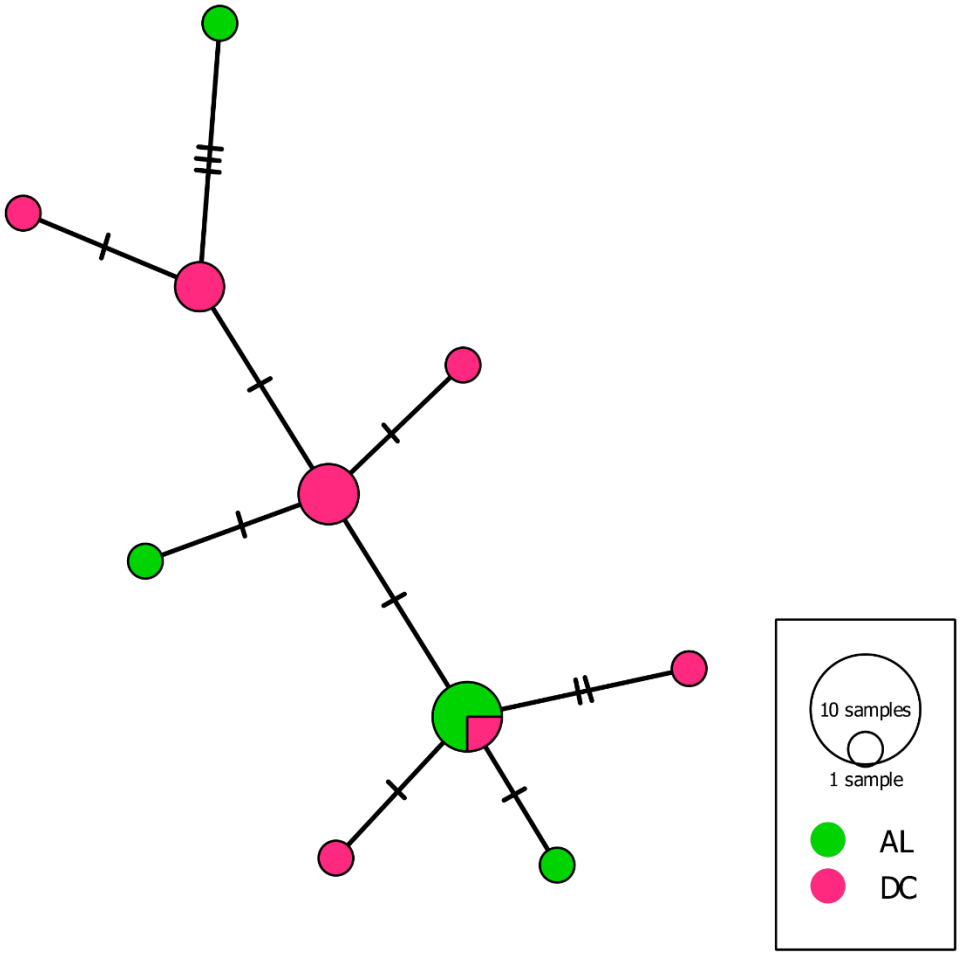
APPENDIX A – CHAPTER 2

Supplemental Table 2.1 Prevalence of trematode infection in each *Littorina* host species at each site sampled in 2011, displayed as both total prevalence and prevalence of infection by the two most common trematode species, *Microphallus similis* and *Cryptocotyle lingua*.

Species	**Site**	**Mean Size**	**N**	**N Total Infected**	**N *C. lingua***	**N *M. similis***	**% Total Infection**	**% *M. similis***	**% *C. lingua***
L. littorea	Appledore	16.13	113	31	31	0	27.43	0.00	27.43
	Smuttynose	15.58	98	23	18	0	23.47	0.00	18.37
	Odiorne	16.96	99	3	3	0	3.03	0.00	3.03
	Boothbay	17.56	103	5	5	0	4.85	0.00	4.85
	Hilton	15.31	78	0	0	0	0.00	0.00	0.00
	Merepoint	16.53	94	1	1	0	1.06	0.00	1.06
L. obtusata	Appledore	10.34	69	11	5	3	15.94	4.35	7.25
	Smuttynose	10.73	111	12	4	6	10.81	5.41	3.60
	Odiorne	9.77	105	0	0	0	0.00	0.00	0.00
	Boothbay	8.55	94	1	0	0	1.06	0.00	0.00
	Merepoint	10.07	105	6	3	1	5.71	0.95	2.86
	N. Hampton	10.57	99	1	0	1	1.01	1.01	0.00
L. saxatilis	Appledore	10.28	69	11	0	10	15.94	14.49	0.00
	Smuttynose	9.30	107	5	1	2	4.67	1.87	0.93
	Odiorne	10.17	78	6	1	4	7.69	5.13	1.28
	Boothbay	9.34	52	2	0	2	3.85	3.85	0.00
	N. Hampton	9.88	81	0	0	0	0.00	0.00	0.00
	Rye	9.96	120	0	0	0	0.00	0.00	0.00



Supplemental Figure 2.1 Haplotype network of *M. similis* sequence data from Appledore ledges (AL) and Damariscove Island (DC). The network was drawn using PopART (http://popart.otago.ac.nz), based on minimum spanning networks (Bandelt et al. 1999)

Bandelt H, Forster P, Röhl A (1999). Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16(1):37–48.

APPENDIX B - CHAPTER 3

3.1 PCR detection of internal rhizocephalan infection

Because rhizocephalan-infected crabs may not show external signs of infection until the rhizocephalan parasite is reproductively mature (i.e. has extruded a virgin externae at least once), and internal phases of early-stage infections are difficult to distinguish microscopically within the crab, we developed an 18s rDNA PCR assay to detect the presence of pre-patent infections within the host tissue. The assay described below was used both for the detection of latent natural infections in 20-30 crabs from each native range site, and to detect pre-patent infections in all sentinel crabs from the common garden exposure experiment.

DNA Extraction

To collect host tissue for PCR detection, crabs were immobilized by freezing and then dissected with sterilized forceps. Because rhizocephalan infections in the family Sacculinidae (encompassing all three potential species known to infect *H. sanguineus*) are reported as proceeding outward from the midgut during development (Hoeg 1995), we focused our tissue collections on the midgut and sections of hindgut and membrane tissue from around the cardiac stomach. DNA extraction was carried out using a CTAB method modified from Doyle and Doyle (1987). Briefly, after the tissue was digested in [CTAB + Proteinase K at 60°C] overnight, the DNA was isolated using 1:1 phenol chloroform and precipitated in EtOH before washing with 75% EtOH and finally rehydrating in deionized distilled water.

PCR assay and Sequencing

Primers for detection of rhizocephalans in the genus *Polyascus* and *Sacculina* were designed using 18s rDNA sequences for three species of *Polyascus* (*P. plana, P. polygenea, and P. gregaria*) and 21 other species of rhizocephalan and non-rhizocephalan barnacles accessed in GenBank. A host (*H. sanguineus*) sequence was also included when designing primers to ensure no cross-amplification of host DNA. After testing several combinations of prospective primer sequences, the following forward and reverse primers gave the highest specificity to parasite DNA without amplifying host DNA: 18Ssac3F, 5'-TTTCTGTTTTGTAACCGTACTCT-3' (forward); 18Ssac1R, 5'-TATCGGCTGGCCGCATTCAA-3' (reverse). PCR was carried out using Tks Gflex DNA Polymerase (Takara, Japan) consisting of a 60 second (s) initial denaturation step at 94°C, 35 cycles of 98°C for 10s, 60°C for 15s and 68°C for 12s, followed by extension at 60°C for 420s. Amplified products were isolated in a 2% AgaroseS (Wako, Japan) gels, and bands were visualized using GelGreen (BioTium, USA). Samples that produced a visible band were scored as positive for parasite infection.

To assess the specificity of our primers for rhizocephalan genus of interest (*Polyascus*), we sequenced a subset of 32 of our PCR-positive samples (from the experiment described here and a separate experiment that was run concurrently using the same PCR detection method). Specifically we used the 18Ssac3F primer for direct sequencing with BigDye Terminator version 3.1 (Applied Biosystems, USA) and an ABI 3130-Avant automated sequencer. Sequence results for these samples showed 100% similarity after exclusion of indels with the congeneric rhizocephalans *P. polygenea* (AY265363) and *P. gregaria* (AY265363) using a BLAST search on Genbank, confirming the amplification of target parasite DNA.

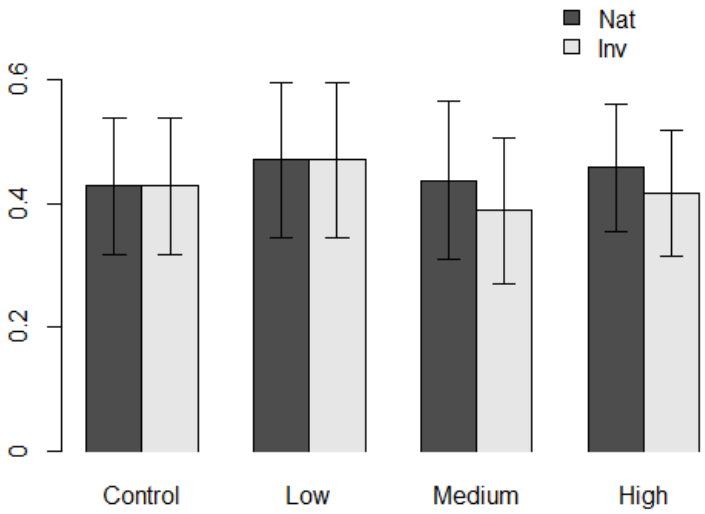
Reducing False Positives

Relying on PCR by itself has the potential to increase the rate of false positives if infecting rhizocephalan larval stages (i.e., vermigon) are successfully sequestered by the host encapsulation response, but persisting parasite DNA gives a positive signal in the PCR assay. To reduce the possibility of this type of false positive, we checked each sample for darkened capsules in the host tissue that could have represented encapsulated parasites, and excluded any capsules from the tissue samples used to assess infection status. Though it has not been previously studied in rhizocephalan infections of *H. sanguineus*, a study by Kuris *et al*. (2007) showed that successful host immune responses against rhizocephalan infection in 3 decapod hosts presented as darkened (via the host's melanization response) capsules embedded in the host tissue (Kuris *et al.* 2007). To explore whether exclusion of potential brown bodies from our tissue samples was effective in excluding DNA from non-viable parasites which *H. sanguineus* had successfully encapsulated, we separated one such capsule or "brown body" found in the midgut tissue of an experimentally infected invasive-range crab from the surrounding host tissue, and extracted DNA separately from the capsule and the surrounding tissue. Our PCR assay revealed a positive signal for the capsule but not for the host tissue in which it had been found embedded. The lack of amplification in the host tissue around the successfully encapsulated parasite supports that the parasite DNA that amplified in our samples should represent live/successful infections that would likely have established to maturity if allowed to progress.

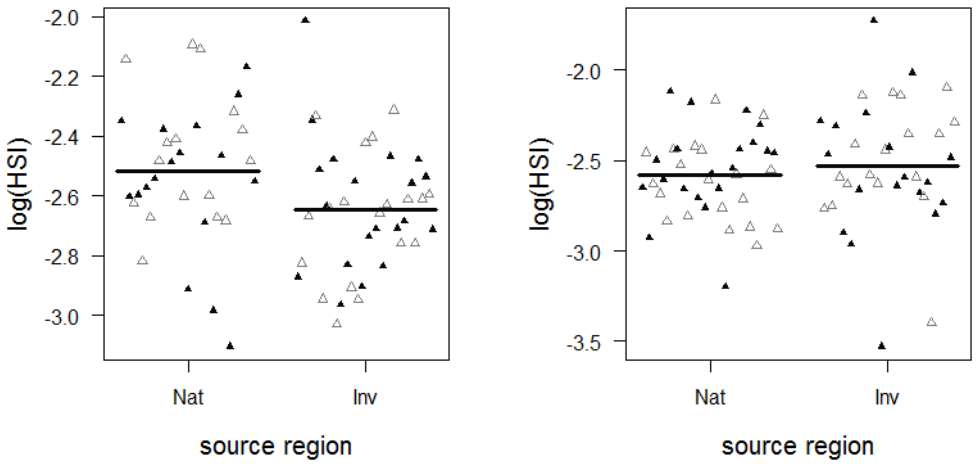
Supplemental Table 3.1 Detailed site locations and site-level sample sizes and prevalence from common-garden experiment

Site ID – City, Prerecture or State (Lat/Lon)	External infection Prev. (N)	Initial PCR Prev. (N)	Exper. N	N Infected	Exper. Prev.	ΔPrev
N1 - Himi, Toyama (36°89'1031" N, 137°00'3503" E)	0.14 (97)	0.47 (30)	24	10	0.42	-0.05
N2 - Hayama, Kanagawa (35°25'9511" N, 139°57'7050" E)	0 (99)	0.20 (20)	4	1	0.25	0.05
N3 - Amakusa, Kumamoto (32°55'0075" N, 130°41'7070" E)	0.10 (211)	0.45 (22)	24	15	0.63	0.18
I1 – Nahant, Massachusettes (42°42'0856" N, -70°91'7183" W)	0 (75)	NA	29	18	0.62	0.62
I2 – Weekapaug, Rhode Island (41°32'6450" N, -71°75'4096"W)	0 (44)	NA	10	7	0.70	0.70
I3/ - Tappen Beach, New York (40°50'2132" N, -73°39'1136" W)	0 (75)	NA	27	26	0.96	0.96
Rhizocephalan source for experiment						
Mitoyo, Kagawa (34°24'3256"N, 133°67'4286"E)	0.54 (185)	NA	NA	NA	NA	NA

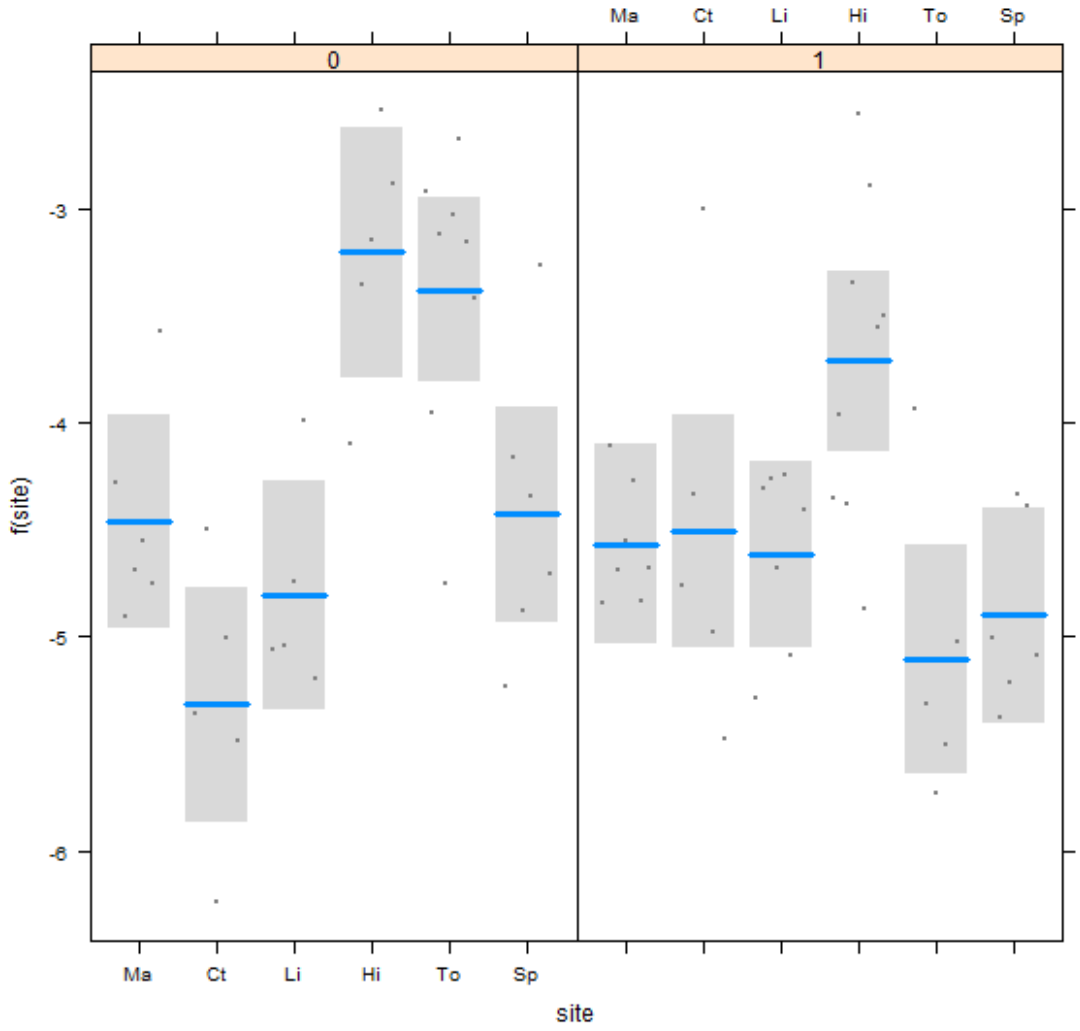
APPENDIX C – CHAPTER 4



Supplemental Figure 4.1 Proportion of individuals with PCR-detected rhizocephalan infections at the end of the exposure and incubation periods, by source region and controlled cyprid exposure level. Error bars represent standard error of the proportion.



Supplemental Figure 4.2 Output from LMER models of HSI by source region for (A) males or (B) females, accounting for size and infection status.



Supplemental Figure 4.3 Female GSI as a function of individual collection site and infection status (1=infected), with size accounted for as a covariate. Sites are arranged from north to south in the invasive range (Ma, Ct, Li) followed by the native range (Hi, To, Sp), corresponding to I1-2 and N1-3 on the map in Figure 1.

Supplemental Table 4.1 Overview of statistical models organized by response variable/overarching question. Models of infection status are generalized linear mixed models with a binomial distribution, and all other models are linear mixed effects models. We used conditional AIC to choose between full and reduced models. If the original full models was preferred, NA is listed in the column for the reduced model. All models include exposure block and collection site within source region as random effects unless otherwise noted. Asterisks indicate interaction effects between variables. Bolded variables were significant ($p \leq 0.05$).

Dependent variable	**Full model**	**Reduced model**
Does experimental cyprid exposure induce infection, immune traits, or ΔSMR?		
1. Infection	~ region*treat(level) + region + treat(level) [1]	region + treat(level)
2. Δhem	~ region*treat + region + treat	region + treat
3. Encaps	~ region*treat(level) + region + treat(level)	region + **treat(**level**)**
4. ΔSMR	~ region*treat(level) + region + treat(level) + size + sex	region + **treat(**level**)**
Is infection status predicted by immune traits, and do these relationship depend on source region?		
5. Infection	~ hemc*region + hemc + region	NA
6. Infection	~ Δhem*region + **Δhem*treat** + Δhem + region + treat	NA
7. Infection	~ encaps*region + encaps*treat + encaps + region + treat	**Encaps** + region
Does SMRc predict (response), and does the relationship depend on source region? [2]		
8. Hem1	~ SMRc*region + SMRc + region + size + sex	**SMRc*region** + SMRc + **region** + size + sex
9. Δhem	~ SMRc*region+ SMRc + region + size + sex + infect + treat	**SMRc*region** + **SMRc** + **region** + size + sex + treat.

10. Encaps	~ SMRc*region + SMRc + region + size + sex + infect + treat	**SMRc** + **region** + **size** + sex + **treat**
Is HSI predicted by immune traits, and do these relationships depend on source region or infection?		
11. HSI	~ hemc*region*infect + hemc*region + hemc*infect + region*infect + hemc + region + infect + size	**hemc*region** + hemc + **region** + infect. + **size**
12. HSI	~ Δhem *region*infect +Δ hem*region + Δhem*infect + region*infect + Δhem + region + infect + size + treat	Δhem*region + Δ**hem*infect** + Δhem + region + infect **+ size** + treat
13. HSI	~ encaps*region*infect + encaps*treat + size	**encaps*region** + encaps*treat + encaps + region + treat + infect + **size**
Is GSI predicted by immune traits, and does this depend on source region or infection status?		
14. Fem. GSI	~ hemc*region + hemc + region + infect + size	**hemc*region** +hemc + region + infect + **size**
15. Fem. GSI	~ Δhem*infect + Δhem*region + Δhem + region + infect + size	Δ**hem*infect** + Δ**hem** + infect **+ region** + **size**
16. Fem. GSI	~ encaps*region + encaps*infect + encaps + region + infect + size	**encaps*region** + encaps + region + infect + **size**
17. Male GSI	~ hemc*region + hemc + region + infect + size	**hemc**+ region + **infect** + **size**
18. Male GSI	~ Δhem*region + Δhem + region + infect + size	Δ**hem** + region + **infect** + **size**
19. Male GSI	~ encaps*region + encaps*infect + encaps + region + infect + size	encaps + region + infect + **size**

Variable abbreviations: treat = experimental treatment (exposed vs. not exposed, unless otherwise noted); region = source region (invasive vs. native); Δhem = proportional change in circulating hemocyte density after cyprid exposure; encaps = encapsulation strength; ΔSMR = proportional change in SMP after cyprid exposure; SMRc = constitutive or baseline SMR measured prior to cyprid exposure

[1]for models with treat(level), the categorical variable "treatment" contained four levels (control, low, medium high) of cyprid doses. In all other instances of "treat", the variable is collapsed into a binary predictor (exposed vs. not-exposed).
[2]In section 2 models where SMRc is a predictor, exposure block is omitted as a random effect because only 2 blocks were included in the experiment