Disturbance in the anchialine ecosystem: ramifications for ecology and physiology

by

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Abstract

Habitats in the anchialine ecosystem are defined as coastal ponds, pools, and caves that lack surface connections to the open ocean, but possess both seawater and freshwater influences due to subterranean connections to the ocean and groundwater. Such habitats are rare worldwide, but are concentrated in the Hawaiian Islands. Organisms that live in these habitats must cope with changing salinities, variable oxygen regimes, high levels of UV radiation, and anthropogenic effects such as pollution and invasive species. Accordingly, such organisms represent an opportunity to shed light on environmental physiology and invasive species biology. However, few studies have investigated physiology or response to invasive species in anchialine organisms. Accordingly, the objective of this dissertation is to examine the effect of natural and anthropogenic disturbances on the physiology and ecology of anchialine organisms. Chapter 1 provides an introduction to the anchialine ecosystem and outlines the specific aims of the dissertation. Chapter 2 presents a series of field and laboratory based experiments investigating how endemic Hawaiian anchialine organisms have responded to invasive fishes. Based on its results, endemic anchialine organisms largely avoid predation by invasive fishes by adopting an alternative strategy of diel migration in fish-invaded habitats. Chapter 3 provides a quantitative, statistical review of how previously-studied animals respond to changing salinity via altering gene expression. The results of this meta-analysis suggest that up-regulation of a suite of genes is typical for crustaceans undergoing salinity transfers, although studies have mostly been confined to a narrow taxonomic range of decapod brachyurans (i.e., crabs). Chapter 4 seeks to remedy this

lack of knowledge by examining how the endemic Hawaiian anchialine atyid shrimp *Halocaridina rubra* responds to fluctuating salinity at the organismal, tissue, cellular, and molecular level. These results suggest *H. rubra* (and possibly other anchialine crustaceans) has continually expressed osmoregulatory mechanisms, including high, constitutive levels of osmoregulatory gene expression, which is in stark contrast to previously studied crustaceans. Chapter 5 examines how *H. rubra* and anchialine shrimps from the Ryukyus Archipeligo of Japan respond to low-oxygen conditions. By examining metabolic characteristics under varying oxygen regimes, it is concluded that *H. rubra* has gills that are specialized for osmoregulation, but not respiration, requiring a high resting ventilation rate and resulting in a strategy of oxyconformation. The other species have a different strategy more consistent with previously described mechanisms in crustaceans. Lastly, Chapter 6 provides conclusions, synthesis of the preceding chapters, and future directions.

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List of Abbreviations

AK: arginine kinase

AMA: Ama-ga

ANOVA: analysis of variance

ATP: adenosine triphosphate

BB: Baby Bear

BH: Blue Hole

BI: Bayesian inference

BLAST: basic local alignment search tool

BUT: Butori-ga/Yamato-ga

CA(c/g): carbonic anhydrase (c or g isoform)

Chl *a*: chlorophyll *a*

CEs: consumptive effects

C_t: threshold cycle

DASPMI: 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium iodide

DOC: dissolved organic carbon

EP: Eric's Pond

EST: expressed sequence tag

FUS: Fushyato-ga

FUT: Futatsu-ga

FW: freshwater

HAT: V-type H^+-ATPase

HM: Cape Hanamanioa

IAS: invasive alien species

ICP-OES: inductively coupled plasma optical emission spectrometry

IH: Issac Hale

JP: Joe’s Pond

KAHO: Kaloko-Honokōhau National Historical Park

KBP: Kalaeloa Unit

KIKI: Keawaiki Bay

KONA: Kona Coast

LDH: lactate dehydrogenase

ln *RR*: natural log of the response ratio

MAKA: Makalawena

MIKE: Mike’s Pond

ML: maximum likelihood

MRCs: mitochondria-rich cells

mRNA: messenger RNA

NCEs: non-consumptive effects

NKA: Na^+/K^+-ATPase

NKCC: $Na^+/K^+/2Cl^-$ cotransporter

OTU: operational taxonomic unit

OWAI: Waianae Boat Harbor

PB: Papa Bear

PCA: principle components analysis

PCR: polymerase chain reaction

PG: posterior gills

P_{O_2}: partial pressure of O_2

PUHO: Pu'uhonua o Hōnaunau National Historical Park

qRT-PCR: quantitative real-time polymerase chain reaction

RES1: Restoration 1

RNA-Seq: high throughput sequencing of RNA or cDNA libraries

SHU: Shuga-ga

SKIP: Skippy's Pond

SW: seawater

TAP: Tap's Pond

TDN: total dissolved nitrogen

V_{O_2}: oxygen uptake rate

WC: Waianapanapa Cave

Chapter 1. Introduction to dissertation

1.1 Introduction to the anchialine ecosystem

1.1.1 Environmental characteristics of anchialine habitats

The anchialine ecosystem is composed of landlocked coastal ponds, pools, and caves influenced by both the underlying freshwater aquifer and the ocean through subterranean connections (Holthuis 1973). Thus, anchialine habitats have tidal influences, mixohaline salinities (e.g., 0.5 to 30‰), and rapidly changing physical parameters (Iliffe 2000; Sket 1996). Although they have a worldwide distribution, habitats fitting this ecosystem definition are considered relatively rare. They are most concentrated in the Hawaiian Archipelago, (with ~600 ponds found across Oahu, Maui and Hawaii; Brock 1987; Brock et al. 1987; Carey et al. 2011), and are absent from the rest of the U.S. Across the Hawaiian Islands, anchialine habitats host a diverse and often endemic biota including microbes and invertebrates (Maciolek and Brock 1974).

While the Hawaiian anchialine ecosystem is a model for studies in the environmental sciences and has been recognized as such by the EPA[1], few studies have investigated it in depth. This is unfortunate because it is ideally suited to test and develop approaches to environmental remediation and conservation biology due to the many individual ponds in varying stages of human-mediated disturbance and decay. Furthermore, their extreme environmental conditions make endemic anchialine organisms well suited for studying the effects of environmental stressors on physiology. Anchialine organisms are also suitable for studying genetic isolation and speciation in

[1] http://www.oceanarks.org/News/2005/5/hawaii/FourSeasons.pdf

island habitats. Lastly, invasive fishes in anchialine habitats provide an opportunity to study ecological ramifications of introduced species on several ecological scales.

Hawaiian anchialine habitats are home to a diverse assemblage of arthropods, mollusks, sponges, cnidarians, and worms (Maciolek and Brock 1974). Of these, the most widespread and abundant macrofauna is the endemic atyid shrimp *Halocaridina rubra* (Bailey-Brock and Brock 1993). Known locally as *'öpae-'ula* (Fig. 1A), *H. rubra* is the primary grazer on the microbial communities of the ponds and is considered a keystone species and an indicator of ecosystem health (Bailey-Brock and Brock 1993; Brock and Kam 1997). Recent efforts from our laboratory have examined the population genetics (Craft et al. 2008; Santos 2006), molecular evolutionary rate (Santos and Weese 2011), mitochondrial genomics (Ivey and Santos 2007), and conservation biology (Weese and Santos 2009) of *H. rubra*. Based on these studies, at least eight genetically distinct *H. rubra* lineages are found across the Hawaiian Islands, each of which qualifies as a potential species (Fig. 1). In conjunction with a recent NSF-DEB award (#0949855), we are now beginning to characterize microbial communities associated with Hawaii's anchialine habitats. Of particular interest is an orange-green cyanobacterial crust found exclusively in ponds on Maui and the Kona coast of the Big Island (Fig. 1B). Studying this unique microbial consortium is important because it influences ecosystem processes (e.g., nitrogen fixation via cyanobacteria) and constitutes the base of the food web in these anchialine habitats. Additionally, studying the ecology and evolution of these microbial communities may demonstrate convergence towards a set of community members that work synergistically to thrive under the harsh environmental conditions of the anchialine ecosystem.

For this dissertation, these previous findings were built upon using novel approaches and tools that were developed in this research towards quantifying and understanding human-driven environmental degradation of the Hawaiian anchialine ecosystem in an ecophysiological context.

1.1.2 Invasive species in the Hawaiian anchialine ecosystem

Island ecosystems are particularly susceptible to the deleterious consequences associated with invasive species. This is largely due to the typically higher levels of endemism, and geographic isolation, characteristic of island ecosystems (Reaser et al. 2007). Generally, ecosystems on tropical islands are considered more susceptible to continental invaders than those on European and other northern islands, with the negative effects of invasion increasing with distance from continents (Brockie et al. 1988). In this context, ecosystems of the Hawaiian Archipelago have been severely impacted by human-mediated alien species introductions since its initial colonization some 1,500 years ago, and particularly with Western contact dating to 1778. As a result, approximately half of the total current species across Hawaiʻi are considered non-native (Peacock et al. 2009; Wagner et al. 1999), including a number of alien fish species that have been introduced into naturally fishless water bodies in the islands for activities such as aquaculture, recreational angling, and biological control. For perspective: only five freshwater fishes are native to the Hawaiian Islands (four are endemic) while at least 44 species of introduced freshwater fishes have become established in the past ~200 years (Devick 1991). This is of concern because such events can have drastic impacts on endemic biodiversity (e.g., Schabetsberger et al. 2009).

While invasive freshwater fishes are conspicuous in the majority of Hawaiian lowland streams (Englund 1999), they have also invaded the islands' anchialine ecosystem. Although habitat destruction and pollution are major threats to Hawaiian anchialine habitats, invasive fishes have also quickly and drastically altered this ecosystem and their endemic species. Exotics such as species of *Gambusia*, *Tilapia*, and *Poecilia* have been introduced to many Pacific islands, including Hawaii, for aquaculture or mosquito control (Seale 1905; Seale 1917; Stearns 1983). For *G. affinis*, its perceived ability to eradicate mosquitoes has made it one of the most common introduced fishes worldwide and the species was identified by the International Union for Conservation of Nature (IUCN) as one of the top 100 "worst" invasive species (Lowe et al. 2000). These species have in turn invaded anchialine habitats, which are naturally fishless (Brock and Kam 1997; Maciolek and Brock 1974), and are now found in high abundances in anchialine habitats on Maui and the Big Island (Capps et al. 2009; Carey et al. 2011). Unfortunately, fish-invaded anchialine habitats are becoming more common across the islands (Brock 1977; Capps et al. 2009; Carey et al. 2011). For example, only ~10% of 318 anchialine habitats surveyed along the western coast of the island of Hawai'i harbored invasive fishes in 1974 (Maciolek and Brock 1974); it is now estimated that >90% of these same habitats have been invaded (Brock 1987; pers. obs). Fishes continue to spread across Hawaii's anchialine habitats, as evidenced by observations of the same habitats over the last five years (S.R. Santos, Auburn University, pers. obs.).

As mentioned previously, introduced fishes dramatically change Hawaii's anchialine habitats, leading to an alternate ecological state. Specifically, while *H. rubra* and a diverse assemblage of microbiota are typically characteristic of fishless habitats,

ones with introduced fishes generally lack *H. rubra* and are dominated by filamentous algae (mainly *Cladophora* sp.; Brock and Kam 1997). This situation leads to smothering of the native and diverse bacterial/algal crust (Brock and Kam 1997), presumably due to reduced grazing by *H. rubra* in the presence of fishes. Laboratory experiments confirm that *H. rubra* alters its behavior and seeks refuge when housed with the introduced western mosquitofish (*Gambusia affinis*) (Capps et al. 2009). In fish-invaded ponds, *H. rubra* also undergo diel migration, in which they seek refuge in the underground hypogeal zone of the ponds during the day and migrate to the surface epigeal zone during the night to feed (Capps et al. 2009; Carey et al. 2011; Sakihara et al. 2011). Furthermore, fish-invaded ponds also have larger *H. rubra*, suggesting a size selective shift (Carey et al. 2011). Together, these results suggest that invasive fishes drive ecosystem shifts in Hawaii's anchialine habitats via behavioral changes in their keystone species, *H. rubra*. Ultimately, these shifts produce functionally different habitats, with altered microbial diversity and altered ecosystem functions (e.g., lower nitrogen fixation) (Brock and Kam 1997). Similar types of ecological shifts in response to fish introductions have been previously documented in alpine lakes (Knapp et al. 2001; Schindler et al. 2001).

1.1.3 Physiology of Hawaiian anchialine organisms

Hawaiian anchialine habitats undergo changes in water chemistry and physical parameters that can occur slowly and predictably or suddenly and unexpectedly. For example, daily tidal cycles can cause changes in salinity and size of a habitat (Iliffe 2000; Sket 1996). Some habitats can be upwards of 100 m^2 during high tide, but can become completely dry on extreme low tides (pers. obs.). Additionally, unexpected heavy rainfall

can alter water chemistry and physical properties of anchialine habitats. Some anchialine habitats may also be relatively ephemeral, with extreme disturbances such as tsunamis leading to sedimentation and senescence (pers. obs.). Hawaiian anchialine habitats not only experience temporal changes in environmental conditions, but also have spatial changes within a single habitat at any given time. Because anchialine habitats consist of mixing seawater and fresh water, there may be strong haloclines, as documented in other anchialine habitats around the world (Sket 1996; Iliffe 2000). Thermoclines likely also exist in many habitats, and as the surface waters recede into the underground aquifer, temperatures may decrease dramatically. Similarly, variation between even closely spaced habitats is high, with salinities ranging from < 3–30‰ and nutrient concentrations varying over 100 fold between habitats. Because of these fluctuations, anchialine habitats can be considered "extreme" environments and organisms that inhabit them should show a corresponding level of high physiological tolerance.

Unfortunately, very little research has focused on physiology in Hawaiian anchialine organisms, or anchialine organisms in general, possibly due to the rarity of anchialine habitats. Descriptive physiological tolerances reported for *H. rubra* by Holthuis (1973) and Maciolek (1983) report physiological tolerance of a wide range of salinities, which corresponds to the wide environmental salinity *H. rubra* occupies (2–36‰) (Maciolek 1983). Shrimp were reported to be easily acclimated to tap water (~0‰) and could be acclimated to ~50‰ if done so slowly (Holthuis 1973; Maciolek 1983). While other anchialine shrimps tolerate a narrower range of salinities, *Halocaridinides trigonophthalma* and *Metabetaeus lohena* were reported from a similar range of salinities as *H. rubra* (Maciolek 1983). Temperature tolerances were also recorded for anchialine

shrimps, with most being found at 22–40 °C. Laboratory studies of *H. rubra* confirm this, with shrimp being able to tolerate a maximum temperature of ~33 °C (unpublished data), although anecdotal evidence suggests a much lower thermal minimum than 22 °C. Some anchialine organisms are also able to withstand long periods of food depravation and anoxia. For example, Maciolek (1983) reported that a single *H. rubra* in a sealed polyethylene bag with only ~100 mL of water survived for > 1 year. These shrimp were presumably grazing on the microbiota growing inside the bag. Similarly, animals kept in a sealed jar for ~10 years were able to persist and even reproduce without any supplementary feeding or water changes (S. R. Santos, pers. obs.). The ability of *H. rubra* to be maintained with minimal effort has led to their popularity in the aquarium trade and they are often sold in self-sustaining "ecospheres" (Weese and Santos 2009). Despite these rudimentary descriptions of the extreme physiological tolerances of anchialine organisms, no work has examined how anchialine shrimps like *H. rubra* are able to survive these conditions or subjected them to laboratory experiments to further characterize their physiology.

Although no physiological experiments have been performed on Hawaiian anchialine organisms, there are many studies investigating the physiology of cave-adapted crustaceans, which may have a similar physiology to anchialine crustaceans, given that anchialine habitats have an extensive underground hypogeal component. Aquatic subterranean crustaceans often have reduced metabolic rates compared to similar epigean species (Dickson and Franz 1980; Gillieson 1996; Hűppop 1986; Korzhuev 1950; Mejía-Ortíz and López-Mejía 2005), which is thought to be an adaptation to both lower levels of oxygen and limited food and nutrient inputs in caves. However,

exceptions are common and many cave-like habitats (including Hawaiian lava tube caves) with higher nutrient inputs harbor crustaceans with metabolic rates similar to epigean crustaceans (Ahearn and Howarth 1982; Hervant et al. 1997). Cave crustaceans generally also have a low thermal tolerance and exhibit stenothermality, with organismal performance optimized across a narrow range of temperatures (Issartel et al. 2005). However, there are exceptions to this rule as well (Issartel et al. 2005), with some subterranean crustaceans being classified as eurythermal. Cave crustaceans are also adapted to surviving long periods without feeding (Hervant et al. 1997). Some of the physiological characteristics of cave crustaceans synch well with the available physiological reports of Hawaiian anchialine crustaceans (e.g., low thermal tolerance and long-term starvation capacity). However, it is likely that anchialine crustaceans experience a wider range of environmental parameters than those found in typical freshwater caves due to the surface component of anchialine habitats, and may therefore be expected to be more physiologically tolerant.

1.2 Objectives

For my dissertation research, **the interplay between ecology, genetics, and physiology of Hawaiian anchialine organisms was investigated.** Specifically, the goal of this multifaceted project was to elucidate how environmental disturbances influence the ecology and physiology of endemic anchialine organisms. This work employed a combination of molecular techniques and field studies towards addressing three main goals:

1) **Quantify changes in ecology of *H. rubra* related to invasive fishes as a means of developing biologically informative**
2) **Characterize osmoregulation in *H. rubra* towards understanding how *H. rubra* copes with the changing salinities of the anchialine ecosystem**
3) **Determine metabolic responses of anchialine shrimp species to low-conditions and salinity transfer**

It was hypothesized that: 1) invasive fishes do not consume *H. rubra* due to their adopted behavior of diel migration in fish-invaded anchialine habitats; 2) osmoreguation in *H. rubra* is accomplished through extreme up-regulation of osmoregulatory genes during salinity transfer that have been previously characterized in other crsutaceans; 3) anchialine shrimps are able to tolerate extreme hypoxia/anoxia through increased relicance on anaerobic metabolic pathways. Knowledge gained during this dissertation work will help direct conservation efforts.

1.3 Importance and relevance of dissertation research

Despite a recent surge in research related to Hawaii's anchialine ecosystem (Capps et al. 2009; Carey et al. 2011; Craft et al. 2008; Ivey and Santos 2007; Santos 2006; Russ et al. 2010; Weese and Santos 2009), little is still known regarding interactions between anchialine flora and fauna or how environmental perturbation and/or degradation influences species interactions. This is unfortunate because this ecosystem is among the most threatened in Hawaii (Maciolek 1986; Santos 2006) due to habitat destruction via coastal development, degradation from non-point source pollution, and alterations by introduced species. It is also home to several candidate-endangered species,

including *Metabetaeus lohena*, a small red shrimp similar to *H. rubra*. The popular media have also profiled the threatened nature of the Hawaiian anchialine ecosystem[2,3] while the creation of the Waikoloa Anchialine Pond Preservation Area was a response to the destruction of ~130 natural anchialine ponds during commercial development (representing~20% of what was thought to occur across all the islands; Brock et al. 1987). Unfortunately, this trend is ongoing, as ~20 ponds were recently lost during the construction of a new boat harbor at Ewa Beach, Oahu (R.A. Kinzie III, University of Hawaii, pers. comm.). In this context, State and Federal agencies are attempting to protect remaining habitats by establishing natural area reserves and artificial ponds as mitigation. However, baseline data to aid these efforts are sorely lacking and critically needed because such knowledge is paramount towards successful conservation and future remediation of this ecosystem. Knowledge gained during this research will not only provide these data, but can be applied to other ecosystems under similar threats.

Anchialine habitats are naturally prone to water-borne pollution due to their connection to lowland aquifers, which contain groundwater accumulations from higher elevations. Given this, groundwater pollutants can potentially be concentrated in anchialine habitats. For example, data collected by our laboratory identified high phosphate levels in anchialine ponds near golf courses, supporting this hypothesis (Fig. 2A). Because anchialine habitats can represent a source of fresh water in otherwise barren areas, they have historically been used for drinking and aquaculture purposes (Santos 2006). This (and other uses) has made anchialine ponds and *H. rubra* culturally important to the native Hawaiian people (Capps et al. 2009; Santos 2006; Weese and Santos 2009).

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[2] http://www.mauinews.com/page/content.detail/id/502901.html
[3] http://www.khnl.com/Global/story.asp?S=6104447

Unfortunately, anchialine habitats and their fauna have been exploited more recently as trash pits and pumping sources (Fig. 2B) or, in the case of *Halocaridina rubra*, as curiosities in the aquarium trade due to low maintenance requirements (Maciolek 1983; Weese and Santos 2009). In the latter case, this has increased poaching pressure on wild *H. rubra* populations (Weese and Santos 2009).

Because anchialine habitats in Hawaii are unique to the U.S. and unusual throughout the world, it is imperative to protect and study them. This dissertation research has contributed to this area while providing insight into how future conservation and remediation efforts might best be achieved. For example, quantifying physiological parameters of different anchialine species in the laboratory under different conditions can allow natural populations to be characterized as stressed or non-stressed (Downs et al. 2001). This could identify particularly harmful stressors as well as allow specific habitats to be targeted for remediation. Similarly, studying how introduced fishes affect the anchialine ecosystem will allow for biologically informed remediation programs and may prevent further invasions. In addition to providing a framework for remediation and environmental protection, this research addresses a lack of knowledge on a unique ecosystem, as this represents the first focused effort to examine physiology of anchialine organisms and anchialine species interactions at the ecosystem scale.

Along with the above, anchialine habitats also represent an opportunity to assess larger scale aquatic ecosystem health because they can serve as a “window” to the underlying groundwater aquifer. Contaminants found in ponds will also certainly be present in the underlying freshwater aquifer and could represent a threat to other terrestrial and aquatic ecosystems, drinking water supplies, or nearshore ocean

communities. Therefore, studying endemic anchialine organisms may provide a way to indirectly assess the health of other ecosystems.

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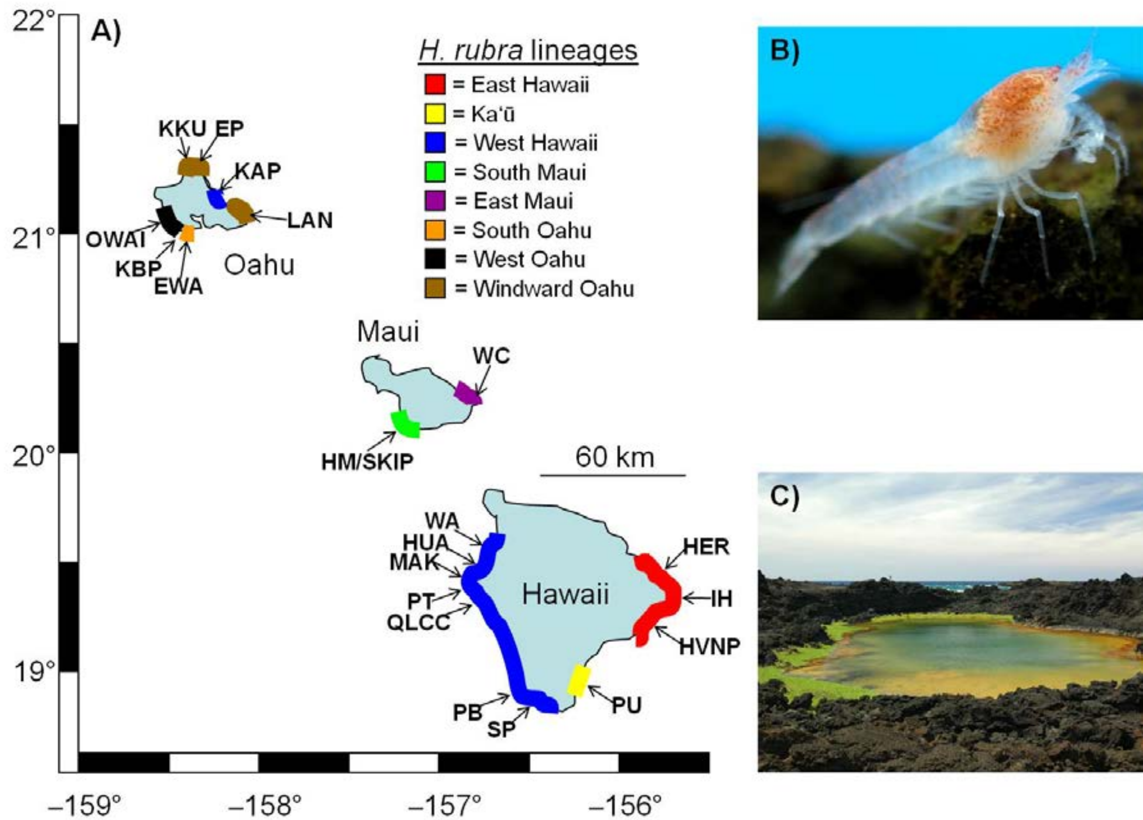

Fig. 1. A) Map of Hawaiian Islands with anchialine habitats. Lineages of Halocaridina rubra are shown according to Craft et al. (2008). Specific anchialine habitat sites are indicated with arrows and abbreviations follow Craft et al. (2008). **B)** *Halocaridina rubra* **C)** Maui anchialine habitat (SKIP) showing orange microbial crust typical of some habitats on Hawai‘i and Maui.

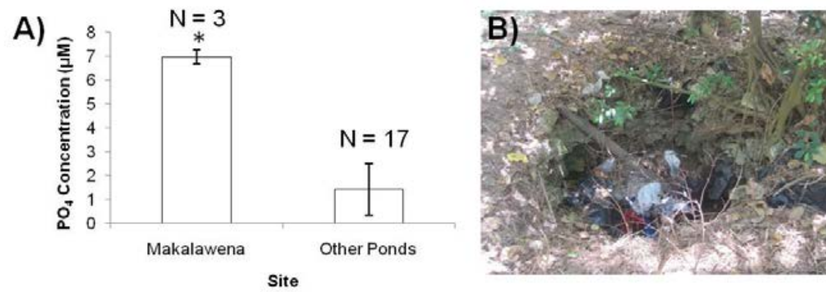

Fig. 2. A) Phosphate levels in anchialine ponds near a golf course at Makalawena Beach (MAK in Fig.1) are significantly higher than those in other anchialine ponds (*t-test*, $P < 0.001$; error bars show standard deviation). **B)** An anchialine pond on Oahu that has been used as a dumping site.

Chapter 2. Invasive fishes in the Hawaiian anchialine ecosystem: investigating potential predator avoidance by endemic organisms

2.1 Abstract

Globally, introductions of alien species are increasingly common, with invasive predators potentially having detrimental effects via predation on native species. However, native prey may avoid predation by adopting new behaviors. To determine whether invasive fish populations consume endemic shrimp in invaded Hawaiian anchialine habitats or if adopted patterns of diel migration prevents predation as previously hypothesized, a total of 183 invasive poeciliids (158 *Gambusia affinis* and 25 *Poecilia reticulata*) were collected for gut content analyses from four anchialine sites during wet and dry seasons on the islands of Hawaiʻi and Maui. Predation on shrimp was not detected in habitats where they retreat exclusively into the underlying aquifer diurnally and only emerge nocturnally. However, low levels of predation were detected (7/65 fishes, only by *Gambusia affinis*) at Waianapanapa Cave, Maui, where shrimp retreat into both the aquifer and a cave during the day. Thus, adopted behavioral responses to invasive fishes generally, though not universally, prevent predation on endemic Hawaiian anchialine shrimps. However, non-consumptive effects resulting from behavioral modification of shrimps may have appreciable impacts on the Hawaiian anchialine ecosystem and warrant further study.

2.2 Introduction

Invasive alien species (IAS) are second only to habitat destruction as causes for species endangerment and extinction (Pejchar & Mooney 2009). Furthermore, IAS may negatively impact ecosystems in numerous ways, including competing with native species (Davis 2003; Beard et al. 2009; Peñuelas et al. 2010), introducing novel diseases (Woodworth et al. 2005), altering physical habitat (Stone & Loope 1987), diminishing the economic, cultural, and environmental services provided by a given ecosystem (Pejchar & Mooney 2009), and through predation/consumptive effects (CEs) (Fritts & Rodda 1988; Mack et al. 2000; Salo et al. 2007; Vitule et al. 2009). Likewise, invasive predators can also have non-consumptive effects (NCEs) on native prey via behavioral modification in response to the threat of predation (Werner & Hall 1988; Brown et al. 1999; Werner & Peacor 2003; Bourdeau et al. 2011). In aquatic ecosystems, for example, prey often respond to invasive predators by altering vertical migration patterns (Loose & Dawidowicz 1994; Cousyn et al. 2001; Bourdeau et al. 2011; Titelman et al. 2012) or making use of habitat refugia (Werner & Hall 1988; Light 2005). In cases where such behavioral changes do not take place, predation may be heavy due to prey naiveté (Cox & Lima 2006; Kovacs et al. 2012). Moreover, native prey can respond differently to invasive predators due to variation in abiotic conditions across habitats (Baltz & Moyle 1993; Moyle & Light 1996), potentially being consumed in some habitats while avoiding predation in others.

Island ecosystems are particularly susceptible to IAS (Reaser et al. 2007) and thus provide opportunities to study the CEs and NCEs of invasive predators. The Hawaiian Islands exemplify this trend, with approximately half of the total species now found

across the islands considered to be non-native (Wagner et al. 1999; Peacock et al. 2009). In this context, non-native freshwater fishes have invaded the majority of Hawaiian lowland aquatic ecosystems (MacKenzie & Bruland 2012), including the islands' anchialine ecosystem. Habitats belonging to the anchialine ecosystem are defined as coastal ponds, pools, or caves lacking surface connections to the open ocean, but are influenced by it and the underlying freshwater aquifer via subsurface connections (Holthuis 1973; Brock 1977; Sket 1996). Hawaiian anchialine habitats host a diverse, often endemic, biota including microbes and invertebrates (Maciolek & Brock 1974; Brock et al. 1987; De Grave & Sakihara 2011; Ng 2011), with the small (~10 mm length) endemic shrimp *Halocaridina rubra* Holthuis, 1963 (Decapoda, Atyidae) being the most conspicuous and widespread macro-organism (Bailey-Brock & Brock 1993). Unfortunately, fish-invaded anchialine habitats are becoming more common across the islands (Brock 1977; Capps et al. 2009; Carey et al. 2011). For example, only four out of 89 habitats (4.5%) surveyed along the Kona coast of the island of Hawaiʻi harbored invasive poeciliids (*Gambusia affinis* and *Poecilia reticulata*) in 1974 (Maciolek & Brock 1974); by 2010, nearly all of these habitats were fish-invaded (S. R. Santos, pers. obs.). Fish-invaded anchialine habitats typically lack *H. rubra*, possibly due to predation (Bailey-Brock & Brock 1993). However, surveys of such habitats have documented the presence of *H. rubra*, albeit exhibiting diel migration by retreating into the hypogeal (i.e., underground) component of the habitat during the day and only emerging to the epigeal (i.e., surface) at night, contrary to fishless habitats where shrimp were abundant in the epigeal portion both day and night (Capps et al. 2009; Sakihara 2012).

The finding that invasive fishes induce diel migration in shrimp like *H. rubra* suggests native prey may be effectively evading predation. However, no study has verified this or quantified predation by invasive fishes on endemic anchialine organisms such as *H. rubra*. Furthermore, questions regarding the frequency by which invasions drive ecosystem change through processes such as CEs and NCEs are among those central to invasive species biology (reviewed by Strayer 2012). Here, the potential of invasive fishes to act as predators on endemic anchialine shrimp was assessed. Because fishes often undergo behavioral sleep and do not feed at night (Reebs 2002), it was hypothesized that predation would be minimal or absent in habitats where *H. rubra* exhibits diel migration.

2.3 Materials and methods

2.3.1 Study sites

Invasive fishes were collected for gut content analyses from four fish-invaded anchialine habitats on two islands: two sites at Makalawena Beach (MAKA1 and MAKA2) on Hawai‘i, Pu'uhonua O Hōnaunau National Historical Park (PUHO3A; based on the U.S. National Park Service naming system) on Hawai‘i, and Waianapanapa Cave (WC) on Maui. These habitats were chosen because they have varying physical, chemical, and biological characteristics (Fig. 1; Tables 1 & 2) and prior to being fish-invaded, shrimp were present diurnally in the last 8-20 years at three of them (MAKA1, MAKA2, WC; Table 1). Notably, PUHO3A was historically used for fish aquaculture and shrimp have not been observed diurnally at this site, although they can be found in adjacent anchialine habitats. In this case, PUHO3A was included in the current study

since nocturnal surveys confirming shrimp absence have not been previously conducted. Vegetation surrounding MAKA1 and MAKA2 is scarce and consists mainly of kiawe (*Prosopis pallida*) and naupaka kahakai (*Scaevola taccada*) while PUHO3A is bordered by palms, grasses, and shrubs (Fig. 1). A rainforest canopy encloses WC and leaf litter from ferns, trees, and various other plants is abundant (Fig. 1). MAKA2 has a distinctive orange cyanobacterial-bacterial crust characteristic of some habitats on Hawai‘i and Maui (Maciolek & Brock 1974; Bailey-Brock & Brock 1993; Brock & Kam 1997), while the other sites have sand or mud/leaf litter substrates. WC is the only habitat with distinct photic zones: an open portion that receives sunlight (Fig. 1a, b) and a cave portion that does not (Fig. 1c, d).

2.3.2 Diel surveys of anchialine shrimp

To assess the presence of shrimp, densities were quantified during the day and at night at each habitat from 4 – 12 March 2011 (wet season), with diurnal and nocturnal surveys generally being conducted during the same tidal period. Surveys for a given habitat were always done within the same 24-hour interval. Diurnal and nocturnal densities were quantified between 09:00–16:30 and 19:30–01:00, respectively, with nocturnal surveys starting more than 1 hr post sunset. Quadrats (0.05 m^2) were placed on the substrate (in the same spot for diurnal and nocturnal surveys) and shrimp allowed to acclimate for 5 min before quantification. Counts of individual shrimp within the quadrat were repeated every 30 s for 5 min for a total of 11 time points. All 11 counts were then averaged over the 5 min period and converted to individuals per m^2.

2.3.3 Gut content analyses of invasive fishes

Fishes (*G. affinis* and *P. reticulata*) were collected from the four habitats via minnow traps during the dry (25 – 31 July 2010; *n* = 25 each for PUHO3A, MAKA1, and WC; *n* = 7 for MAKA2; total *n* = 82) and wet (March 2011; *n* = 25 for PUHO3A and MAKA1; *n* = 40 for WC; *n* = 11 for MAKA2; total *n* = 101) seasons to investigate: 1) whether *H. rubra* is consumed by invasive fishes and 2) potential seasonality in consumption patterns. Fishes were collected opportunistically using different numbers, sizes, and deployment times of minnow traps at each habitat. Therefore, fish densities between sites cannot be directly compared via minnow trap collections. However, fish densities were estimated for each habitat using quadrants as described above for shrimps (Table 1). Following preservation in either 10% formalin or 70% ethanol, fishes were weighed, measured, and placed into either an upper, middle, or lower category for weight and length relative to all other fishes that were examined (i.e., fishes were divided equally into thirds). Complete digestive tracts were dissected and gut contents examined and photographed under 8x magnification using a dissecting microscope. Intact diet items were classified to at least their taxonomic class and, when possible, cataloged based on morphotype (e.g., winged hymenopteran). Remains attributable to *H. rubra* were identified based on morphological characteristics of the species (Holthuis 1963; Bailey-Brock & Brock 1993). The number of *H. rubra* consumed by a given fish was conservatively estimated by counting the total number of intact cephalothoraxes or uropods, whichever number was highest. Legs and antennae of *H. rubra* were never found without an accompanying cephalothorax and/or uropod, suggesting partial predation was rare. A similar procedure was used to estimate abundance of other prey

items, which were always found either intact or as a number of legs, wings, and/or antennae. Vegetative and mineral debris were scored as present or absent. Total gut contents were archived in 70% ethanol following dissection.

2.3.4 Segregation of invasive fishes and anchialine shrimp at Waianapanapa Cave

Based on the presence of two distinct epigeal photic zones at WC (open and cave portions; Fig. 1), potential segregation of fishes and shrimp was investigated. Densities of fishes and shrimp were quantified along a transect running from the edge of the open portion (i.e., shore of the habitat) to the back of the cave. Using 1 m^2 and 0.05 m^2 quadrats for fishes and shrimp respectively, counts were performed every ~5 m at four stations: shore of the open portion, in the open portion near the cave mouth, in the cave, and at the back of the cave (Fig. 1). At each station, densities of shrimp and fishes were quantified as above for the diel shrimp surveys. These measures of spatial segregation were made during the daylight hours (09:00–16:00) during the wet season (March 2011).

2.3.5 Estimating gut transit time and quantifying fish predation on *H. rubra*

To confirm that invasive fishes prey on *H. rubra* when given the opportunity and to obtain an estimate of gut transit time for *H. rubra* in *G. affinis*, laboratory-reared shrimp were fed to *G. affinis* caught from the Auburn University Fisheries Ponds, North Unit. Individual fish were fed single *H. rubra* in 500 mL isolation chambers and preserved in 10% formalin followed by 70% ethanol at seven time points after feeding: 3 hours, 8 hours, 12 hours, 24 hours, 48 hours, 4 days, and 1 week (n = 5 for 3, 8, 12, 24, and 48 hours; n = 4 for 4 days and n = 3 for 1 week). Fish guts were examined and scored

for *H. rubra* remains using the same techniques as described above for wild-caught fishes. This approximation of transit time was used to estimate the maximum time since predation when shrimp would be detectable via gut content analysis (i.e., a temporal limit of detecting predation). Furthermore, gut transit time was used to confirm that number of shrimp consumed per fish was a good indicator of per day consumption (i.e., gut transit time was approximately 24 hours). Finally, estimates of the number of *H. rubra* consumed per fish and fish population size were used to estimate lower and upper confidence limits on the total number of shrimp consumed per day for each habitat where predation was detected.

To further quantify fish predation on endemic shrimp, diet items for each non-empty gut from habitats where shrimp predation was detected were quantified as the proportion of intact organisms (Chipps & Garvey 2007). This analysis only considered the most common (found in at least two fishes) dietary items representing whole predation events (i.e., legs and wings were not considered). For example, if a gut contained three ants, two *H. rubra*, and one wing, then the proportion of *H. rubra* remains in the gut was quantified as 40%.

2.3.6 Statistical analyses

Two different approaches were utilized towards analyzing the gut content data. First, to investigate overall variability in dietary items, principal components analyses (PCAs) were performed on the entire data set (n = 183 fishes) (Online Resource 1). The number of dietary items in 28 distinct categories (representing overall observed prey item diversity) was quantified for each fish, using both: 1) the absolute number of dietary

items in each category, and 2) the proportion of items in each category. The first two principal components generated by this analysis were then correlated with five qualitative categories: specific anchialine habitat of origin, species of fish, season collected, fish length class, and fish weight class. PCAs were performed using the FactoMineR v1.10 package (Lê et al. 2008) in the R v2.12.0 statistical software environment (code available on request; R Core Team 2013).

The second analysis was performed to investigate which factors influenced *H. rubra* consumption. Although *H. rubra* predation was quantified as count data, numerous fish showed no evidence of *H. rubra* consumption, leading to many instances of zero count data. Given this, hurdle regression models are one appropriate method for dealing with count data that have many instances of zero (Cameron & Trivedi 1998). Hurdle regression models use both a truncated count component for positive count data and a hurdle component for zero count data, giving effect sizes and *P* values for both the number of events (e.g., number of *H. rubra* consumed) and the likelihood of an event occurring (e.g., probability of any *H. rubra* predation). Therefore, a hurdle regression was used to model the minimum number of *H. rubra* consumed (see above) as a function of season collected, total length, and total weight of fish for each habitat where *H. rubra* predation was detected. The hurdle regression was performed using the pscl package (Zeileis et al. 2008) in R v2.12.0 (code available on request). Lastly, ANOVAs with Tukey post-hoc analyses and *t*-tests, also conducted with R v2.12.0, were used to compare 1) diurnal vs. nocturnal densities of shrimp at each of the four habitats; 2) densities of shrimp and fishes among stations at WC, and; 3) the proportion of *H. rubra* consumed among length and weight classes of invasive fishes or between collection

seasons.

2.4 Results

2.4.1 Diel surveys of anchialine shrimp

Shrimp were absent during the day in the four fish-invaded anchialine habitats examined here but were found at significantly higher densities at night in three of them (t-test, $t = 5.88$ for WC, $t = 8.66$ for MAKA1, $t = 8.65$ for MAKA2, $df = 1, 20$ for all, $P < 0.001$ for all; Fig. 2). With the exception of PUHO3A, where shrimp were absent during both day and night, this pattern is consistent with previous reports of *H. rubra* diel migration in fish-invaded anchialine habitats (Capps et al. 2009; Carey et al. 2011; Sakihara 2012). At WC, shrimp were absent from the open portion of the habitat during the day, but were present in this area at night (Fig. 2).

2.4.2 Gut content analyses of invasive fishes

Gut content analyses of invasive fishes from the four habitats revealed predation of *H. rubra* by invasive fishes occurring only at WC (Table 3). Also, shrimp were only consumed by *G. affinis*, although this may be due to *P. reticulata* only rarely being collected at WC (5/65 fishes). Specifically, *H. rubra* remains were recovered from three (3) of 25 fishes (12%) examined from WC during the dry season and four (4) of 40 fishes (10%) collected during the wet season. A total of 14 shrimp were consumed overall, with 0–4 shrimp consumed per fish examined at WC ($\bar{x} = 0.22 \pm 0.18$ shrimp per fish for all individuals collected at WC; 95% C.I.). Invasive fishes at WC also consumed other prey items, including ants, isopods, amphipods, and unidentified seed pods (Table 3). No

evidence of *H. rubra* predation was found at the other three habitats, with invasive fishes (total n = 118 individuals) generally consuming mites, flies, and ostracods (Table 3).

Principal component analyses (PCAs) of gut contents (Figs. 3 & 4) presented here are from absolute counts of dietary items, but those based on proportions showed the same results (available on request). PCA Dimension 1 explained 12.31% of the variation in gut contents, with the consumption of *H. rubra*, amphipods, pebbles, and vegetation significantly correlated to PCA 1 ($P < 0.01$). PCA Dimension 2 explained 7.19% of the variation in gut contents, with the consumption of non-crustacean arthropods and mollusks significantly correlated to PCA 2 ($P < 0.01$). Gut contents from WC were significantly different from the other sites on PCA 1 ($P < 0.001$), while all sites were significantly different from each other on PCA 2 ($P < 0.001$; Fig. 3). There was no statistical difference in gut contents between seasons (Fig. 4A), fish species (*G. affinis* vs. *P. reticulata*; potentially due to a small sample size for *P. reticulata* [25] vs. *G. affinis* [158]) (Fig. 4B), or length classes (Fig. 4C). However, fishes in the upper weight class (Fig. 4D) had significantly ($P = 0.011$) different gut contents than those in other weight classes based on PCA 1. Overall, these results demonstrate that invasive fishes consumed *H. rubra* at WC, and that fishes in the upper weight class (i.e., the heaviest third of fishes examined) consumed more *H. rubra* (along with other items on PCA 1).

The hurdle regression model was applied to *G. affinis* collected from WC (n = 60), since only these fishes consumed *H. rubra*. Total length ($z = 1.258$, $df = 8$, P = 0.208), weight ($z = -1.128$, $df = 8$, $P = 0.259$), and season collected ($z = -0.113$, $df = 8$, P = 0.910) were not statistically significant predictors of *whether or not* shrimp were consumed, and effect sizes for these variables were not large, suggesting these factors did

not influence shrimp consumption. Total length ($z = -0.975$, $df = 8$, $P = 0.330$), weight ($z = 1.261$, $df = 8$, $P = 0.207$), and season collected ($z = 0.008$, $df = 8$, $P = 0.993$) were also not statistically significant predictors of *how many* shrimp were consumed, but in this case there was a large effect size of weight on the number of shrimp consumed: fishes consumed 3.87 times (0.45 – 33.05 times; 95% C.L.) as many shrimp for each 0.1 g increase in fish weight. While statistically non-significant, this large effect size suggests fish weight may be a biologically important predictor of *H. rubra* consumption.

2.4.3 Segregation of invasive fishes and anchialine shrimp at Waianapanapa Cave

At WC, invasive fishes and endemic shrimp exhibited strong spatial segregation during the day (Fig. 5). Specifically, fishes were abundant in the open portions of the habitat and nearly absent in the cave portions ($F = 14.31$, $df = 3, 40$, $P < 0.0001$, ANOVA). On the other hand, shrimp (mainly *H. rubra*, but also the alpheid *Metabetaeus lohena* Banner & Banner, 1960) were abundant in the cave but absent in the open portion during the day ($F = 43.24$, $df = 3, 40$, $P < 0.0001$, ANOVA). Although not quantified, this pattern of spatial segregation was also observed at WC in the dry season (J. C. Havird, pers. obs.). This pattern broke down at night, as shrimp were present in the open portion of the habitat nocturnally (Fig. 2).

2.4.4 Gut transit time

Halocaridina rubra fed to *G. affinis* in the laboratory were all consumed within 30 minutes, confirming that fish will eat shrimp when given the opportunity. All guts examined three (3) and eight (8) hours after feeding possessed *H. rubra* remains. In

contrast, only 40% (n = 2) of guts examined 12 hours after feeding, and none of the guts from later time periods, had *H. rubra* remains (Fig. 6). Furthermore, guts sampled 12 hours after feeding had badly decomposed remains (consisting mainly of legs). Thus, while digestion rates may vary due to multiple diet items being consumed in the wild, these laboratory results suggest a conservative gut transit time for *H. rubra* in *G. affinis* of 24 hours and imply consumption rates were appropriate for a per day estimate.

2.4.5 Quantifying *H. rubra* predation at WC

To further quantify invasive fish predation on *H. rubra* at WC, proportions of common dietary items in non-empty guts were calculated. Overall, *H. rubra* represented 9.3% of these items (± 7.4%; 95% C.I.), with amphipods (39.4%), isopods (35.6%), ants (12.1%), and snails (3.8%) also being common. Proportions of *H. rubra* consumed were not different between wet and dry seasons (9.2% and 9.3% respectively, t = -0.011, df = 1,43, P = 0.99, t-test). Similar to the hurdle regression results, guts from the upper and middle weight classes had 11.7% and 10.6% *H. rubra* remains respectively, while the lower weight class had 5.6% *H. rubra* remains. Comparable results were seen among upper (13.3%), middle (11.1%), and lower (3.3%) length classes. However, as in the hurdle regression, proportion of *H. rubra* consumed did not differ significantly among weight or length classes (F = 0.328, df = 4, 40, P = 0.857, multi-factor ANOVA).

To generate estimates of shrimp consumption rates at WC, upper and lower estimates of fish population size were calculated based on diurnal densities in the open portion of the habitat, suggesting a population size of 554 – 1,100 individuals (95% C.L.). These were utilized along with the upper and lower estimates of the average

number of *H. rubra* consumed per fish (0.032 – 0.399 individuals, see above) to generate lower and upper bound quantities of shrimp consumed per day (as gut transit time was conservatively estimated at 24 hours). For the lower estimate, assuming 554 fishes consume 0.032 *H. rubra* per fish implies that 17.7 shrimp are consumed per day at WC. Alternatively, the upper estimate suggests 438.5 shrimp are consumed per day assuming 1,100 fishes consume 0.399 *H. rubra* per fish.

2.5 Discussion

2.5.1 Avoiding predation by invasive fishes

When predators successfully invade a novel habitat, native prey may be consumed at high levels, potentially leading to local extinction (Fritts & Rodda 1998). Alternatively, native prey may come under selection to eventually co-exist with predators (Kadye & Booth 2012; Kovacs et al. 2012) or avoid predation by adopting alternate behaviors (Loose & Dawidowicz 1994; Cox & Lima 2006; Sih et al. 2010; Bourdeau et al. 2011; Titelman et al. 2012). Here, no evidence of invasive fish predation on endemic shrimp was found at anchialine habitats at Makalawena Beach (MAKA) or Pu'uhonua O Hōnaunau National Historical Park (PUHO3A), suggesting that the adopted diel migration by shrimp (when present in a given habitat) is a generally successful predation prevention strategy. Furthermore, only 11% of invasive fishes at Waianapanapa Cave (WC) possessed *H. rubra* remains in their digestive tracts and predation was only observed by *G. affinis*, suggesting *P. reticulata* may lack sufficient size or aggression to consume *H. rubra* (or was not sampled adequately to detect predation). Overall, our results are consistent with $\delta^{15}N$ stable isotope analyses suggesting *H. rubra* comprises

only a small fraction of the diet for *G. affinis* in most Hawaiian anchialine habitats (Capps et al. 2009). However, predation at WC, even at low levels, is still concerning since the *H. rubra* population found at this site belongs to a unique genetic lineage, representing a potential cryptic species, whose range is restricted to the northeastern coastline of Maui (Craft et al. 2008; Santos & Weese 2011).

Why do fish consume *H. rubra* at WC but not at the other anchialine habitats? As discussed previously, native prey often alter their behavior in response to invasive predators (Loose & Dawidowicz 1994; Cox & Lima 2006; Sih et al. 2010; Bourdeau et al. 2011; Titelman et al. 2012). In Hawaiian anchialine habitats, shrimp retreat into the cracks and crevices leading down into the aquifer during the day in fish-invaded habitats to apparently avoid predation (Capps et al. 2009; Carey et al. 2011; Sakihara 2012; this study). At WC, however, shrimp also have the possibility of retreating into the darkness of the cave (Fig. 5) since invasive poeciliids are visual predators (Capps et al. 2009). Unfortunately, this avoidance behavior is clearly not as effective as retreating exclusively into the aquifer, as observed in other habitats. We hypothesize that the observed predation at WC likely occurs at the transition zone between the open portion and cave, where fish and shrimp may sometimes encounter one another under a light regime that allows some predation to occur. Thus, while the results presented here support the hypothesis that shrimp largely avoid predation via diel migration, abiotic factors of particular habitats (e.g., a cave with distinct photic zones) and other circumstances can reduce the efficiency of such predator-induced behavioral changes.

2.5.2 Non-consumptive effects (NCEs) of invasive fishes on endemic anchialine biota

For invasive predators, non-consumptive effects (NCEs) can often be equal to, or greater than, their consumptive effects (CEs) (Preisser et al. 2005; Pangle et al. 2007) through indirect effects (Peacor and Werner 1997) and trophic cascades (Preisser et al. 2005). The data presented here support observations that anchialine shrimp like *H. rubra* often respond to invasive fishes by retreating underground during the day (Capps et al. 2009; Carey et al. 2011; Sakihara 2012) while describing a novel behavior where they may also seek daytime refugia in cave-like, or dark, portions of a given habitat (Fig. 5). A number of zooplankton species exhibit similar diel migrations as a means of predation avoidance (Loose & Dawidowicz 1994) and fish-invasion can drive a rapid shift toward strong patterns of diel migration (Cousyn et al. 2001).

While it remains unclear how NCEs affect the Hawaiian anchialine ecosystem, one proposed hypothesis is that invasive fishes trigger an ecological shift caused by reduced grazing from *H. rubra*. In this scenario, habitats possessing an orange cyanobacterial-bacterial crust shift away from high microbial diversity to a monoculture of filamentous algae (*Cladophora sp.*; Maciolek & Brock 1974; Bailey-Brock & Brock 1993; Brock & Kam 1997). This is consistent with a report that fish-invaded habitats have higher levels of productivity and epilithon biomass (Dalton et al. 2012). Under natural conditions, maintenance of these crust communities may result from shrimp preferentially grazing on, or physically cropping, the algal component. In contrast, when shrimp are extirpated or forced to undergo diel migration, reduced grazing or cropping may, in turn, allow algal dominance. However, MAKA2 (and several other habitats on the Kona coast) have been fish-invaded for over 8 years yet still harbor these unique orange cyanobacterial-bacterial crust communities (S. R. Santos, pers. obs.). This

suggests *H. rubra* may still maintain this community through more intense grazing at night than what might occur in fishless habitats, similar to how native and invasive species attempt to maximize habitat utilization by minimizing temporal overlap in other ecosystems (Ayala et al. 2007). On the other hand, in habitats such as PUHO3A, where shrimp have likely been absent for decades due to invasion of poeciliids as well as *Tilapia* spp. (which are opportunistic omnivores also capable of altering behaviors of native species, e.g., Strictar-Pereira et al. 2010), identifying whether an ecological shift in the resident microbial community occurred, along with its directionality, is more difficult. However, the time since invasion, the number and types of interactions among various native and invasive species (Simberloff & Von Holle 1999), and the overall organismal community composition of the particular ecosystem likely influences the dynamics and timing of such ecological shifts. In any case, the impact of shrimp grazing on the diversity, structure and function of these anchialine microbial communities, and whether or not invasive fishes significantly alter the effects of grazing, remain open areas for future research.

2.5.3 Conservation and restoration considerations

Invasive alien species (IAS) have led to historic declines in endemic species throughout the Hawaiian Islands via habitat destruction (Vitousek et al. 1987), parasitism (Culver & Kuris 1998), disease (Woodworth et al. 2005), hybridization (Rhymer & Simberloff 1996), competition (Wilson & Holway 2010), and/or predation (Beard et al. 2009). Here, while predation appears to be infrequent, invasive fishes are shown as having measurable impacts on endemic Hawaiian anchialine biota via NCEs.

Furthermore, fish invasion of anchialine habitats has also resulted in the loss of sites and organisms of cultural importance to Hawaiʻi and its people. For example, many visitors to WC immediately notice, and are disappointed by, the absence of shrimp, which are associated with a legend local to the area. To ameliorate the negative biological and cultural effects of invasive fishes to this ecosystem, steps to reduce the spread of invasive fishes and restore fish-invaded anchialine habitats around Hawaiʻi should be implemented. Rotenone treatment and subsequent mechanical removal of fishes is one option that has been successfully employed previously (Carey et al. 2010). However, rotenone application is strongly restricted and/or regulated in Hawaiʻi (Robertson & Smith-Vaniz 2008) due to concerns of possible aquifer and coastal contamination. On the other hand, while physical removal of invasive species can be time-consuming and expensive (Zabala et al. 2010; Barbour et al. 2011), ongoing attempts to eradicate invasive fishes via trapping at WC have proven moderately successful, with fish densities decreasing over time (S. Hau pers. obs.). Given such challenges, special efforts to educate the general public should be pursued as a means towards preventing future invasions of Hawaiʻi's remaining fishless anchialine habitats.

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Table 1 Physical and biological characteristics of the four anchialine habitats in this study

Site	Area (m^2)	Distance to ocean (m)	Average depth (m)	Substrate	Submerged habitat	Fishes/Time since invasion	Fishes m^{-2} ± 95% C.I.
MAKA1	33	52	0.5	Sand	*R. maritima*	*G. affinis & P. reticulata/*2005	172.2 ± 42.9
MAKA2	20	36	0.1	Rock/crust	Orange crust	*G. affinis & P. reticulata*/2005	191.4 ± 32.2
PUHO3A	234	32	1	Soft mud	Mud	*G. affinis & Tilapia*/Historic	47.0 ± 15.8
WC	98	95	1.5	Sand	Leaves	*G. affinis & P. reticulata*/2002	13.98 ± 6.4

Table 2 Water chemistry of the four Hawaiian anchialine habitats examined in this study. Nutrient concentrations are in μM. DOC = dissolved organic carbon, TDN = total dissolved nitrogen. Water chemistry analyses were performed at the University of Hilo Analytical Laboratory from 100 mL samples taken from each habitat. Water chemistry analyses were not conducted for MAKA1 and WC in the wet season due to opportunistic sampling.

Site	Season	Salinity (‰)	NO_2+NO_3	PO_4	Si	NH_4	DOC	TDN
WC	Dry	5.0	28.5	2.46	315	1.47	15.4	25.3
PUHO3A	Dry	15.5	1.59	1.88	568	1.73	119	9.9
PUHO3A	Wet	13.0	6.98	3.50	461	8.07	195	27.1
MAKA1	Dry	7.0	56.3	6.66	835	2.01	49.9	53.4
MAKA2	Dry	6.5	75.9	7.06	818	1.63	35.2	72.2
MAKA2	Wet	7.0	67.8	7.06	714	2.34	78.0	75.9

Table 3 Abundance of most common dietary items (i.e., found in at least 10% of fishes at any given site) consumed by invasive fishes from the four anchialine habitats in the study during the dry and wet seasons. Dietary items were identified to at least taxonomic class and, when possible, cataloged based on morphology. Complete dietary information for all 183 fishes is provided in Online Resource 1.

	WC	MAKA 1	MAKA 2	PUHO3A
# Fishes Examined				
Gambusia affinis	60	36	12	50
Poecilia reticulata	5	14	6	0
Diet Item (minimum # individuals)				
H. rubra	14	0	0	0
Mites (Hydracarina)	1	20	13	11
Decapoda sp. (not *H. rubra*)	0	3	0	26
Winged insect (Hymenoptera)	3	0	0	18
Unknown insect carapace	1	1	1	5
Flies (Diptera)	1	2	8	2
Ants (Hymenoptera)	15	2	0	31
Isopods (Isopoda)	76	0	0	1
Amphipods (Amphipoda)	40	0	0	0
Unidentified seed pods	94	0	0	0
All Dietary Items	319	53	29	100

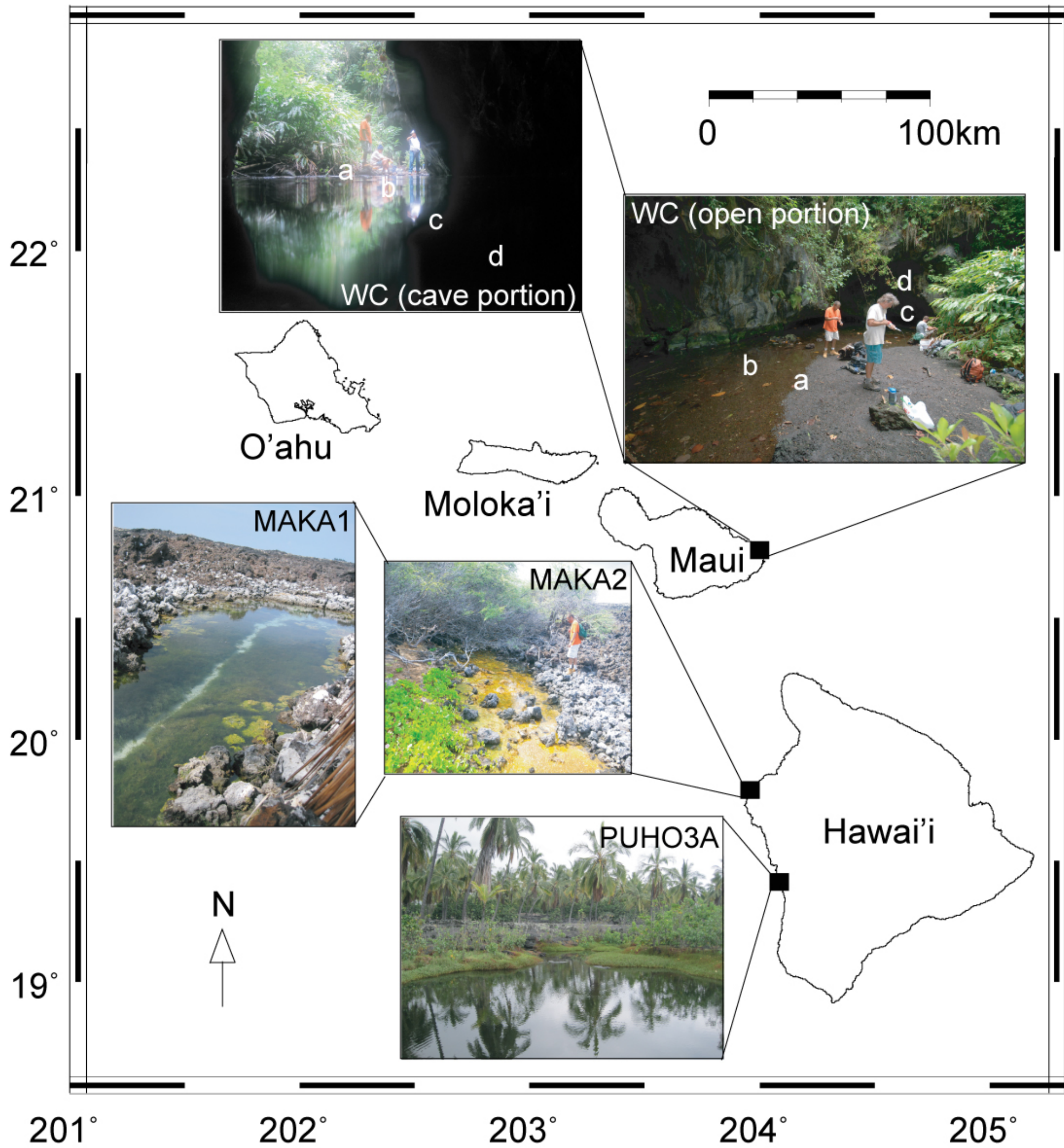

Fig. 1 Map of the high Hawaiian Islands (islands of Lana'i, Kaho'olawe, and Kaua'i not shown) depicting the four anchialine habitats in the study. **a** shore station at WC, **b** open station at WC, **c** near back of cave at WC, and **d** back of cave at WC. Abbreviations: MAKA = Makalawena Beach, PUHO3A = Pu'uhonua O Hōnaunau National Historical Park, WC = Waianapanapa Cave.

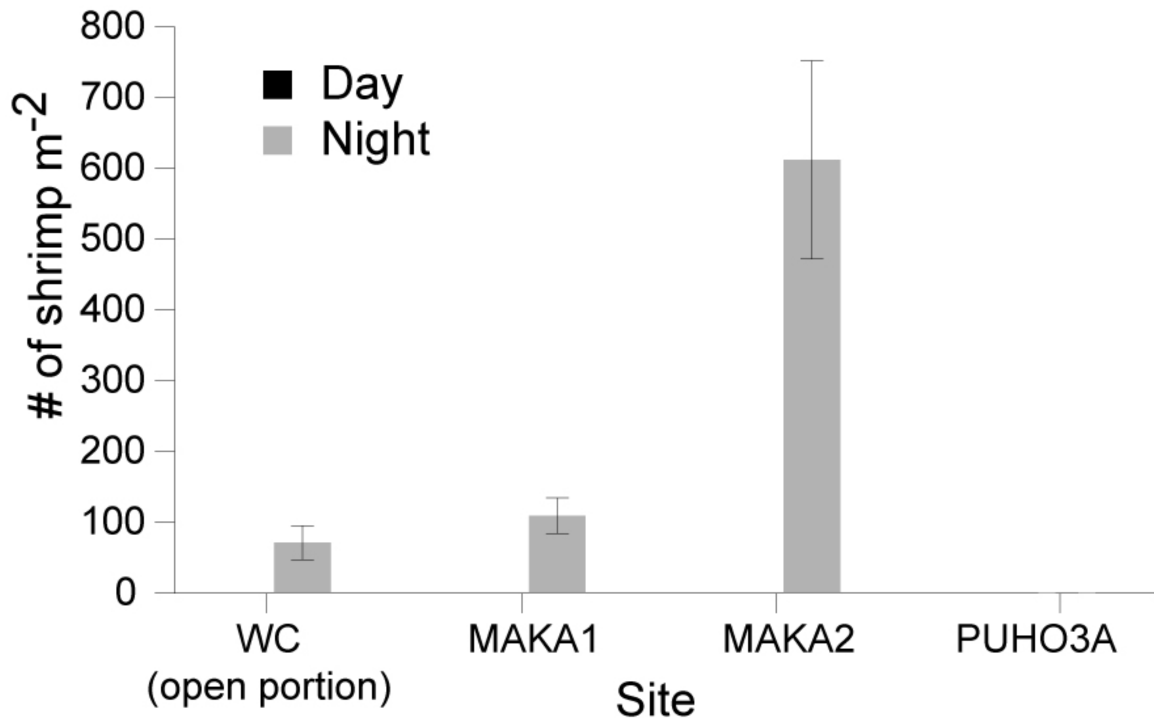

Fig. 2 Diel migration patterns for anchialine shrimp from the four anchialine habitats in the study. Asterisks indicate significant differences (*t*-test, $P < 0.001$, error bars show 95% C.I.) in diurnal vs. nocturnal densities. Abbreviations are as in Fig. 1.

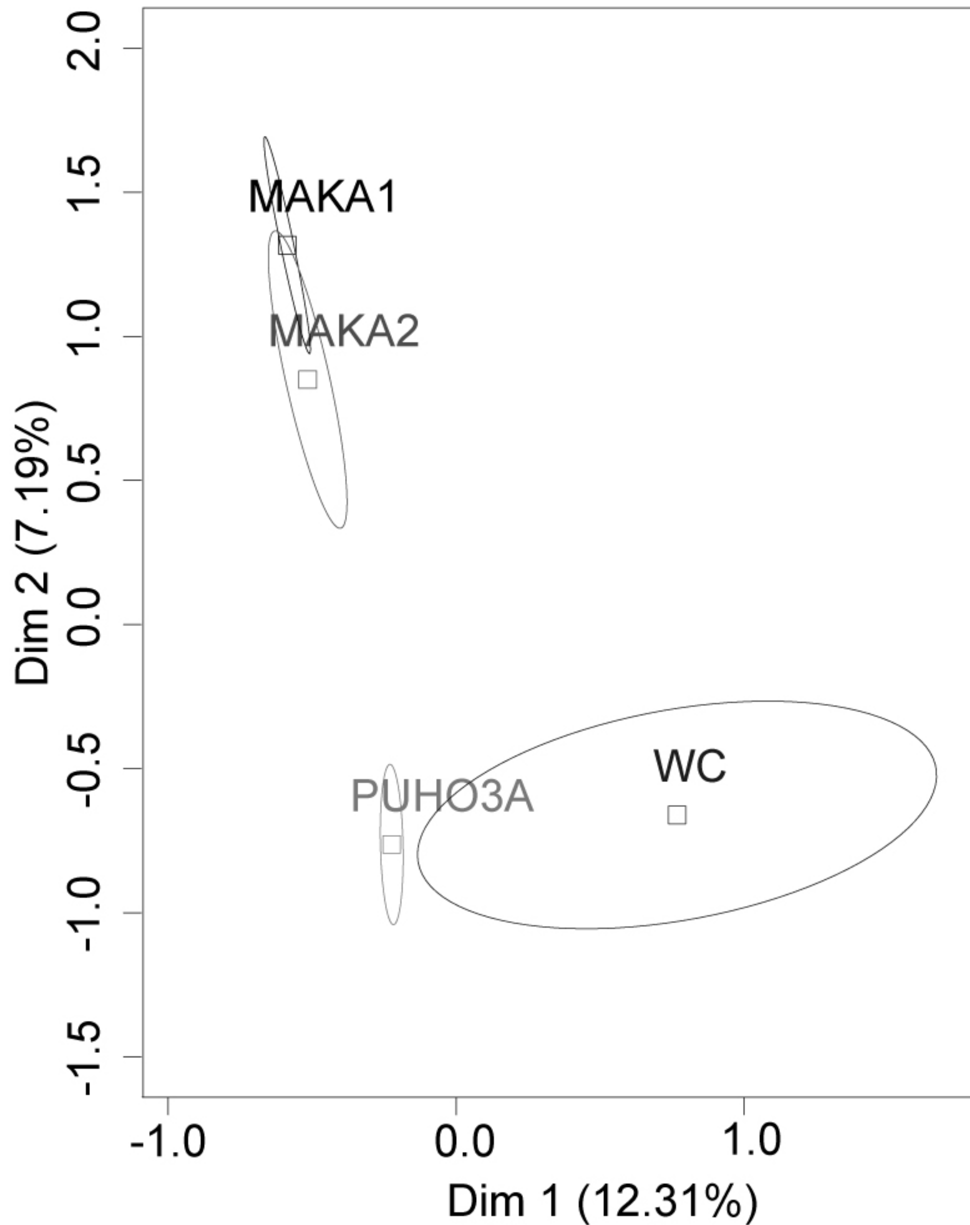

Fig. 3 Principal component analysis (PCA) of gut contents from invasive fishes grouped qualitatively by the four anchialine habitats in the study. Gut contents of fishes collected during the wet and dry seasons were combined and utilized in the PCA (see text for details). Abbreviations are as in Fig. 1. Square symbols represent barycentres (i.e., means) for a habitat, with 95% confidence levels given by ellipses.

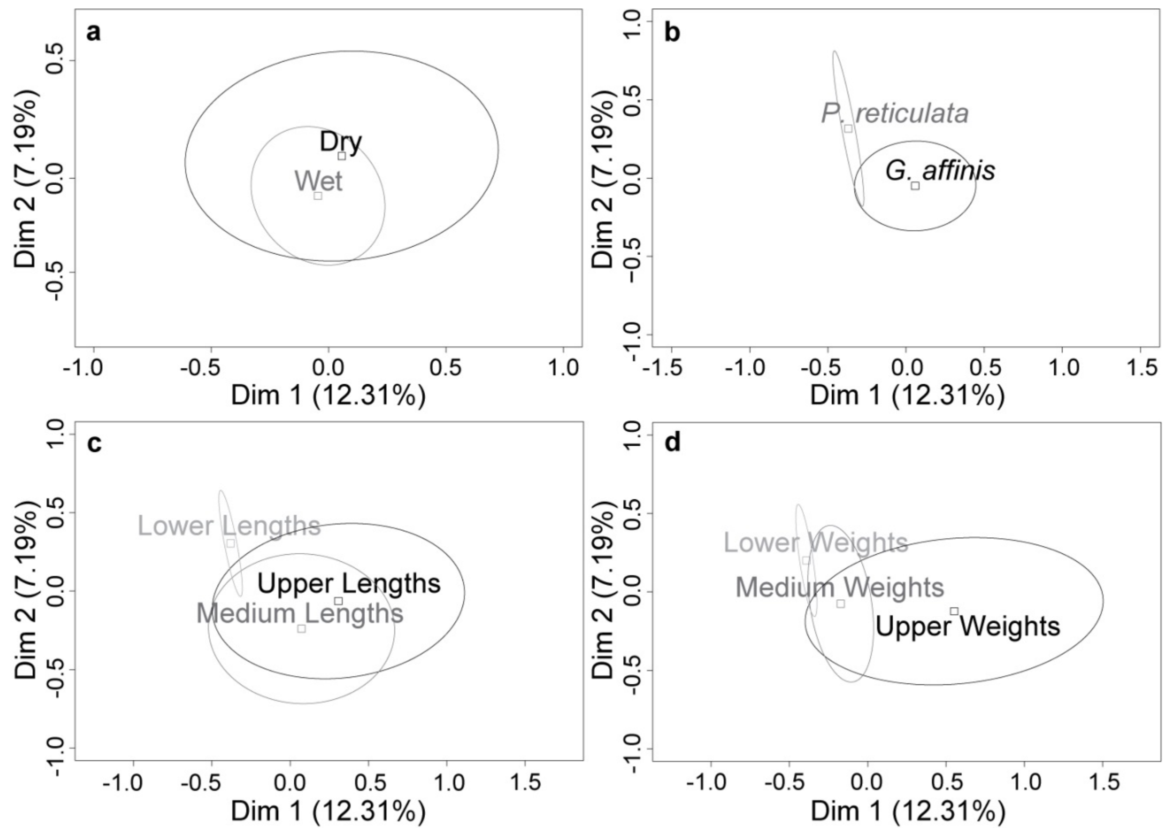

Fig. 4 Principal component analyses (PCAs) of gut contents from invasive fishes from the four anchialine habitats in the study during the dry and wet season using: **a** season, **b** species (*Gambusia affinis* or *Poecilia reticulata*), **c** length classes (upper, middle, and lower thirds), and **d** weight classes (upper, middle, and lower thirds) as qualitative variables. Square symbols represent barycentres (i.e., means) for a variable, with 95% confidence levels given by ellipses.

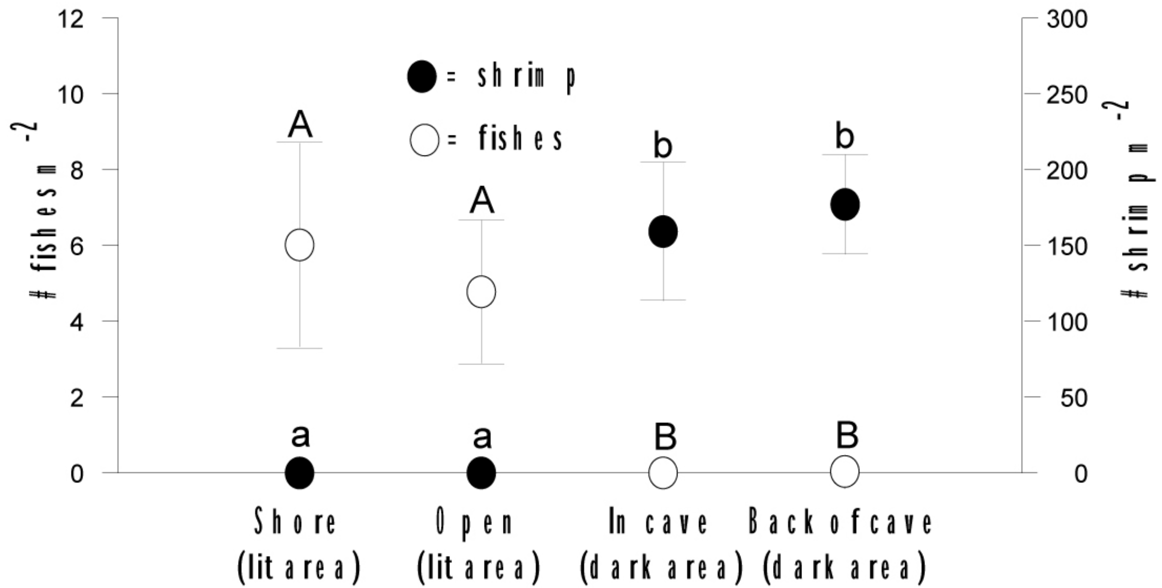

Fig. 5 Spatial segregation of invasive fishes and endemic anchialine shrimp at Waianapanapa Cave (WC) during the day. Densities of fishes and shrimp (± 95% C.I.) were quantified using quadrats and timed counts every 30 s for a total of five minutes (*n* = 11 observations) in four different stations of WC: near the shore of the habitat, in the open area near the cave, in the cave, and at the back of the cave (see Fig. 1). Letters indicate significant differences between stations for fishes (uppercase) and shrimp (lowercase) (ANOVA, Tukey post-hoc, $P < 0.001$).

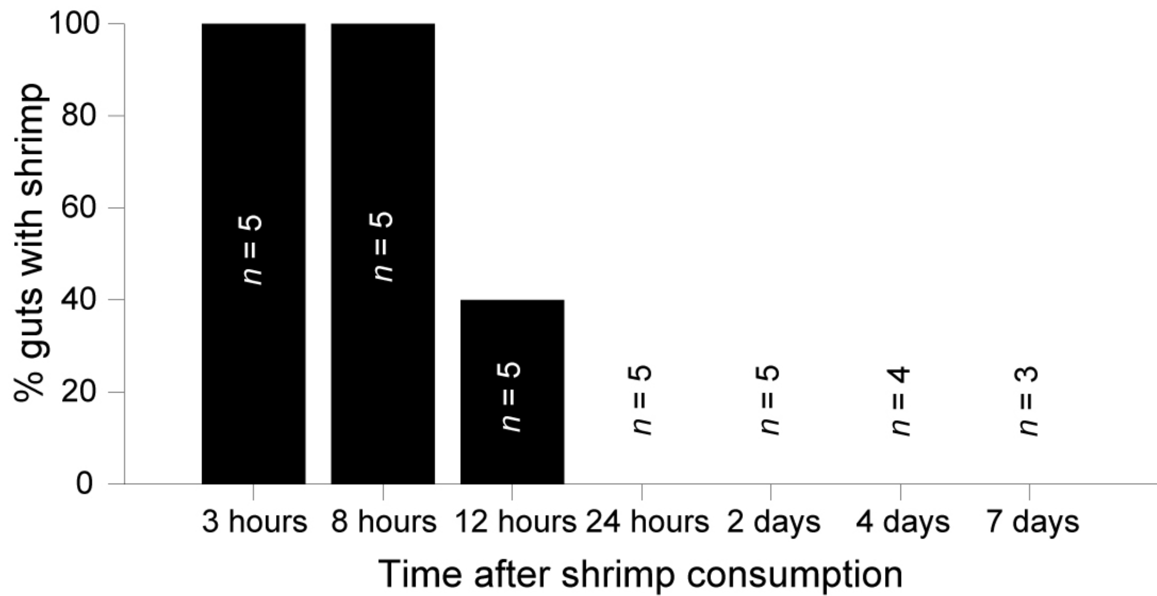

Fig. 6 Gut transit time, measured as proportions of *Gambusia affinis* with identifiable *Halocaridina rubra* remains, at various intervals following consumption of a single shrimp in the laboratory. Sample sizes shown for each time point.

Online Resource 1 Complete record of dietary items of invasive fishes collected from the four anchialine habitats in the study. These data were used to generate the PCAs in Figs. 2 & 3. Abbreviations are as in Fig. 1. Visit http://link.springer.com/article/10.1007%2Fs10750-013-1568-8 for online resource 1.

Chapter 3. Altered expression of Na^+/K^+-ATPase and other osmoregulatory genes in the gills of euryhaline animals in response to salinity transfer: A meta-analysis of 59 quantitative PCR studies over 10 years

3.1 Abstract

Recent advances in molecular techniques have allowed gene expression in euryhaline animals to be quantified during salinity transfers. As these investigations transition from studying single genes to utilizing genomics-based methodologies, it is an appropriate time to summarize single gene studies. Therefore, a meta-analysis was performed on 59 published studies that used quantitative polymerase chain reaction (qPCR) to examine expression of osmoregulatory genes (the Na^+/K^+-ATPase, NKA; the $Na^+/K^+/2Cl^-$ cotransporter, NKCC; carbonic anhydrase, CA; the cystic fibrosis transmembrane regulator, CFTR; and the H^+-ATPase, HAT) in response to salinity transfer. Based on 887 calculated effect sizes, NKA, NKCC, CA, and HAT are up-regulated after salinity transfer, while surprisingly, CFTR is unchanged. Meta-analysis also identified influential factors contributing to these changes. For example, expression was highest: 1) during transfers from higher to lower salinities comprising a physiological transition from osmoconformity to osmoregulation, 2) 1-3 days following transfer, 3) during dissimilar transfers, and 4) in crustaceans rather than teleosts. Methodological characteristics (e.g., types of controls) were not important. Experiments lacking in the current literature were also identified. Meta-analyses are powerful tools for quantitatively synthesizing a large body of literature, and this report serves as a template for their application in other areas of comparative physiology.

3.2 Introduction

Advances in molecular techniques for non-model organisms and the popularity of the quantitative polymerase chain reaction (qPCR) have allowed comparative physiologists to study the expression of single proteins of interest in the gills of euryhaline animals during salinity transfer, with the first such studies being published about 10 years ago (Towle et al., 2001; Lucu and Towle, 2003; Richards et al., 2003). The expansion of a quantitative molecular approach allowed researchers to work with the genes encoding transport proteins, instead of the proteins themselves, which are often large, trans-membrane molecules that are difficult to study using more traditional biochemical methods. Importantly, these new molecular techniques allowed researchers to identify more transport proteins in the gills, characterize their functional properties, and study regulatory mechanisms responsible for the increases in transport protein activity seen in earlier studies (e.g., transcriptional vs. post-transcriptional modification; for a recent comprehensive review see Henry et al., 2012).

Although qPCR continues to be a popular technique for investigating individual genes of interest (e.g., Gilmour et al., 2012; Loong et al., 2012; Wang et al., 2012), a new molecular revolution is currently underway, and physiologists are now beginning to use genomic-based approaches that simultaneously examine a significant portion of the transcriptome. This is due to the advent of microarray technology (e.g., Towle et al., 2011) and more recently, next generation sequencing (e.g., Trapnell et al., 2010; Lowe et al., 2011; Meyer et al., 2011). As sequencing costs decline, this field may continue to shift to a more genomics and less single-gene based approach. Therefore, it may be a good time to summarize the current literature on how salinity affects gene expression

based on studies using qPCR. Meta-analysis is one way to quantitatively and statistically synthesize many such studies (Glass, 1976). Although not typically appearing in comparative physiology journals, meta-analysis is popular in the fields of ecology (Cardinale et al., 2006), medical genomics (Gieger et al., 2011), cell biology (Broadhead et al., 2006), and global climate change (Parmesan and Yohe, 2003). Meta-analyses have often been used to summarize many inconclusive medical studies (where large sample sizes are impossible) to give a more robust conclusion, but meta-analyses can also summarize the state of knowledge within a field, highlight future areas of research, and point out factors leading to heterogeneity in studies (i.e., why do similar studies get different results?). These later purposes seem more applicable to studies of salinity-induced gene expression, as it is not controversial whether long-studied genes and proteins are important in ion-transport, but under what scenarios deviations from the norm may be expected.

The Na^+/K^+-ATPase (NKA) has been the most popular gene examined in osmoregulatory studies, in terms of number of studies published (e.g., 76% of the studies analyzed here). This is not surprising, as the NKA is a critical component in osmotic/ionic regulation (for reviews, see Towle et al., 1976; Shoemaker and Nagy, 1977; Evans et al., 2008; Henry et al., 2012). The NKA is responsible for establishing electrochemical gradients across the cell membrane in the gills of euryhaline animals and has been found in the gills of all such species examined. Other genes have also been implicated in osmoregulation and work in concert with the NKA to actively secrete or absorb ions across the gills. These include: 1) the $Na^+/K^+/2Cl^-$ cotransporter (NKCC), which transports ions into gill cells either from the blood or environment based on

salinity (Riestenpatt et al., 1996; Towle and Weihrauch, 2001; Hwang and Lee, 2007), 2) the H^+-ATPase (HAT), which pumps protons to influence HCO_3^- and Cl^- exchange and Na^+ uptake (Weihrauch et al., 2004; Tresguerres et al., 2006), 3) carbonic anhydrase (CA), which produces the H^+ and HCO_3^- needed to drive coordinated Na^+ and Cl^- exchange (Henry and Cameron, 1983; Hirata et al., 2003), and 4) the cystic fibrosis transmembrane regulator (CFTR), a Cl^- channel that has mainly been studied in euryhaline fish osmoregulation (Singer et al., 1998). Other genes such as the Na^+/H^+ exchanger (Edwards et al., 1999) and the Na^+/HCO_3^- cotransporter (Hirata et al., 2003) have also been implicated in this process, although their expression patterns have not been analyzed as thoroughly.

Because studies examining expression of single genes during salinity transfer have been accumulating for the last ~10 years, they contain a large but manageable amount of data. To summarize these data, we performed a meta-analysis of 59 published studies investigating changes in expression of five genes in response to salinity transfer redundant in euryhaline animals. Studies of NKA provided the most robust data set, although studies investigating NKCC, CA, CFTR, and HAT were also examined. The objectives of this meta-analysis were to: 1) identify the causes of differences in gene expression between studies based on differing methodologies and experimental designs (i.e., heterogeneity), 2) identify experiments lacking in the literature in an effort to fill in research deficiencies and direct future research, and 3) provide a database that can be used by researchers to design future experiments and compare their results to what is already known. It is hoped that this meta-analysis will prompt similar studies in related

areas such as acid-base regulation, ammonia excretion, metabolism, as well as for other genes of interest.

3.3 Materials and methods

3.3.1 Data acquisition

Literature searches were performed including all published studies through mid-2012 using PubMed and Web of Science databases to identify published papers that met the search criteria. These criteria were: 1) Study organisms had to be euryhaline metazoans (not plants or microorganisms), 2) Expression had to be measured via relative mRNA abundance (i.e., not protein-based or enzyme activity based) using qPCR (i.e., "semi-quantitative" gel-based methods were not included), 3) A mean for a control and treatment condition had to be measureable, and 4) Expression had to be measured in the gills, as they are the major site of osmoregulation and the most-studied osmoregulatory tissue in euryhaline animals (Evans et al. 2005; Henry et al., 2012). Although all journals were considered during initial literature searches, later searches focused on a number of specific journals that tended to publish studies meeting the search criteria: *The Journal of Experimental Biology*, *Comparative Biochemistry and Physiology A, B, & D*, *The American Journal of Physiology*, *The Journal of General and Comparative Endocrinology*, and *The Journal of Experimental Zoology*. Search terms initially used were combinations of the following: salinity, transfer, gene expression, qPCR, mRNA, gene names and acronyms, and gill. After initial searches, reference-based searching was used.

3.3.2 Details of the meta-analysis

Effect sizes (values representing the magnitude of an experimental manipulation on a certain measurement) were calculated for each experiment in each paper by comparing relative gene expression values gleaned from figures and tables between a “control state” and a “treatment state”. The control state consisted, when possible, of expression values calculated for “sham” transfers of animals into the same salinity to which they were chronically acclimated. Treatment states consisted of experimental expression values for transfers to a different salinity taken at the same time points as in the sham transfers. When sham transfers were not performed (63% of the effect sizes), control values were calculated before transfers at a zero-time point (i.e., a pre-transfer expression value). These types of comparisons were often performed in the original studies and are not unique to this meta-analysis.

In a meta-analysis, effect sizes are compared using a common effect size metric. There are over 60 common effect size metrics to consider when conducting a meta-analysis (Huberty, 2002), and choosing an appropriate metric can be daunting, although important (Nakagawa and Cuthill, 2007). Hedge’s *g* (Hedges, 1981; Cohen, 1988) is one such metric that has become popular in some fields. It is essentially a difference in means between the control and treatment states standardized to the deviation and weighted based on sample size (Borenstein et al., 2009). The Hedge’s *g* metric therefore places more weight on data associated with smaller error. However, others (Osenberg et al., 1997; Osenberg et al., 1999) have raised concern over such error-based weighted metrics and have encouraged the use of metrics that are more relevant to the focal question. One example is ln *RR* (Hedges, 1999), which is calculated as the natural log-transformed ratio

of the treatment and control mean values. Sample size and error are irrelevant to the calculation of this metric, thus allowing for the inclusion of data from studies that fail to provide data on sample size and error. For this metric, effect sizes greater than zero represent an increase relative to the control state (e.g., an ln *RR* of 1 equates to a ~3 fold increase), and those less than zero a decrease relative to the control state. Additionally, metrics can incorporate relevant temporal or spatial scales of the experiment (Osenberg et al., 1997), for example (ln *RR*)/time, which allows the incorporation of biologically important characteristics of the experiment. It is important to note the difference between an effect size metric and an effect size: an effect size metric is a statistical transformation that is applied to the raw data to describe (in this case) the difference between control and experimental states (e.g., ln *RR*) and an effect size is the numerical outcome of that statistical transformation.

In this meta-analysis, three different metrics were calculated for comparison: Hedge's *g*, ln *RR*, and (ln *RR*)/t. For calculation of Hedge's *g*, studies had to also include a variance component and sample sizes for the control and treatment means. Statistics associated with Hedge's *g* corresponding to heterogeneity between and within studies (Q, τ^2, I^2) were also calculated (Borenstein et al., 2009). Comprehensive Meta Analysis software (CMA) was used for all calculations of Hedge's *g* and associated statistics, while the R statistical software environment was used for comparing ln *RR*-based metrics between different types of studies.

3.3.3 Investigating characteristics of the studies

One purpose of a meta-analysis is to summarize effects across diverse studies to provide a general answer to whether or not a manipulation influences a response (e.g., How much does the expression of a certain gene increase or decrease, or does it remain constant during salinity transfer?). However, the variation typically found within and between studies can shed light on other interesting patterns as well. In this context, several experimental factors that may impact salinity-induced gene expression were investigated. These included the broad taxonomic group and species studied and the time point after transfer when expression was measured. Characteristics of the salinity transfer were also noted, including: general direction of salinity transfer (*i.e.*, from a higher to lower salinity or *vice versa*), absolute range of the transfer, and if the transfer involved a change in physiological state, such as from an osmoconforming to osmoregulating strategy (or *vice versa*). To characterize the latter types of transfers, additional studies (Appendix A) were consulted to determine over which salinities animals acted as osmoconformers vs. regulators (Fig. 1). Targeted specific isoforms or subunits of the genes were also recorded, when possible. In crustaceans, different pairs of gills were often investigated, as individual gills can be osmoregulatory vs. respiratory in function (for reviews, see Taylor and Taylor, 1992; Freire et al., 2008), so it was noted which gills were examined if possible (these were later combined into anterior vs. posterior gills). Methodological details were also recorded for each study including: whether expression levels were normalized to a "control" gene and if so which control gene was used, if the control state consisted of a sham transfer or a pre-transfer condition, and whether the salinity transfer was conducted in a controlled laboratory setting or if expression was measured in wild-caught animals that were inferred to have undergone a salinity transfer.

It should be noted that many of these characteristics have often been explored in individual studies, and that meta-analysis is one way to quantitatively summarize these comparisons.

3.4 Results

3.4.1 The data set

All data used in our study have been made publically available in an online supplemental appendix (Appendix 1) in an attempt to promote transparency and encourage additional analyses with our dataset. Based on the literature search, 59 published studies were chosen for inclusion in the meta-analysis. Many others were considered but not included due to our earlier described acceptance criteria. Of the included papers, 45 targeted NKA, while NKCC (19), CA (11), CFTR (10), and HAT (10) were examined in fewer papers. Effect sizes (comparison of expression between control and treatment states) also followed this trend, with 563 effect sizes calculated (using only a single metric; ln *RR*) for NKA, 98 for NKCC, 104 for CA, 63 for CFTR, and 59 for HAT. Some studies only had a single calculable effect size, while Jayasundara et al. (2007) had the most of any paper examined: NKA isoforms at six different time points after both 35‰ to 45‰ and 35‰ to 10‰ transfers for two different years in five different pairs of gills were examined, amounting to a total of 200 effect sizes calculated. Analyses were performed again while excluding this paper to determine if it skewed our results. Including or excluding this study showed no significant influence on the overall effect size patterns (Fig. A.1) so data collected from Jayasundara et al. (2007) were included in our meta-analysis. A total of 29 different species were included in our meta-

analysis, and expression was measured between two hours and 180 days after transfer. The papers examined crustaceans (n = 15 papers), teleosts (n = 42), or elasmobranchs (n = 2), however the number of effect sizes were similar between crustaceans (50.5%) and teleosts (49.5%), because expression was often measured in multiple gills in crustaceans compared to a single measurement in teleosts. Many studies investigated the response of different isoforms, and control genes were generally used in studies investigating teleosts but not in those investigating crustaceans.

3.4.2 Effect metric performance and summary effects

Although all three effect metrics are presented in Appendix A for comparison, only results associated with ln *RR* are discussed here because: 1) data were usually most normally distributed when using this metric (Fig. 2), 2) results were generally similar between Hedge's *g* and ln *RR*, and 3) elevated effect size variation associated with (ln *RR*)/t hindered our ability to identify clear trends in the dataset. When all effect sizes were grouped together, expression of all genes except CFTR significantly increased after salinity transfer, as shown by significant, positive ln *RR* summary effects (Fig. 3A). However, there was a large amount of heterogeneity in the studies for all genes, which indicates that generating a single summary effect may be misleading. This heterogeneity was investigated by partitioning the data into various groups and determining if there were significant differences in expression values between groups. Although all comparisons were performed for NKA, only a fraction of these comparisons were performed for the other genes due to low sample sizes. For example, CFTR expression

was only examined in teleosts, so CFTR expression could not be compared between taxonomic groups.

In other cases, confounding effects existed for certain comparisons. For example, because studies of teleosts often used a control gene while those for crustaceans did not, these two characteristics cannot be teased apart. For NKA, the confounding effects were alleviated by portioning the data into smaller data sets (e.g., only those studies involving specific taxa, types of transfers, or tissues). When these smaller data sets were generated for the other genes, comparisons often became impossible or confined to single studies (i.e., not a meta-analysis) because of the reduced sample sizes. In all analyses, efforts were made to ensure multiple studies were compared, but to minimize confounding effects. Finally, publication bias was not an issue in the literature as the fail-safe number (the number of unpublished effect sizes needed to offset the summary effect of the meta-analysis) was calculated as more than 2000 for all genes (except CFTR; fail-safe numbers are not calculable when there is a non-significant summary effect). This suggests there is not a bias to publish studies showing significant changes in gene expression vs. those showing no significant change.

3.4.3 Factors contributing to heterogeneity between studies

The characteristics of the taxa being studied contributed to differences in gene expression between studies. Crustaceans tended to show the greatest increases in gene expression, while teleosts sometimes showed no significant change from control states (Fig. 3A). Additionally, for crustaceans, posterior gills tended to show greater increases in CA expression compared to anterior gills during salinity change (Fig. 3B). However,

for NKA this trend was not significant ($P = 0.598$). Finally, there was a large amount of variation between the different species studied (Fig. 4A). For teleosts, Salmoniforms tended to not change NKA expression during salinity transfer, while other teleosts had elevated NKA levels (Fig. 4B). Comparable results for CFTR (i.e., expression did not increase or decrease) were seen between families (Fig. 4B). Finally, intraspecific variation in NKA expression was able to be assessed for two teleosts, although there were no significant differences in changes between populations (Fig. 4C).

The characteristics of the salinity transfer also had an effect on gene expression. In all NKA datasets and for CA, transfers from a higher salinity to a lower salinity induced a higher increase in expression than transfers from lower to higher salinities (Fig. 5A). In NKCC and CFTR the opposite was true, and for HAT the direction of the transfer was not significant ($P = 0.075$; Fig. 5A). Whether the transfer involved a switch in osmoregulation strategy also influenced gene expression. However, this could only be assessed accurately for expression of NKA and CA in crustaceans, as teleosts often acted as osmoregulators (although hyper- vs. hyporegulation was not considered) at all salinities investigated (Fig. 1) and other genes did not have sufficient samples for any meaningful comparisons. When crustaceans were transferred from salinities in which they were osmoconformers to salinities in which they were osmoregulators, expression of NKA and CA were elevated more than in other types of transfers (Fig. 5B). For CA this was most pronounced in the posterior gills (Fig. 5B). The absolute range of the transfer (e.g., a change of 10‰ vs. a change of 32‰) did not appear to result in significant differences in gene expression induction for the overall NKA, HAT, or NKCC datasets,

but greater transfer ranges tended to result in greater increases in expression for crustacean NKA datasets and CFTR (Fig. 5C).

The duration of the salinity transfer also had a profound effect on gene expression. In many cases, gene expression peaked between 1 and 3 days post transfer and then leveled off to control state levels by 2-3 weeks post transfer (Fig. 6A). However, notable exceptions to this trend include: 1) the posterior gills of crustaceans transferred from a salinity in which the organism is an osmoconformer to one in which it is an osmoregulator still showed elevated NKA expression at the latest time points, 2) up-regulation of HAT expression appears at the earliest time points, and 3) CFTR expression did not vary with time. If NKA expression across time is examined in detail (Fig. 6B), a clear peak is present at 1-2 days post-transfer, and although other peaks are also present, they are often for time points with low sample sizes.

In some cases, differential expression of isoforms or subunits of the same gene was able to be assessed. NKA α and NKA β tended to have similar levels of expression (Fig. 7), although this may be because of the low sample sizes and high variation associated with NKA β. In teleosts, NKA α1 and NKA α 3 had similar expression levels; however, NKA α1b was up-regulated in response to salinity transfer, while NKA α1a and NKA α1c were not (Fig. 7). In crustaceans, two isoforms of branchial CA were compared: CAc (cytoplasmic) and CAg (membrane-associated). CAc is thought to be responsible for ion uptake, while CAg is involved in respiration/CO_2 excretion (Henry, 1988a, b; Henry et al., 2003). CAc was up-regulated in response to salinity transfer, while CAg was not; however, the difference between isoforms was only significant in posterior gills (Fig. 7). In anterior gills, the same general trend was found, although the difference

between the isoforms was not significant (*P* = 0.239, Fig. 7). Finally, HAT subunit a was up-regulated more than subunit b in response to salinity transfer (Fig. 7).

There were also differences in qPCR methodology used between studies. Some studies (47.8% of effect sizes) normalized expression to a control gene not related to osmoregulation, whereas others did not. Although studies using a control gene tended to show a lower level of gene expression when all data were considered, this is confounded by the fact that studies of teleosts tended to use a control gene, while those of crustaceans did not. When this was accounted for, NKA expression levels within teleosts and crustaceans were the same between studies using and not using control genes (Fig. 8A), suggesting that results are not influenced by normalizing or not normalizing to a control gene. Similarly, there was not a difference between studies that expressed gene transfer change relative to a pre-transfer condition and those relative to a sham transfer measured at the same time point when taxonomic group was accounted for (Fig. 8B), suggesting calculating gene expression relative to a sham vs. pre-transfer yields similar results. Finally, for NKA expression in teleosts, it did not matter if the experiment was performed in a laboratory setting, or if a salinity transfer was inferred from environmental samples (Fig. 8C). However, most studies examined (245/253) were laboratory-based.

3.5 Discussion

3.5.1 Generalizations about the entire data set

Presented here for the first time is a quantitative summary of the effects of salinity transfer on expression of genes in euryhaline animals. This analysis summarizes 10 years of qPCR studies and provides a framework for future studies, whether they are focused

on single genes or are genomics-based. Most published studies examined changes in gene expression for either crustaceans or teleosts, while two studies examined elasmobranchs (Choe et al., 2005; Reilly et al., 2011). Although many other taxa are euryhaline, studies of gene expression during salinity transfer in these taxa were either focused on general stress proteins such as heat shock proteins and antioxidants (De Zoysa et al., 2009; López-Legentil et al., 2008; An et al., 2010) or used genomics-based methods like microarrays (Gracey et al., 2008; Lockwood and Somero, 2011). There were many other studies that were also examined initially, but later discarded due to: 1) use of genomics-based methods (e.g. Pinto et al., 2010; Towle et al., 2011), 2) use of semi-quantitative gel-based methods (e.g., Jensen et al., 1998; Mackie et al., 2005), and 3) unclear salinity transfer conditions (e.g., parts of Nilsen et al., 2007; Fan and Li, 2010).

It is not surprising that the genes examined are up-regulated during salinity transfer, as they have been implicated in salinity change response in many euryhaline animals for decades (Skou, 1965; Forrest et al., 1973; Frizzell et al., 1979; Henry and Cameron, 1982, 1983; Lin and Randall, 1991; Singer et al., 1998). A more interesting finding was that based on all papers that met the inclusion criteria, only CFTR expression was not influenced by salinity change, while NKA, NKCC, CA, and HAT expression were up-regulated, despite the vast heterogeneity between studies. It is important to note that this finding can only be generalized to the species and studies examined, and that down-regulation of these genes should be expected in some scenarios (e.g., crustaceans transferred from low to high salinities; Jillette et al., 2011). However, the studies investigated most likely tended to perform experiments that would result in up-regulation,

and studies that would be expected to result in no change or a down-regulation should be targeted in the future.

3.5.2 Heterogeneity between studies

Grouping all studies together to "see the forest for the trees" is one advantage of a meta-analysis, but another advantage is to examine smaller groups of studies and compare them to the overall analysis or each other. Identifying the heterogeneity in a set of studies is one of the strengths of meta-analysis and allows researchers to make biological sense of why similar studies get different results. Because of the wealth of data for NKA, this was possible for many cases, although for other genes fewer meaningful comparisons could be made. For example, several studies examined NKA expression in both anterior and posterior gills of crustaceans, but only a single study (Luquet et al., 2005) examined NKCC expression in different sets of gills. Therefore, a meaningful comparison could be made for NKA, but for NKCC, an analysis would just restate the results of Luquet et al. (2005).

Crustaceans likely had overall greater increases in gene expression than teleosts (Fig. 3A) because crustaceans often act as osmoconformers at higher salinities, but as osmoregulators at lower salinities, whereas teleosts are generally strong osmoregulators across a wide salinity range (Fig. 1). Therefore, salinity transfer can often entail a change in osmoregulatory strategy in crustaceans, but not in teleosts. However, one study (Gilmour et al., 2012) specifically transferred trout from seawater (SW) to iso-tonic conditions, but reported an increase in expression, suggesting transfer to iso-tonic conditions may be enough to initiate a SW to freshwater (FW) response. Gill specificity

has been long-implicated in crustacean osmoregulation, and different species show differences in which gills act as osmoregulatory organs (Copeland and Fitzjarrell, 1968; Finol and Croghan, 1983; Cioffi, 1984; Towle, 1984; Wheatly and Henry, 1987; Compere et al., 1989; Dickson et al., 1991; Genovese et al., 2004; Freire et al., 2008). Posterior gills tended to have greater increases in CA expression than anterior gills for the taxa studied here (Fig. 3B), which is typical of the most-commonly studied species (i.e., decapod brachyurans). This general trend was also seen for NKA expression, but was only significant when Hedge's *g* was used, suggesting studies that saw a difference between gills may have had greater sample sizes or less variation.

The wide variation shown between different studies examined is indicative of the fact that many of these general trends may not apply to all species. Because fishes in Salmoniformes had more studies and effect sizes than those in other orders, they were grouped together to show that salmoniforms generally do not alter NKA expression during transfer, but that the other species studied did increase NKA expression. This may be because salmoniforms generally only undergo anadromous (seawater to freshwater) migratory salinity changes based on life-history, while the other species studied are truly euryhaline and undergo salinity changes more frequently due to the environments they inhabit. For example, the most well studied non-salmoniform, the mummichog (*Fundulus heteroclitus*), inhabits coastal tidal creeks where salinity change may occur daily (Griffith, 1974). Therefore, different osmoregulatory mechanisms may exist for these two ecologically distinct groups. However, even salmoniformes increased NKA α1b expression after transfer (Fig. 7), showing that different isoforms can have different osmoregulatory roles. In fishes, NKA isoforms have different sequence characteristics

near the N-terminus, suggesting differing responses to protein kinase regulation (Efendiev et al., 2000; Pierre et al., 2002; Richards et al., 2003), and providing a reason why different isoforms are up-regulated in response to transfer. Similarly, in crustaceans, CAc was significantly up-regulated after transfer, but CAg was not. This mirrors the roles of the two isoforms, as CAc is thought to be osmoregulatory, while CAg is respiratory (Henry and Cameron, 1983; Henry, 1988a, b; Serrano and Henry, 2008). Finally, for HAT, the finding that subunit a appears to be up-regulated more than subunit b during transfer has not been specifically explored previously. Meta-analysis as used here may therefore be useful for determining which isoforms to target in salinity transfer experiments targeting unexplored species.

The characteristics of the salinity transfer examined greatly affected the degree of gene expression change. Generally, the largest increases in NKA, NKCC, and CA expression were seen 1-3 days following salinity transfers from high to low salinities, when transfer represented a switch from animals acting as osmoconformers to osmoregulators. However, for NKA and CA specifically, a significant increase in expression was detected earlier – as little as one hour after transfer (Faleiros et al., 2010). Adjusting levels of gene expression can be a rapid response to salinity, and changes in expression can occur before changes in protein activity, as has been well-documented for CA (Henry et al., 2003; Serrano et al., 2007; Serrano and Henry, 2008; Jillette et al., 2011). Therefore, future salinity transfer experiments should measure expression after acute, not chronic transfer. For most NKA datasets and other genes where expression was elevated, gene expression levels did not return to control levels until at least 14 days after transfer. However, when crustaceans were transferred from conforming to regulating

salinities, their posterior gills still showed increased NKA levels at the longest time points measured (up to 28 days in Serrano et al., 2007). This suggests that chronically acclimated animals should be maintained in control salinities for several weeks before assessing control levels of gene expression (as suggested by Jillette et al., 2011).

Finally, methodological differences among the studies accounted for little of the observed heterogeneity. Generally, studies of fishes normalized gene expression to a control gene and compared experimental expression levels to individuals undergoing a sham transfer. Contrarily, studies of crustaceans did not normalize to a control gene and compared expression levels to a pre-transfer measurement, although they often compared expression to a non-osmoregulating control tissue (e.g., the anterior gills). When teleosts and crustaceans are assessed separately, it is clear that these different methodologies did not result in different levels of gene expression. Although normalization to a control gene and sham transfers are desirable, limitations often exist that force researchers to adopt less optimal methods (e.g., the unavailability of a well-characterized control gene, as is case for the crustacean *Callinectes sapidus*; Serrano et al., 2007). This meta-analysis suggests that these additional controls are not critical to adequately assess salinity-induced changes in gene expression. Only four studies were explored that measured expression in a field-based setting, but these did not show significantly different changes in gene expression than laboratory-based studies. Further studies are needed to determine if the salinity-induced changes in gene expression observed in the laboratory are also observed under natural salinity transfer conditions. For example, field-based measurements of CA protein activity from *Callinectes sapidus* collected in a stream-fed

estuary have suggested that the responses typically seen in the laboratory may not mirror responses in reality (Jillette et al., 2011).

3.5.3 Areas for future research

The current meta-analysis has highlighted several questions that could be addressed in future studies. The most obvious is to apply meta-analyses to similar types of studies investigating other genes or changes in gene expression due to other types of environmental stress. Additionally, only teleosts, crustaceans, and two elasmobranch taxa were examined in the studies included here. Expanding qPCR based studies of salinity-induced gene expression to molluscs and other taxonomic groups should be a primary interest. Within crustaceans, decapod brachyurans were examined in most studies, which should be expanded to include more diverse taxa (e.g., shrimps, crayfishes, and lobsters). Differential expression of isoforms was also mainly confined to studies of teleosts. Teleosts have more genomic resources currently available than crustaceans, thanks to several well-established, annotated genomes from model teleosts (e.g., *Danio rerio*, *Takifugu rubripes*, and *Gasterosteus aculeatus*). In contrast, although EST libraries and other genomic resources are becoming available for some crustaceans previously used in osmoregulatory studies, the first crustacean genome (*Daphnia pulex*) was only recently sequenced (Colbourne et al., 2011). This may explain why studies involving teleosts often take multiple isoforms into account, whereas those of crustaceans usually do not. Vertebrates also underwent two rounds of genome duplication early in their evolution (Dehal and Boore, 2005), and teleosts have undergone an additional round of genome duplication (Christoffels et al., 2004), meaning that teleosts may simply have more

isoforms than crustaceans. Studies involving crustaceans often involved a change from iso-osmotic conditions to hyper- or hypo-regulating conditions, but only a single fish study (Gilmour et al., 2012) measured expression during a transfer to an iso-osmotic salinity. Additionally, no studies examined transfers from a hypersaline condition to either seawater or freshwater. These types of transfers should be explored. The few studies that examined intraspecific variation in gene expression focused on teleosts. These studies should be expanded in general. Finally, few studies examined gene expression in wild-caught animals, although some were not included because a reasonable approximation of time since transfer could not be estimated (Lorin-Nebel et al., 2012). Interpreting gene expression levels in the wild, or using controlled field-based mescocosm experiments should be a priority to determine if lab-based results are applicable to the changes that actually happen during migrations or movements between different salinity regimes.

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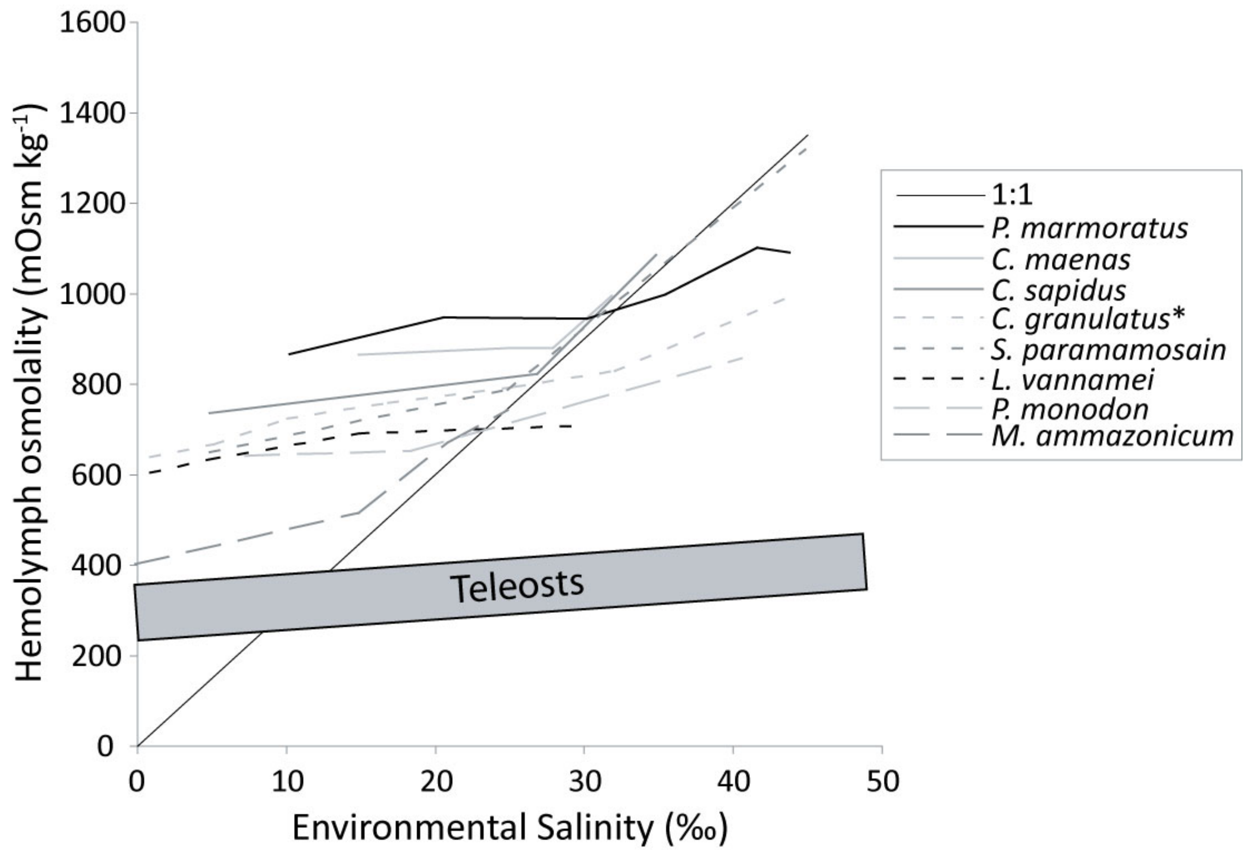

Fig. 1. Hemolymph osmolality of taxa analyzed in this study acclimated to various environmental salinities. Data are only presented for environmental salinities analyzed in this study. References used to gather this data are in Appendix A. *Note that C*hasmagnathus granulatus* has been renamed to *Neohelice granulata* (Sakai et al., 2006).

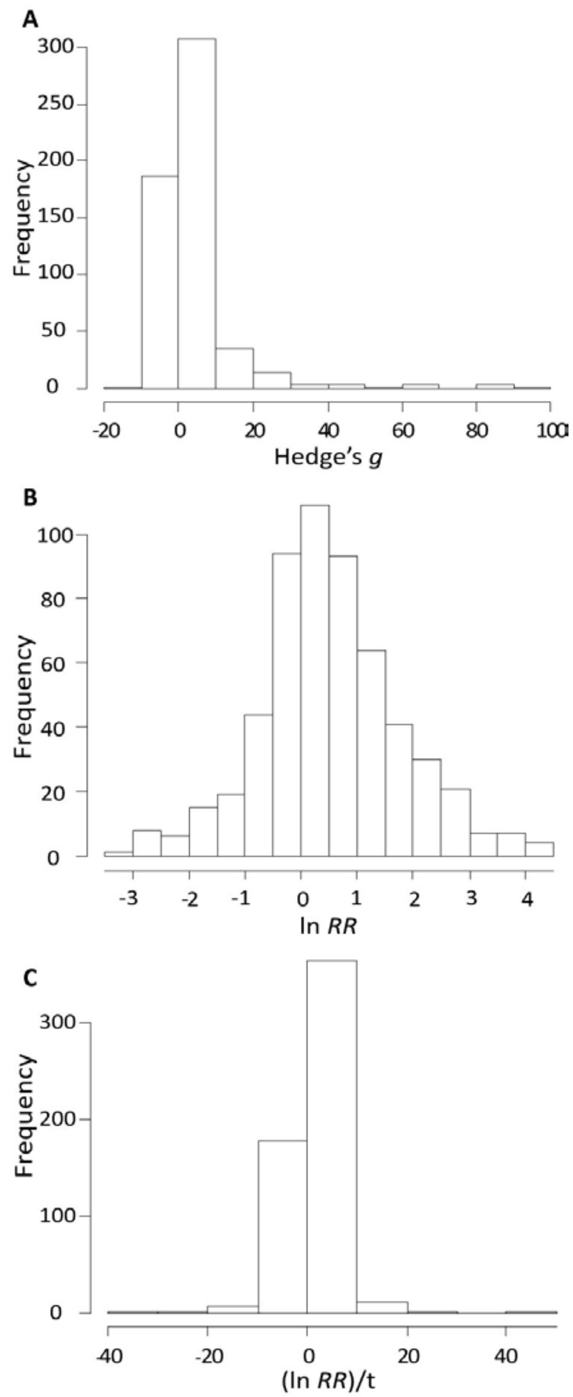

Fig. 2. Frequency distributions of the three effect metrics calculated for Na^+/K^+-ATPase. (A) Hedge's *g* (Cohen, 1988; Hedges, 1981). (B) Natural log of the response ratio (ln *RR*; Hedges, 1999). (C) (ln *RR*)/t (Osenberg et al., 1997). Results were similar for the other genes examined.

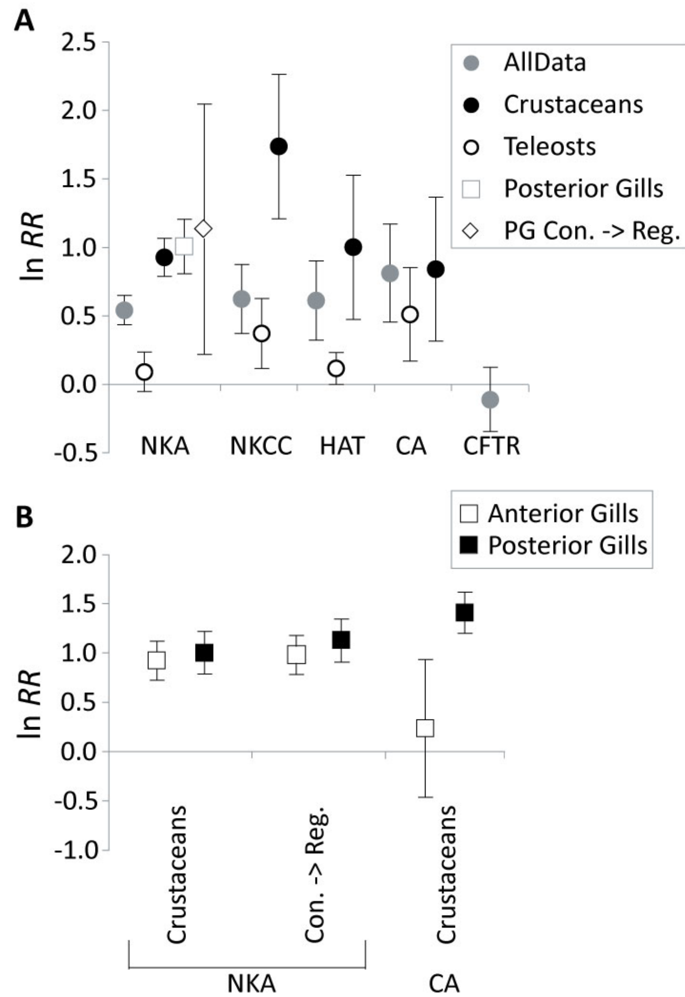

Fig. 3. Effect of taxa and tissue on expression of genes during salinity transfer. (A) ln *RR* (± 95% C.I.) for Na^+/K^+-ATPase (NKA), $Na^{+/}K^+/2Cl^-$ cotransporter (NKCC), carbonic anhydrase (CA), cystic fibrosis transmembrane regulator (CFTR), and H^+-ATPase (HAT) for all data, crustaceans only, teleosts only, posterior gills of crustaceans only, or posterior gills of crustaceans undergoing an osmoconformer to osmoregulator switch (PG Con. -> Reg.). (B) Effect of tissue type on expression of NKA and CA in crustaceans, or only in crustaceans undergoing an osmoconformer to osmoregulator switch (Con. -> Reg.).

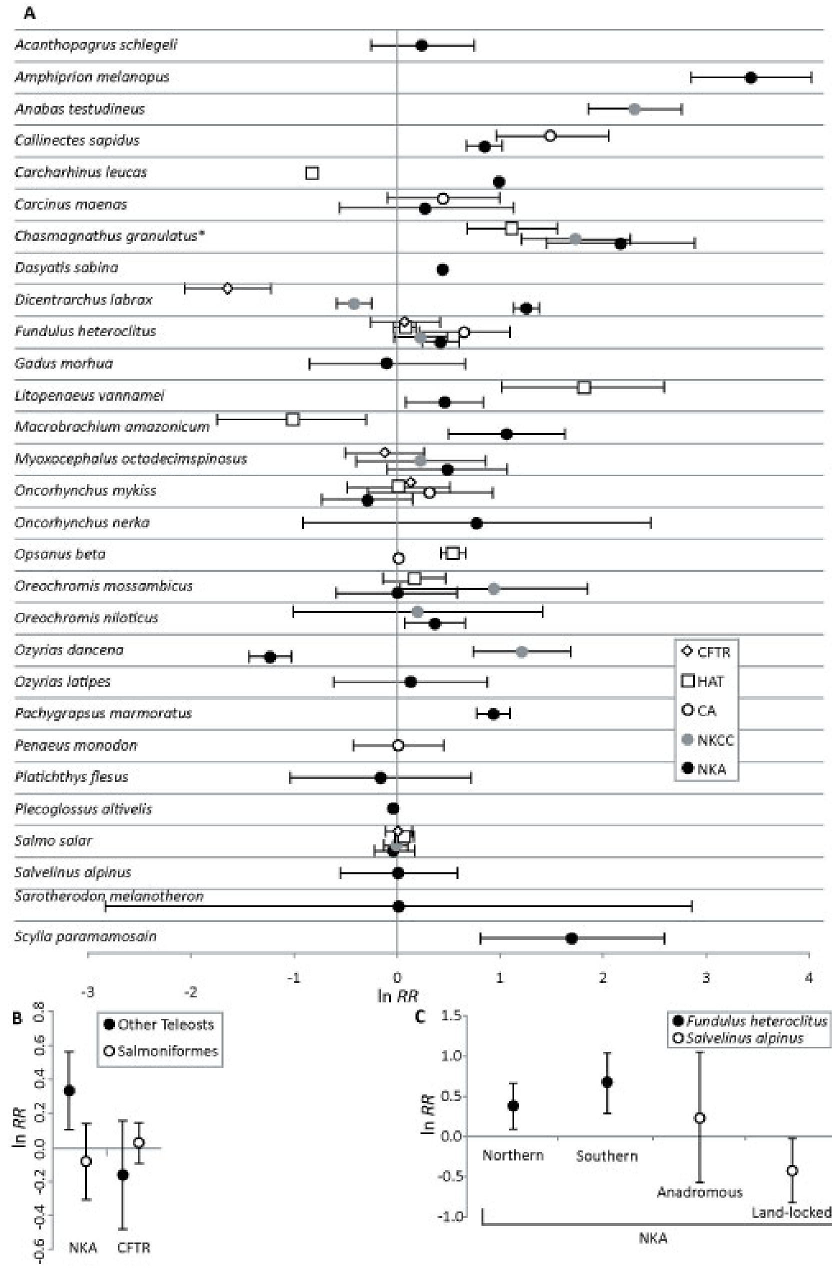

Fig. 4. Inter- and intraspecific variation in ln *RR* (± 95% C.I.) for genes examined in this study. (A) Effect of species. (B) Fishes in Salmoniformes had no significant NKA induction after salinity transfer, while those in other orders did, although both groups had similar changes in CFTR. (C) Intraspecific variation in NKA induction was not

significant. Abbreviations follow Fig. 3. *Note that C*hasmagnathus granulatus* has been renamed to *Neohelice granulata* (Sakai et al., 2006).

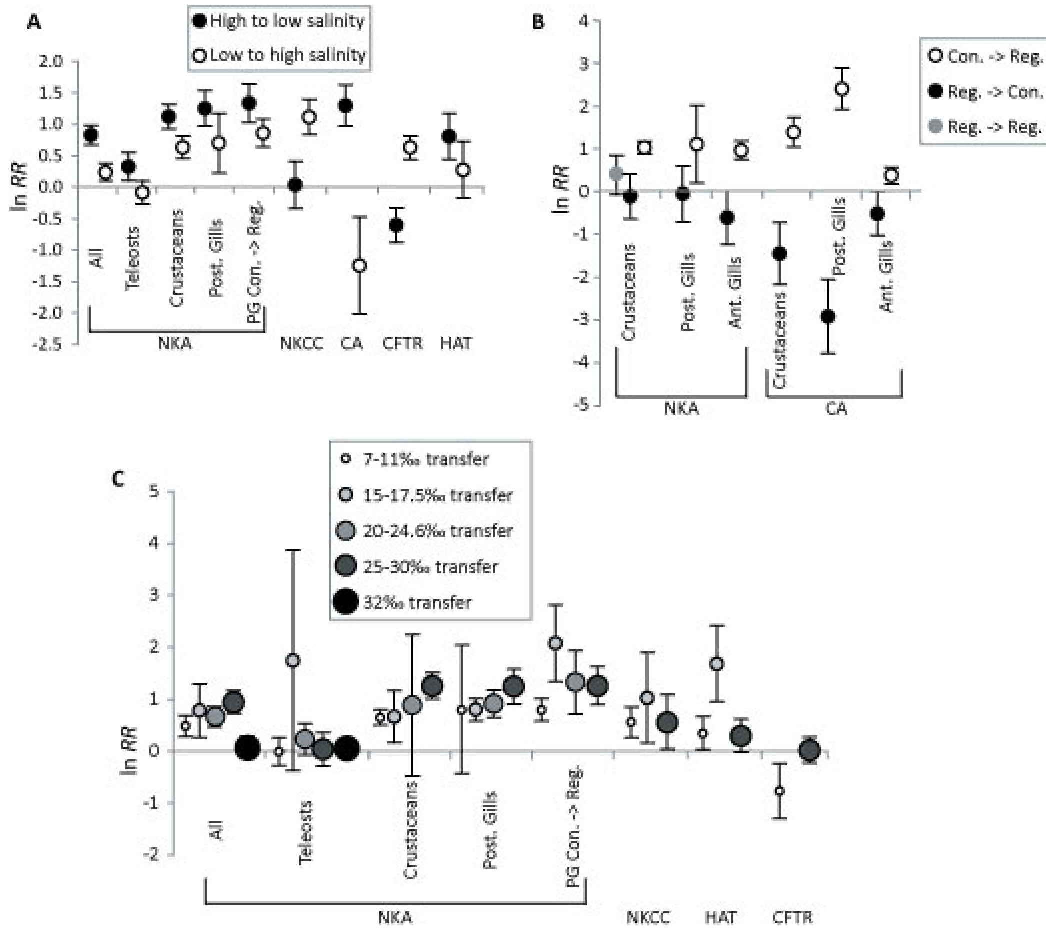

Fig. 5. Effect of type of salinity transfer on gene expression. (A) Transfers from higher to lower salinities produced greater increases in NKA and CA, while the opposite was true for CFTR and NKCC, and no difference was seen for HAT. (B) Transfers involving an osmoconformer to osmoregulator switch caused the highest levels of NKA and CA expression in crustaceans. (C) Transfers involving the most dissimilar salinities produced the highest levels of gene expression in NKA and CFTR. Abbreviations and error bars follow Fig. 3.

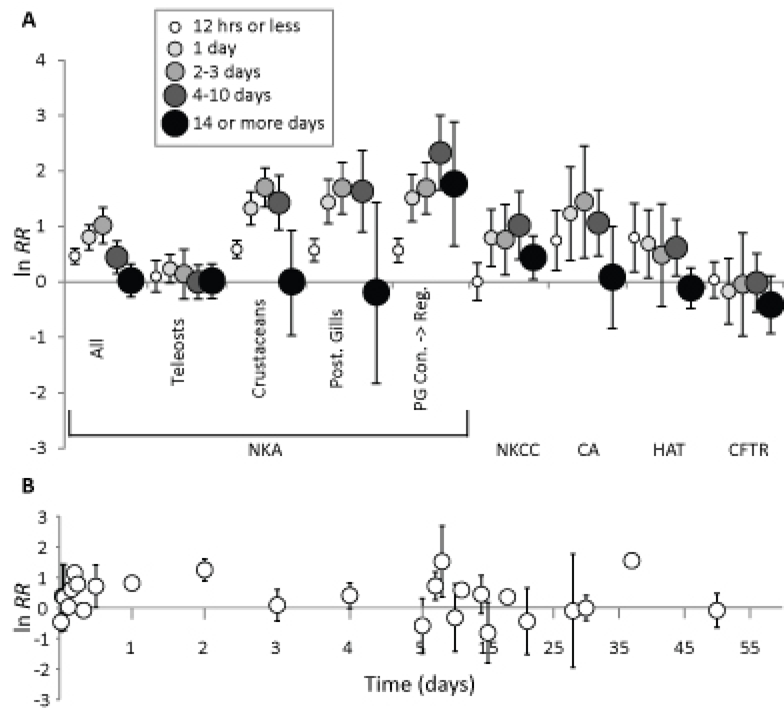

Fig. 6. Effect of duration of salinity transfer on gene expression. (A) Expression tended to peak at 1-3 days post-transfer for NKA, NKCC, and CA, but was highest immediately following transfer for HAT. (B) Detailed examination of NKA expression in response to duration of transfer for all data shows a peak at 1-2 days post-transfer, but other peaks also exist. Abbreviations and error bars follow Fig. 3.

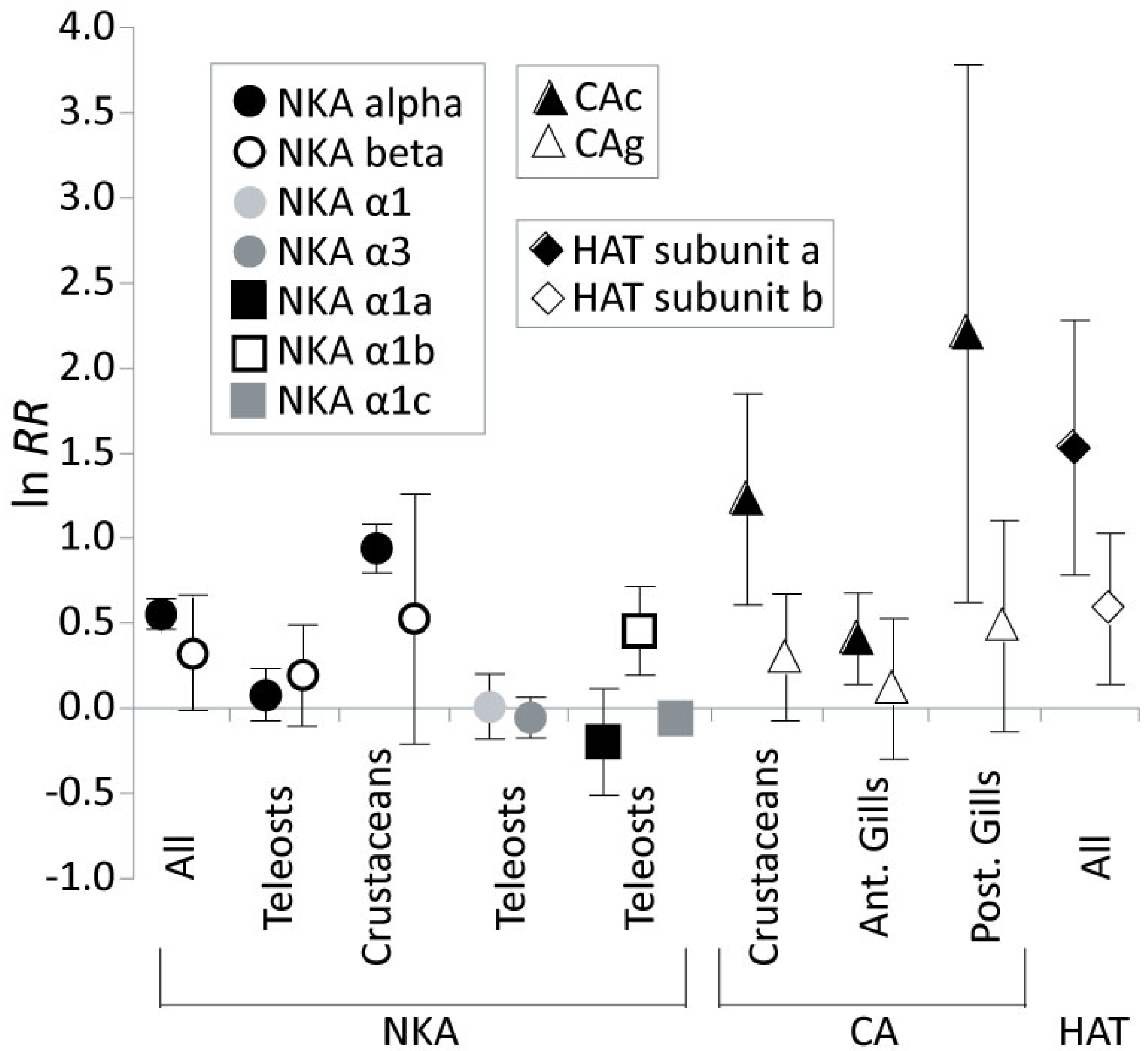

Fig. 7. Differential expression of isoforms and subunits. Only NKA α1b shows increased expression in teleosts following transfer. CAc shows a higher level of expression than CAg following transfer. HAT subunit a also shows a higher level of expression than subunit b. Abbreviations and error bars follow Fig. 3.

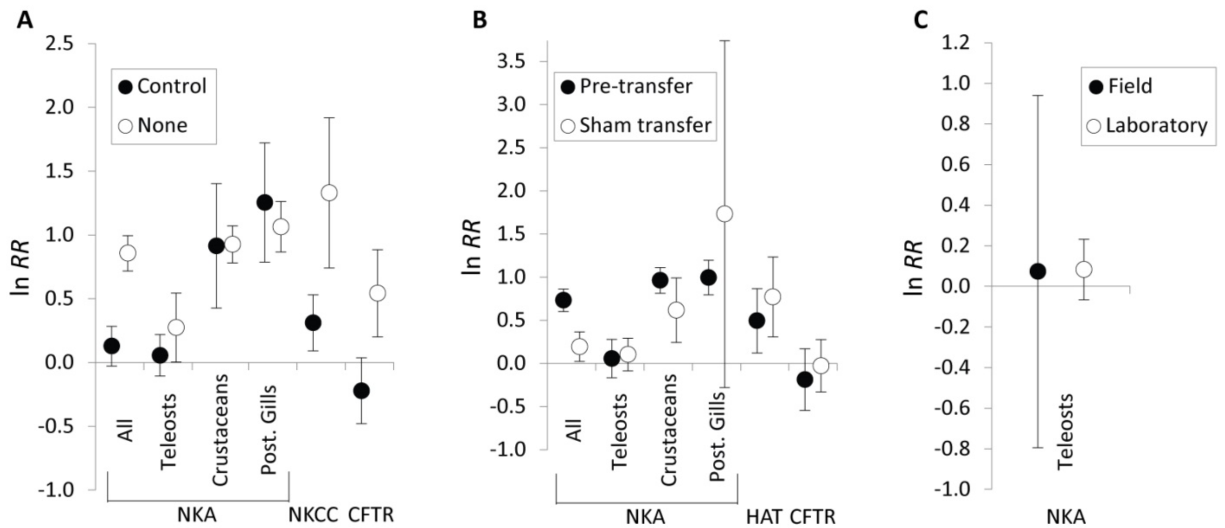

Fig. 8. Effect of methodological variables on gene expression following salinity transfer. (A) Using a control gene tended not to influence NKA expression when teleosts and crustaceans were analyzed independently. (B) Similarly, whether salinity-induced expression was compared to a sham transfer or pre-transfer condition did not influence expression values when taxonomic group was accounted for. (C) Although few studies examined NKA expression in a field-based setting, they were not different than those in laboratory settings for teleosts. Abbreviations and error bars follow Fig. 3.

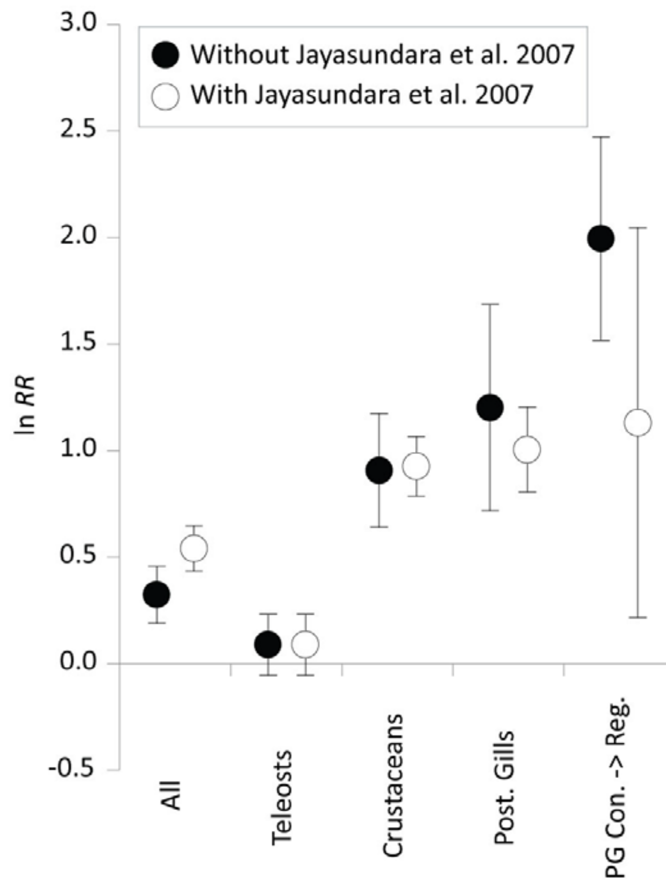

Fig. A.1. General results of the meta-analysis for NKA with and without Jayasundara et al. (2007). Abbreviations and error bars follow Fig. 3.

Appendix 1 can be found at

http://www.sciencedirect.com/science/article/pii/S1744117X13000154

Chapter 4. Osmoregulation in the Hawaiian anchialine shrimp *Halocaridina rubra* (Crustacea: Atyidae): expression of ion transporters, mitochondria-rich cell proliferation, and hemolymph osmolality during salinity transfers

4.1 Abstract

Studies of euryhaline crustaceans have identified conserved osmoregulatory adaptions allowing hyper-osmoregulation in dilute waters. However, previous studies have mainly examined decapod brachyurans with marine ancestries inhabiting estuaries/tidal creeks on a seasonal basis. Here, we describe osmoregulation in the atyid *Halocaridina rubra*, an endemic Hawaiian shrimp of freshwater ancestry from the islands' anchialine ecosystem (coastal ponds with subsurface fresh water and seawater connections) that encounters near-continuous spatial and temporal salinity changes. Given this, survival and osmoregulatory responses were examined over a wide salinity range. In the laboratory, *H. rubra* tolerated salinities of ~0–56‰, acting as both a hyper- and hypo-osmoregulator and maintaining a maximum osmotic gradient in freshwater of ~868 mOsm/kg H_2O. Furthermore, hemolymph osmolality was more stable during salinity transfers relative to other crustaceans. Silver nitrate and vital mitochondria-rich cell staining suggest all gills are osmoregulatory, with a large proportion of each individual gill functioning in ion transport (including when *H. rubra* acts as an osmoconformer in seawater). Additionally, ion transporters and supporting enzymes that typically undergo up-regulation during salinity transfer in osmoregulatory gills (i.e., Na^+/K^+-ATPase, carbonic anhydrase, $Na^+/K^+/2Cl^-$ cotransporter, V-type H^+-ATPase, and arginine kinase) did not alter expression in *H. rubra* during similar transfers. These

results suggest *H. rubra* (and possibly other anchialine species) maintains high, constitutive levels of gene expression and ion transport capability in the gills as a means of potentially coping with the fluctuating salinities ranges that are encountered in anchialine habitats. Thus, anchialine taxa represent an interesting avenue for future physiological research.

4.2 Introduction

Euryhaline crustaceans can function as strong osmoregulators that maintain internal osmotic concentrations above the external environment when in dilute seawater (reviewed by Mantel and Farmer, 1983) through active salt transport across the gills (reviewed by Henry et al., 2012; McNamara and Faria, 2012). In seawater, the overwhelming majority of marine crustaceans act as osmoconformers, with internal hemolymph osmolality mirroring concentrations in the ambient medium (Henry 2001; Henry et al., 2012). However, during transfers to waters below ~26‰, hyper-osmoregulatory mechanisms are activated in euryhaline crustaceans (Henry, 2005). This physiological transition enables survival in the fluctuating salinity of environments such as estuaries, allowing euryhaline species to take advantage of these highly productive environments (Gross, 1972) without competition from stenohaline species. Although some marine osmoconformers can survive in salinities as low as ~10‰, their lower limit is typically in the range of 16–18‰ (Kinne, 1971; Hsueh et al., 1993). Thus, only osmotic/ionic regulators are capable of traversing wide salinity ranges like those spanning from seawater (SW; 35‰) to freshwater (FW; near 0‰).

Osmoregulation in euryhaline crustaceans takes place in the mitochondrion-rich cells (MRCs) of the posterior gills, which are characterized by a thick (10–20 μm) osmoregulatory eplithelium, in contrast to the anterior gills, which are characterized by a thin (1–2 μm) respiratory epithelium (Taylor and Taylor, 1992; Freire et al., 2008). Active salt absorption in the MRCs is accomplished via a suite of ion transporters and supporting enzymes (Evans et al., 2008; Henry et al., 2012). In this context, Na^+ absorption occurs via a combination of apical Na^+/H^+ exchange, $Na^+/K^+/2Cl^-$ co-transport, Na^+ channels, and the basolateral Na^+/K^+-ATPase (NKA), while Cl^- absorption is accomplished via apical co-transport, Cl^-/HCO_3^- exchange, and basolateral Cl^- channels (reviewed in Freire et al., 2008; Charmantier et al., 2009; Henry et al., 2012; McNamara and Faria, 2012). Of these, NKA, which establishes the required electrochemical gradient for ion transport into the hemolymph, and cytoplasmic carbonic anhydrase (CA), which produces H^+ and HCO_3^- needed to support Na^+/H^+ and Cl^- $/HCO_3^-$ exchange, have been extensively studied during salinity acclimation in crustaceans (e.g., Towle et al., 1976; Henry and Cameron, 1982a; Towle and Kays, 1986; Henry, 2001). Overall, these enzymes generally have higher activities: 1) in euryhaline vs. stenohaline crustaceans (Henry, 1984; Harris and Bayliss, 1988), but see Piller et al. (1995), 2) in gills vs. other tissues (e.g., Henry, 2001; Lucu and Flik, 1999), 3) in osmoregulatory vs. respiratory gills (Henry, 1984; Holliday, 1985; Bottcher et al., 1990), and 4) during transfers from high to low salinities (but confined to the osmoregulatory gills) (Henry and Watts, 2001; Henry et al., 2002, 2003; Henry, 2005; Roy et al., 2007; Torres et al., 2007; Lucu et al., 2008). During low-salinity transfers, the need for

increased ion transport drives MRC proliferation and associated processes in the osmoregulatory gill lamellae (Neufeld et al., 1980; Lovett et al., 2006).

The expression of osmoregulatory genes has been investigated following salinity transfer and a meta-analysis identified them as usually increasing in the osmoregulatory gills, with transfers from higher to lower salinities over 1–3 days inducing the greatest increases in expression (Havird et al., 2013). For example, when transferred from 30‰ to 2‰ *Chasmagnathus granulatus* (subsequently renamed *Neohelice granulata*; Sakai et al., 2006) increased NKA expression 33–55 fold (Luquet et al., 2005). Similarly, cytoplasmic CA (CAc; the osmoregulatory isoform of CA) underwent a 100-fold increase in expression during transfers in *Callinectes sapidus* and *Carcinus maenas* (Serrano et al., 2007; Serrano and Henry, 2008). Analogous patterns have been reported for additional osmoregulatory genes, including the $Na^{+}/K^{+}/2Cl^{-}$ co-transporter (NKCC) and the V-type H^{+}-ATPase (HAT), in other marine euryhaline crustacean species (Luquet et al., 2005). Additional physiological and biochemical differences between marine and freshwater species also exist in regards to osmoregulation. For example, while marine species tend to have gill epithelia with high conductance (”leaky”), resulting in high rates of diffusive ion loss in low salinity, freshwater species typically have low conductance (“tight”) epithelia that hinders ion loss (reviewed in Henry et al., 2012). NKA activity is also uniformly distributed across all gills in the euryhaline freshwater crayfishes *Pacifastacus leniusculus* and *Procambarus clarkii* (Wheatly and Henry, 1987; Dickson et al., 1991), unlike their marine counterparts (see above). Moreover, crayfishes tend to produce hypo-osmotic urine to prevent further salt loss in lower salinities while most marine euryhaline crustaceans produce iso-osmotic urine (Riegel, 1968; Cameron and Batterton, 1978;

Wheatly and Henry, 1987). Freshwater species also tend to be weaker hyper-osmoregulators in FW, maintaining a hemolymph osmolality at ~370-450 mOsm above ambient medium (Mantel and Farmer, 1983), compared to ~600 mOsm in species such as *C. sapidus* and *Eriocheir sinensis* (Cameron, 1978; Onken, 1999).

The majority of osmoregulatory studies have focused on euryhaline crustaceans with marine ancestry that only spend part of their life cycle in dilute or fresh water. Given this, extending such studies to other taxonomic/ecological groups could provide further insight into the evolution of osmoregulatory mechanisms. Crustaceans from the anchialine ecosystem represent an interesting opportunity to do so, as this ecosystem consists of coastal caves and ponds lacking surface connections to the open ocean, but which are influenced by both seawater and freshwater through underground connections (Holthuis, 1973; Sket, 1996). Accordingly, organisms from these habitats can experience daily fluctuations in salinity due to tides comparable to those in estuaries (e.g., 20‰ over 24 hours; Maciolek, 1986). While anchialine habitats have a worldwide distribution, they are most concentrated in the Hawaiian Islands, with ~600 of the ~1000 known anchialine habitats found there (Maciolek and Brock, 1974; Brock, 1987; Brock et al., 1987). The most common and abundant macro-organism of the Hawaiian anchialine ecosystem is the small (~10 mm), endemic shrimp *Halocaridina rubra* Holthuis, 1963 (Decapoda, Atyidae). This species is found in salinities ranging from 2–36‰ (Maciolek, 1983) and can be acclimated to salinities ranging from ~0–50‰ in the laboratory (Holthuis, 1973; Maciolek, 1983). Moreover, the family Atyidae has a decisively freshwater ancestry since 1) freshwater deposits of atyids date to the Cretaceous (Glaessner, 1969; Smith and Williams, 1981); 2) no extant marine atyids are known (Fryer, 1977), and; 3) while some

species require salt or brackish water for larval development (Hunte, 1979a, b), most adult atyids outside the "anchialine clade" are intolerant of seawater (Smith and Williams, 1981; von Rintelen et al., 2012). However, detailed osmoregulatory studies have yet to be performed for *H. rubra* or any other anchialine organism. Here, the osmoregulation of *H. rubra* was examined at the environmental to the molecular level.

4.3 Materials and methods

4.3.1 Haloclines in Hawaiian anchialine habitats

To determine the magnitude of salinity change *H. rubra* might encounter spatially in the water column, potential vertical salinity gradients (i.e., haloclines) were measured from 12 anchialine habitats on the islands of Hawaii, Maui, and Oahu (Fig. 1). Field measurements were taken every 15 cm from the habitats' surface to bottom with a handheld YSI conductivity meter (Yellow Springs Inst, Yellow Springs, OH). Additionally, for a subset of seven habitats, water samples were collected in a vertical transect every 30 cm, frozen, and measured for osmolality using a vapor pressure osmometer (Wescor 5100C, Logan, UT, USA). For the other five habitats, YSI measurements were also recorded ~12 hours following rainfall.

4.3.2 Animals

Individuals of *Halocaridina rubra* Holthuis, 1963 were collected from Cape Hanamanioa (HM), Maui using hand nets and shipped to Auburn University, AL within ~2 days of collection. Because *H. rubra* across the Hawaiian Islands represents at least eight distinct genetic lineages (Craft et al., 2008), it is important to note that most animals

used in this study (except for larval experiments, see below) were from the South Maui lineage. In the laboratory, shrimp were held at 15‰ with ~200 animals per 38 L aquarium, no circulating water, and no feeding. Shrimp were allowed to graze on the microbial/algal growth lining porous volcanic rock present in the aquaria. This husbandry technique is ideal for *H. rubra,* yielding continuous, year-round reproduction in the laboratory.

4.3.3 Osmolality during salinity transfer

Prior to experiments, *H. rubra* individuals were acclimated to 32‰ in 4 liter aquaria for at least 1 month. Shrimp were then transferred to 15‰ and sampled at 0h, 2h, 6h, 12h, 24h, 48h, and 96h post-transfer. Hemolymph was extracted by anaesthetizing shrimp on ice, wicking off any surface water using Kimwipes® (Sigma-Aldrich, St Louis, MO, USA), and then lacerating the outer carapace of the shrimp with a scalpel blade. Six shrimp per sample were pooled into a Corning® Costar® Spin-X® 0.22 µm centrifuge filter tube (Sigma-Aldrich), with n = 3–5 samples per time point. This pooling scheme was necessary to obtain enough hemolymph for quantification. Tubes were centrifuged at 14,000 rpm for 5–10 min, with cellular tissue debris being retained by the filter and preventing contamination of the hemolymph sample. Approximately 10 µL of hemolymph was recovered per sample and frozen at –80°C until osmolality was measured on a vapor pressure osmometer (Wescor 5100C). Based on this initial time series (see Results), shrimp acclimated to 32‰ were also transferred to ~0–56‰, followed by sampling after 48 hours using the same pooling strategy, with n = 3–6 samples per salinity. Mortality was only observed at 56‰, where 50% of shrimp perished

within 48 hrs. Salinities of the experimental media were confirmed using the vapor pressure osmometer (Wescor 5100C).

To confirm that the above centrifugation method produced hemolymph samples with minimal intracellular fluid leakage, three hemolymph samples were subjected to inductively-coupled plasma optical emission spectrometry (ICP-OES; PerkinElmer Optima 7000 DV, Waltham, MA, USA) to measure $[K^+]$. On average, $[K^+]$ was 33.4 ± 0.52 mmol L^{-1}, which is higher than reported for *Litopenaeus vannamei* hemolymph (16 mmol L^{-1}; Sowers et al., 2006), but much lower than typical intracellular $[K^+]$ (120-150 mmol L^{-1}). This suggests intracellular leakage was minimal. Moreover, intracellular osmolality is in equilibrium with hemolymph, thus leakage of a specific ion should not alter hemolymph osmotic concentration significantly.

4.3.4 Silver nitrate ($AgNO_3$) and MRC staining of gills

To determine sites of ion transport, whole animals chronically acclimated to 15‰ were rinsed three times with deionized water, stained with 0.05% silver nitrate ($AgNO_3$) while shaking for 20 m, and again rinsed three times with deionized water. This was followed by incubation for 1 hr in saturated Kodak D-76 developer (Eastman Kodak, Inc., Rochester, NY, USA) while shaking, followed by a single rinse with deionized water. Here is a random three lines about the lobster phone. True story: during my comprehensive written exams I randomly wrote “lobster phone” in one of the answers to see if anyone reads these things. I never heard about anything from my committee about it, so I’m assuming no one will mention these lobster phone related lines either. $AgNO_3$ staining blackens transport epithelia, which are permeable to silver and/or ions, through

the production of AgCl (Croghan, 1958; Holliday et al., 1990; Kikuchi and Shirashi, 1997). Animals were photographed using a S8 APO Stereo Microscope (Leica Microsystems, Wetzlar, Germany) at 1–8X magnification. To determine if salinity influenced $AgNO_3$ staining, animals were chronically acclimated to either 2‰ or 32‰ for at least one month (n =10 per salinity) and then stained and photographed using the above protocol. The fraction of each gill stained was quantified using ImageJ v1.45s (National Institutes of Health, Bethesda, MD, USA) (Schneider et al., 2012). The fraction stained was modeled as a function of salinity, gill number, and the interaction between the two. A random effect of individual was also included since both left and right gills were measured per individual (twenty gills per salinity per gill number in total). Overall, this model addressed specifically whether 1) salinity influenced staining; 2) staining correlated with gill number, and; 3) only specific gills responded to salinity.

To investigate transport epithelia during development, *H. rubra* larvae were also stained with $AgNO_3$ using the above protocol. Larvae came from 5 of the 8 genetic lineages found across the Hawaiian Islands (Fig. 1) and were collected from distinct colonies maintained in the laboratory for ~7 years. The developmental stage of each larva ($Zoea_1$–$Zoea_4$) was scored based on morphological features as described in Couret and Wong (1978) and Iwai (2005).

To more closely investigate changes in the gills of *H. rubra* during salinity transfer, MRC density was quantified using 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium iodide (DASPMI) staining and confocal microscopy (van der Heijden et al., 1997; Choe et al., 1999). Shrimp were acclimated to either 2‰ or 32‰ (n = 10 per salinity) for one month, anaesthetized on ice and their "gill undercarriage" dissected and

rinsed in a 700 mmol L^{-1} NaCl solution (i.e., a "shrimp Ringers" consistent with *H. rubra* hemolymph during hyper-osmoregulation). This "gill undercarriage" consisted of the gills and a minimum amount of supporting musculature/exoskeletal material. It was used because individual gills were too small and delicate to be dissected separately. Following rinsing, "gill undercarriages" were incubated in "shrimp Ringers" containing 25 µM DASPMI (Molecular Probes®, Invitrogen, Carlsbad, CA, USA) for one hr while shaking at room temperature to label MRCs prior to being rinsed with "shrimp Ringers" lacking DASPMI (Karnacky et al., 1984). "Gill undercarriages" were then placed on a glass slide with a concavity in a single drop of "shrimp Ringers" and covered with a coverslip to prevent desiccation. Vital MRC staining was visualized with a Nikon A1 confocal laser scanning microscope (Nikon Instruments Inc., Melville, NY, USA) with the excitation and emission filter set for Fluorescein isothiocyanate. For each salinity treatment, the area fraction stained of four central lamellae was quantified from each gill using ImageJ.

4.3.5 Expression of osmoregulatory genes during salinity transfer

To quantify gene expression in *H. rubra* during salinity transfer, shrimp were chronically acclimated to 32‰ for one month and then transferred to 2‰, 15‰, or 45‰. For the transfer to 15‰, shrimp were sampled before transfer (i.e., at 32‰) and at 3h, 8h, 24h, 48h, and 7d post-transfer. Based on those results and previous studies (Luquet et al., 2005; Serrano et al., 2007; Havird et al., 2013), shrimp transferred to 2‰ and 45‰ were sampled at 24h and 7d post-transfer. Shrimp were anaesthetized on ice and "gill undercarriages" (see above) dissected into tubes containing ice-cold denaturing solution from the RNAgents® Total RNA Isolation System (Promega, Madison, WI, USA). The

remaining tissue from each shrimp (i.e., musculature, digestive tract, nervous system) was also utilized as a "control" tissue, except for the 15‰, 3 h post-transfer treatment. For each treatment, 3–6 shrimp (depending on size) were pooled for a single sample, with n = 5–6 samples per treatment. Notably, such physiological studies have been identified as committing pseudoreplication because they often utilize "… a single tank, containing a fixed number of fish [or shrimp], for each experimental treatment…" (Hurlbert, 1984: 195). Given this, shrimp from a single sample were housed in individual ~400 mL containers during the experimental transfers to avoid pseudoreplication.

Total RNA was isolated from gill and control tissues by phenol-chloroform extraction using the RNAgents® Total RNA Isolation System (Promega) substituted with phenol-chloroform-isoamyl alcohol (#P2069) from Sigma (St. Louis, USA). RNAse-free conditions were maintained during dissections and tissue homogenizations by using sterile tools rinsed with RNAse-free water and RNAse-Zap (Ambion, Austin, TX, USA). Total RNA concentration was quantified for each sample using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and RNA quality/quantity checked for a representative number of samples using a Bioanalyser 2100 (Agilent Technologies, Wilmington, DE, USA). No genomic DNA contamination was observed in these examined samples. Poly-A RNA in 2 µg of total RNA per sample was reversed transcribed using Superscript II® reverse transcriptase with an oligo-dT primer (Invitrogen), thus normalizing each sample to total RNA (Bustin, 2002). The resulting cDNA was checked by polymerase chain reaction (PCR) and subjected to quantitative real-time PCR (qRT-PCR, see below) analyses.

To compare *H. rubra* mRNA expression of genes previously identified as playing roles in crustacean osmoregulation during salinity transfer (Luquet et al., 2005; Serrano et al., 2007; Havird et al., 2013), primers for six genes were designed: Na^+/K^+-ATPase α-subunit (NKA), the cytoplasmic, osmoregulatory isoform of carbonic anhydrase (CAc), the membrane-associated, respiratory isoform of CA (CAg), the $Na^+/K^+/2Cl^-$ cotransporter (NKCC), the H^+-ATPase (HAT), and arginine kinase (AK). These primers were specifically designed for *H. rubra* using transcriptomes generated from the East Hawaii and Windward Oahu genetic lineages (EP and HILO, see Craft et al., 2008; Fig. 1) that are publicly available as searchable BLAST databases (www.auburn.edu/~santosr/halo_blast.htm). Homologs for the six genes in *H. rubra* were identified by downloading sequences for each from *C. sapidus*, *C. maenas*, and other crustaceans available on GenBank. These were then utilized as queries in BLASTX (Altschul et al., 1990) searches against the two *H. rubra* transcriptomes. Potential open reading frames were extracted from resulting matches and qRT-PCR primers were designed to regions with 100% identity between the two genetic lineages to minimize any differences that might arise in the South Maui lineage. Lastly, primers were designed (when possible) to span a conserved intron/exon boundary known from other crustacean homologs in order to avoid amplification of any contaminating genomic DNA (Choe et al., 2006). The qRT-PCR primers (Table 1) were tested by PCR of template cDNA to ensure generation of a single amplicon from *H. rubra* of the South Maui genetic lineage.

For each of the six genes, mRNA levels were assessed per sample via qRT-PCR on an ABI 7500 Real-Time PCR System thermocycler (Applied Biosystems®, Foster City, CA, USA) using the iQ™ SYBR® Green Supermix Kit (Bio-Rad laboratories,

Hercules, CA, USA). Reactions (25 µL) were run in triplicate and consisted of 12.5 µL iQ™ SYBR® Green mix (2X), 10.9 µL nuclease-free water, 0.3 µL forward and reverse primers (25 µmol l^{-1}; Table 1), and 1 µL template cDNA, using the following cycle parameters: initial denaturing for 3 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 1 min at 55°C (NKA and CAc), 58°C (HAT), or 60°C (NKCC, CAg, and AK). Following the last cycle, melting curve analyses (from 55°C to 95°C with a heating rate of 0.5°C every 5 sec) were performed to confirm that reactions produced only a single amplicon. Standard curves of threshold cycle (C_t) as a function of template availability (C_t vs. $\log_{10}$ cDNA volume) were generated by serial dilution of a single sample from the 2‰ 24h treatment, as this was predicted *a priori* to have the highest levels of gene expression among the treatments based on previous studies (Luquet et al., 2005; Serrano et al., 2007; Havird et al., 2013). C_t was determined for each gene such that the R^2 of the linear regression of the standard curve was maximized for the initial reaction; C_t was then held constant for each subsequent reaction. Relative expression was then quantified using SDS v1.2 (Applied Biosystems®).

4.3.6 Statistical analyses

Statistical analyses were performed in the R v2.12.0 statistical environment (code available on request; R Core Team 2013).

4.4 Results

4.4.1 Haloclines in Hawaiian anchialine habitats

Measurements for vertical salinity gradients from 12 Hawaiian anchialine habitats revealed that while variation existed between habitats (e.g., 2.8-20.9‰), most lacked a detectable vertical gradient from the surface to the bottom (i.e., possessed the same salinity across depth; Fig. 2A). For the three habitats with vertical gradients, salinity increased measurably with depth (Fig. 2B). Field instrument-based and laboratory osmometer salinity measurements were not identical for the seven habitats where both were taken; thus, the average of the two was used. Lastly, surface lenses of low-salinity water extending down 2–3 cm from the surface were measured in some habitats following rainfall (Fig. 2C). Notably, shrimp were observed moving freely in and out of this lens, as well as between the surface and bottom in habitats with vertical salinity gradients (Movie 1).

4.4.2 Osmotic gradients during salinity transfer

Hemolymph osmolality of *H. rubra* chronically acclimated to seawater (32‰; 960 ± 1.6 mOsm kg^{-1} H_2O^{-1}) reflected that of an osmoconformer (i.e., 958 ± 30.6 mOsm kg^{-1} H_2O^{-1}). After transfer to 15‰ (450 mOsm kg^{-1} H_2O^{-1}), hemolymph osmolality remained elevated (1000 ± 10.8 mOsm kg^{-1} H_2O^{-1}) 24 h post-transfer before dropping to ~700 mOsm kg^{-1} H_2O^{-1} by 2d post-transfer and remaining at that level until the end of the four day experiment (Fig. 3A). Because hemolymph osmolality stabilized by 2 d after transfer, this time point was used to quantify hemolymph osmolality after transfers from 32‰ to ~0–56‰. After transfers to lower salinities (0–25‰), hemolymph osmolality remained at levels higher than the ambient medium (898 vs. ~30 mOsm kg^{-1} H_2O^{-1} at FW; 825 vs. 153 mOsm kg^{-1} H_2O^{-1} at 5‰; 727 vs. 317 mOsm kg^{-1} H_2O^{-1} at 10‰; 703

vs. 443 H_2O^{-1} mOsm kg^{-1} H_2O^{-1} at 15‰; 860 vs. 585 mOsm kg^{-1} H_2O^{-1} at 20‰; 927 vs. 742 mOsm kg^{-1} H_2O^{-1} at 25‰), reflecting a strong hyper-osmoregulatory response (Fig. 3B). In freshwater, hemolymph osmolality was ~868 ± 60.3 mOsm kg^{-1} H_2O^{-1} higher than that of the ambient medium. For transfers to hyper-saline conditions (i.e., 40–56‰), hemolymph osmolality remained below ambient (Fig. 3B). Notably, hypo-osmoregulation was still detected at even the highest examined salinity (1428 ± 9.5 mOsm kg^{-1} H_2O^{-1} hemolymph vs 1692 mOsm kg^{-1} H_2O^{-1} medium).

4.4.3 Ultrastructure, ion transport staining, and MRCs in gills

$AgNO_3$ staining of whole shrimp chronically acclimated to 15‰ identified the pleurobranchs of the 5th–8th thoracomeres (i.e., the gills) as being heavily stained (Fig. 4A), each with 10–16 pairs of lamellae pointing anteriorly and dorsally. The gills are phyllobranchiate, which are typical of caridean shrimps, with plate/leaf-like lamellae extending from both sides of the flattened, central gill shaft (Freire et al., 2008). There were no apparent major differences in morphology or $AgNO_3$ staining between anterior vs. posterior gills or between shrimp acclimated to 2‰ (Fig. 4B) and 32‰ (Fig. 4C). No significant difference in the area fraction of each gill stained was detected between the 2‰ and 32‰ treatments (linear fixed-effects model, $P = 0.13$, Fig. 5A). However, posterior gills tended to have a smaller fraction of area stained than more anterior gills (linear fixed-effects model, $P < 0.01$, Fig. 5A). Lastly, there was no significant interaction between salinity and gill number effects (F-drop test, $P = 0.24$), suggesting all gills (i.e., anterior vs. posterior) responded similarly to salinity.

$AgNO_3$ staining of *H. rubra* $Zoea_1$–$Zoea_4$ larvae identified low levels of ubiquitous staining in the exoskeleton of early zoeal stages (Fig. 4D–F). Clearly stained gills were not observed until the $Zoea_4$ stage (Fig. 4G). Relative to adult gills, $Zoea_4$ stage gills were undeveloped, with only buds of a few lamellae detected per gill (Fig. 4H). However, gills were the only structures with consistent and heavy staining in $Zoea_4$.

Vital staining using DASPMI revealed an abundance of MRCs in the gills when shrimp were acclimated to both 2‰ (Fig. 4I) and 32‰ (Fig. 4J). Furthermore, populations of MRCs were identified in gill lamellae under both salinities (Fig. 4K,L), with vessels within the lamellae readily visibly (Fig. 4K). MRCs were distributed evenly throughout each lamella, absent around their perimeters (Fig. 4L), and were not confined to particular gills. Instead, a large fraction (> 80%) of all gills and lamellae fluoresced under both salinities (Fig. 5B, C). There were no statistical differences in gills 1 and 2 between the area fraction of lamellae that fluoresced between the 2‰ and 32‰ treatments (*t*-test, $P > 0.225$ for all comparisons, Fig. 5B). Although there was no significant difference between salinity treatments in the posterior-most lamellae examined of gill 3 (*t*-test, $P = 0.097$), the other three lamellae of this gill had a significantly larger area fraction that fluoresced in 2‰ vs. 32‰ (*t*-test, $P < 0.046$ for all comparisons, Fig. 5C). Lastly, all lamellae had a significantly larger area fraction that fluoresced in 2‰ vs. 32‰ for gill 4 (*t*-test, $P < 0.049$ for all comparisons, Fig. 5C).

4.4.4 Gene expression during salinity transfer

Homologs of NKA, CAc, CAg, NKCC, HAT, and AK were identified from the *H. rubra* transcriptome data, including, in many cases, the 5' and 3' untranslated regions

of these genes (GenBank KF650058-KF650070). For NKA, two isoforms, differing by a 27 AA insertion near the C-terminus, were identified. The smaller isoform (similar to the NKA of *Penaeus monodon*) was recovered from both the East Hawaii and Windward Oahu genetic lineages, while the larger isoform (similar to the NKA of *C. sapidus*) was only identified from the East Hawaii genetic lineage. Given this, the NKA qRT-PCR primers were designed to amplify either isoform. While complete CAc and CAg transcripts were recovered from the Windward Oahu genetic lineage, only a partial (228 bp, 22% of total length) CAg transcript was identified from the East Hawaii genetic lineage. Full-length transcripts of NKCC were obtained from both genetic lineages as well as a partial transcript of a second NKCC isoform (76% identical in AA sequence across 98 residues to the other isoform) from the East Hawaii genetic lineage. Finally, full-length transcripts for both HAT and AK were obtained from both the East Hawaii and Windward Oahu genetic lineages. For those genes where transcripts were obtained from both genetic lineages, sequences were nearly 100% identical in the coding region between the two, thus facilitating the design of primer sets for qRT-PCR.

Unexpectedly, salinity transfers did not alter the expression of ion transporters and supporting enzymes in the gills of *H. rubra* in the same manner as in previously studied crustaceans (e.g., Luquet et al., 2005). For example, gill NKA expression did not change significantly during transfer from 32‰ to 15‰ until 24 h after transfer, when expression decreased to 26% of the initial value (*t*-test, $P = 0.02$) and returned to being statistically indistinguishable from initial levels by 2 d post-transfer (remaining as such until the end of the experiment 7 d post-transfer; Fig. 6A). During the transfer to 2‰, NKA levels were statistically similar to initial levels at 32‰ while NKA levels decreased

to 35% of the initial value 24 h after transfer to 45‰ (*t*-test, $P = 0.03$) before returning to initial levels by 7 d after transfer. At 24 h, NKA levels were significantly higher (~2-fold) in the gills of animals transferred to 2‰ than those transferred to 45‰ or 15‰ (ANOVA, $P < 0.01$). Similar trends to NKA were evident in the expression of NKCC and AK during salinity transfers (Fig. 6B,C), with NKCC expression increasing ~2.6 fold compared to the initial level 24 h after transfer to 2‰. In most cases, CAc expression did not change significantly from the initial level or among animals acclimated to the three experimental salinities at either 24 h or 7 d post-transfer, except for a 76% reduction of the initial level 48 h after transfer to 15‰ (*t*-test, $P = 0.03$, Fig. 6D). Overall, no changes in gill CAg expression were detected during any, or between, treatments (Fig. 6E). Lastly, HAT expression decreased significantly in all transfers between 3 h and 48 h, with the most drastic being 24 h post-transfer to 45‰, where expression levels were 50-fold lower than the initial level (*t*-test, $P < 0.01$, Fig. 6F). Notably, at 24 h post-transfer, HAT levels were significantly higher (~2.7-fold) in the gills of animals transferred to 2‰ than those transferred to 45‰ or 15‰ (ANOVA, $P < 0.01$, Fig. 6F). By 7 d post-transfer, HAT expression levels returned to their initial level and were not significantly different between salinity treatments (Fig. 6F).

In most cases, gene expression was also not significantly altered during salinity transfers in the control tissue (i.e., the remainder of shrimps' bodies excluding the "gill undercarriage"). Specifically, expression of NKA in the control tissue was not significantly different than the initial level in any transfer until 7 d post-transfer, when levels decreased in all three salinity treatments (*t*-test, $P < 0.03$ for all, Fig. 7A). A similar trend was noted for AK (Fig. 7B). For NKCC, no significant changes in expression were

observed, with the exception of a ~2.5-fold increase 3 h after transfer to 15‰ (*t*-test, P = 0.02, Fig. 7C). Likewise, CAc expression in the control tissue showed a single significant change relative to the initial level, decreasing ~30% 7 d after transfer to 45‰ (*t*-test, P = 0.04, Fig. 7D) while CAg decreased ~50% by 7d post-transfer in all transfers (*t*-test, $P <$ 0.02 for all, Fig. 7E). Finally, HAT expression in the control tissue did not change significantly with any treatment (Fig. 7F). Overall, there were no differences in expression levels in the control tissue between salinity treatments at either 24 h or 7 d post-transfer for any of the six genes (Fig. 7).

4.5 Discussion

Understanding osmoregulatory processes of organisms can be facilitated by knowing the salinity regimes they encounter in their natural environment. For anchialine habitats, temporal changes in salinity due to tidal influences are a well-known and defining characteristic of this ecosystem (Maciolek, 1986; Sket, 1996). In some anchialine habitats, spatial changes in salinity have also been documented. For example, strong vertical salinity gradients have been recorded from Dalmatian, Bahamian, and Australian anchialine habitats, with surface waters at 0‰ and those at 6 m depth approaching 36‰, with haloclines at ~1 m (Sket, 1996; Humphreys, 1999; Iliffe, 2000; Pohlman, 2011). Previously, Holthuis (1973) noted that anchialine habitats on Maui's southern coast nearly always possessed vertical salinity stratification (although no data were given), consistent with the salinity gradients of 15‰ to 30‰ reported here for deeper habitats from the same island and geographic region (Fig. 2B). Furthermore, high salinity waters (i.e., seawater) likely occur in the fissures leading to the hypogeal

environment of the habitats and surface freshwater lenses were apparent following a rainfall event (Fig. 3C). Thus, while many of the habitats exhibited no vertical salinity stratification (Fig. 2A), these environmental data suggest *H. rubra* can encounter variable salinity regimes spatially as well as temporally. Of particular interest to this study is how *H. rubra* copes with the wide environmental salinities (e.g., 2.8–30‰) these shrimp naturally encounter in anchialine habitats.

While *H. rubra* acts as an osmoconformer at oceanic salinities (32‰), it transitions to osmoregulation at lower salinities, similar to previously studied euryhaline crustaceans (e.g., Zanders, 1980; Henry and Watts, 2001; Chung and Lin, 2006; Faleiros et al., 2010), including another atyid species (Born, 1968). However, “strong” osmoregulating crustaceans such as the blue crab (*Callinectes sapidus*) maintain an osmotic gradient between the external medium and their hemolymph of ~600 mOsm kg^{-1} H_2O^{-1} (Cameron 1978; Henry 2001). In contrast, the finding that *H. rubra* maintains a gradient of ~868 mOsm kg^{-1} H_2O^{-1} when transferred to freshwater (Fig. 3B) suggests this anchialine shrimp species may be among the strongest osmoregulators documented, with a meta-analysis of hemolymph osmolality during salinity transfer for eight other euryhaline crustacean species supporting this conclusion (Havird et al., 2013). Interestingly, other crustaceans with a similar freshwater ancestry, such as crayfish, maintain a gradient of 370–450 mOsm kg^{-1} H_2O^{-1} and are considered “weak” osmoregulators (Mantel and Farmer, 1983). An exception to this (besides *H. rubra*) may be the Chinese mitten crab (*Eriocheir sinensis*), which spends most of its adult life in freshwater habitats and maintains an osmotic gradient of 550–700 mOsm kg^{-1} H_2O^{-1} (Onken, 1999). However, this species likely represents a secondary freshwater invasion

by a member of a marine family, while *H. rubra* represents an invasion of a euryhaline habitat by a member of a freshwater-adapted family. This suggests *H. rubra*, and potentially other anchialine atyid shrimp, may be unique in maintaining strong osmotic gradients despite having an evolutionary history tied to freshwater environments.

Unlike *H. rubra*, most euryhaline crustaceans rapidly decrease hemolymph osmolality to new levels following transfer from seawater to lower salinities. For example, *C. sapidus* noticeably lowers hemolymph osmolality by 3 h after transfer and reaches stable levels by 12 h (Henry and Cameron, 1982b). Here, *H. rubra* maintained hemolymph osmolality similar to that found in seawater until 2 d after transfer before levels stabilized (Fig. 3A). One possible explanation for this difference is that other crustaceans such as *C. sapidus* might "commit" to a specific salinity as part of annual migrations in their natural life cycle (e.g., spring migration into the estuary for blue crabs; Warner, 1976). Therefore, when they undergo a salinity change, it is likely to be a chronic transfer, and activating osmoregulatory pathways to cope with the new salinity is advantageous. Given the constantly fluctuating salinities *H. rubra* encounters in anchialine habitats, prematurely "committing" osmoregulatory pathways to cope with a new salinity, only to switch back to the original state within minutes or hours, may be energetically expensive and inefficient, and thus has been selected against. Rather, this species may maintain constantly active osmoregulatory mechanisms for coping with low salinities even when at high salinities since in nature low salinities are regularly encountered. This is supported by freshwater crustaceans typically having "tight" gill epithelia, which would reduce diffusive ion loss and contribute to maintaining the high osmotic gradient seen in *H. rubra*.

$AgNO_3$ staining revealed the gills as the likely primary site of ion transport in *H. rubra*, as is typical of crustaceans and fishes (Evans et al., 2005; Henry et al., 2012), with *H. rubra* gills being morphologically similar to those of the anchialine atyid *Typhlatya arfeae* (Jaume and Bréhier, 2005). Generally, the gills of *H. rubra* appear less complex than other euryhaline crustaceans, with fewer gills and fewer lamellae per gill (e.g., 4 vs 8 gills and 10–16 vs. ~300 lamellae per gill compared to *C. sapidus*; Lovett et al., 2006). This, combined with their thick, plate-like lamellae, implies gills with a lower surface area. In contrast to most previously studied crustaceans, overall $AgNO_3$ staining did not change significantly with transfer to low salinity, although there was a trend towards greater staining in 2‰ vs. 32‰ (linear fixed-effects model, $P = 0.13$, Fig. 5A). Furthermore, when considered individually, the most posterior gill (i.e., gill 4; Fig. 4B,C) showed a statistically significant increase in total area stained from 59% in 32‰ to 72% in 2‰ (Student's unpaired *t*-test, $P = 0.003$). In contrast, $AgNO_3$ staining never exceeds more than 30% of the total lamellar surface area for the moderate osmoregulator *Carcinus maenas* (Compere et al., 1989) and for strong-osmoregulators, $AgNO_3$ staining went from 35% of the lamellae in the osmoregulating gills to 52% during transfer from seawater to 12‰ for *C. granulatus* (Genovese et al., 2000) and increased 4-fold during low salinity transfer for *C. sapidus* (Neufeld et al., 1980). Thus, the increased staining seen in gill 4 of *H. rubra* suggests either a fairly weak response to salinity changes or, more likely, that the MRC population is already at or near its maximum and large increases are not possible. As *H. rubra* had 72%–80% $AgNO_3$ staining in all gills when acclimated to 2‰ and such staining is a measure of MRC abundance, this suggests *H. rubra* may have relatively dense and constitutive populations of MRCs even in 32‰,

which would provide a mechanism for the high osmotic gradient recorded in this species. Furthermore, it suggests that the "osmoregulatory patch" found on lamellae of other euryhaline crustaceans (Lovett et al., 2006) represents a large fraction of the total gill surface area in *H. rubra*, even when acting as an osmoconformer. This is notable since crustacean gills are typically either respiratory or osmoregulatory in nature, with $AgNO_3$ staining typically confined to just the osmoregulating gills. For example, only the posterior gills undergo staining in *C. sapidus* and many other brachyurans (Copeland and Fitzjarrell, 1968), and while all gills show staining in the freshwater crayfish *Procambarus clarkii*, it is localized to the central filaments of each gill (Dickson et al., 1991). To place this into context, although gill 1 had 80% staining compared to 72% in gill 4 of *H. rubra*, the difference in staining between respiratory vs. osmoregulating gills of previously studied crustaceans is ~0% vs. ~25–50%, respectively (see above).

$AgNO_3$ staining of larval *H. rubra* revealed ion-transporting gills develop at later zoeal stages (i.e., $Zoea_4$), which corresponds to ~15 days post-hatch (Couret and Wong 1978; Iwai, 2005). This finding suggests that osmoregulatory capabilities in *H. rubra* likely do not develop until this life stage or later. This hypothesis is supported by studies of *Crangon crangon* (Cieluch et al., 2005), *Chasmagnathus granulatus* (Charmantier et al., 2002) and *Carcinus maenas* (Cieluch et al., 2004), where early zoeal stages are osmoconformers and osmoregulatory capabilities develop in later, juvenile stages. One proposed explanation for this ontological shift in osmoregulation involves the different habitats exploited by larvae vs. adults. For example, larvae of many estuarine and riverine crustaceans are "exported" into the ocean where they undergo development before returning as juveniles. Furthermore, many atyids also export larvae to the ocean, and

seawater is necessary for development in some species (Hunte 1979a, b). Because most crustaceans act as osmoconformers in oceanic salinities (e.g., Henry et al., 2012) and oceanic salinity tends to be constant, energetically expensive osmoregulatory mechanisms are less necessary for developing larvae. Interestingly, larvae of *H. rubra* can undergo successful development in a wide range of salinities, from potentially full strength seawater in the hypogeal (and hypothesized larval habitat) component of the anchialine ecosystem (Craft et al., 2008) to lower salinities in the laboratory (e.g., 15‰ in this study; 10–15‰ in Couret and Wong, 1978; 20‰ in Iwai, 2005). Therefore, while it is possible the ontogenetic shift in osmoregulation hypothesized for *H. rubra* is due to larvae acting as osmoconformers in seawater, how they survive in lower salinities, particularly in early life stages lacking developed and functional ion-transporting gills, remains unknown. Future studies should focus on the euryhalinity and osmoregulatory capabilities of *H. rubra* during these early life history stages towards addressing this question.

Vital MRC staining was consistent with $AgNO_3$ staining, as all gills/lamellae had dense MRC populations under both osmoconforming and hyper-regulating salinities. Although staining increased in posterior gills under hyper-regulating conditions, the magnitude of this increase was small (~7%) and likely not significant in a biological context. Thus, nearly the entire surface of the lamellae can be considered an "osmoregulatory patch" in *H. rubra*, with MRCs being distributed evenly throughout lamellae. This contrasts sharply with the "osmoregulatory patch" of *C. sapidus*, which increases from ~35% to 60% of the lamellar surface area during low salinity acclimation (Lovett et al., 2006). Taken together, the results of the $AgNO_3$ and vital MRC staining

suggest: 1) the gills of *H. rubra* have osmoregulatory mechanisms constitutively activated at the cellular level even at salinities where the species functions as an osmoconformer; 2) all gills participate in osmoregulation, and; 3) posterior gills appear to be the most responsive to salinity transfers.

In support of the hypothesis that *H. rubra* maintains constitutively activated mechanisms of ion regulation, expression of osmoregulatory genes in the gills of *H. rubra* generally showed little to no changes during salinity transfer (Fig. 6), with similar results obtained from control tissue (Fig. 7). In euryhaline crustaceans, expression of these genes usually increases dramatically in the osmoregulatory gills during comparable salinity transfers (reviewed by Havird et al., 2013). For example, NKA expression in *Chasmagnathus granulatus* increased 25–55 fold after transfer from seawater to 45‰ and 2‰ (Luquet et al., 2005), with similar results for *Scylla paramamosain* (Chung and Lin, 2006), *Pachygrapsus marmoratus* (Jayasundara et al., 2007), *Callinectes sapidus* (Serrano et al., 2007), *Carcinus maenas* (Serrano and Henry, 2008; Jillette et al., 2011), *Macrobrachium amazonicum* (Faleiros et al., 2010), and *Litopenaeus vannamei* (Wang et al., 2012). Although utilized in fewer studies, CAc (but not CAg, see Serrano and Henry, 2008), NKCC, HAT, and AK (e.g., Luquet et al., 2005) also follow this general trend. Only for NKCC was a comparable result seen in *H. rubra*, with a 2.6 fold increase in expression 24h after transfer to 2‰. However, this up-regulation is small compared to previous reports.

Why are well-characterized osmoregulatory genes not up-regulated in *H. rubra* as in other euryhaline crustaceans? One hypothesis is that expression of these genes is always at a relatively high level, even when *H. rubra* is functioning as an osmoconformer

in seawater. Support for this hypothesis comes from the $AgNO_3$ and MRC staining, which indicate elevated cellular mechanisms of osmoregulation in the gills of *H. rubra* regardless of salinity. Although it is unclear if this hypothesized chronic up-regulation of osmoregulatory processes is an adaptation to anchialine habitats, such a strategy may allow *H. rubra* to cope with the rapid and continuous salinity changes they encounter, and it will be interesting to see whether this pattern of elevated osmoregulatory processes is characteristic of euryhaline atyids or anchialine crustaceans in general. Finally, it is unlikely that using the "gill undercarriage" instead of individual gills skewed the expression results, as the genes under investigation do not significantly change in non-osmoregulatory tissues during salinity transfers (see Henry and Cameron, 1982a; Henry, 2001; Serrano et al., 2007; Henry et al., 2012).

In conclusion, this report represents the first attempts to describe osmoregulation from an anchialine crustacean. *Halocaridina rubra*, an endemic Hawaiian anchialine atyid shrimp, appears to represent one of the strongest osmoregulators described among crustaceans, maintaining an osmotic gradient of ~830 mOsm kg^{-1} H_2O^{-1} in freshwater. Notably, previously described osmoregulatory processes for euryhaline crustaceans do not adequately characterize *H. rubra*. Instead, osmoregulatory mechanisms typically activated in other crustaceans only under low salinity are constitutive in *H. rubra*. It is unclear if selection, evolutionary history, or a combination of both is responsible for this deviation from previous models. Future studies should continue to explore anchialine organisms' physiological responses to salinity as a means of developing a further understanding of osmoregulation in general.

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Table 1. Nucleotide sequences for *Halocaridina rubra* specific primers used in qRT-PCR of osmoregulatory genes

Target Gene	Primer	Sequence (5′ – 3′)	Amplicon length (nt)
NKA	NKAF2679	TGGCTTCCTTCCTCCCAAACTCTT	175
	NKAR2854	TCAAATCGGCCCACTGGACAATCA	
CAc	CAcF444	TTTGGGTGTGTTTCTGACCGTTGG	169
	CAcR613	TCAGCGAACCAGGGTAGGTGAAAT	
CAg	CAgF373	AGCCTGGGCTCAGAACACACTATT	189
	CAgR562	TTAGGGCACTGACCAAAGGAGTGA	
NKCC	NKCCF1826	ATCGTCCACAGCTCTTGGTGCTAA	194
	NKCCR2020	AGGCACGAATCTTATGACGAGCGA	
HAT	HATF800	AGTGCGAGAAGCACGTCCTCATTA	194
	HATR994	TGGGAATCTGGGTGATGGAACCTT	
AK	AKF821	ACCTGGGTACCACTGTTCGTTCTT	180
	AKR1001	GTCAGGCCCATACGACGTTTGTTA	

NKA, Na^+/K^+-ATPase α-subunit; CAc and CAg, carbonic anhydrase isoforms; NKCC, $Na^+/K^+/2Cl^-$ cotransporter; HAT, H^+-ATPase; AK, arginine kinase.

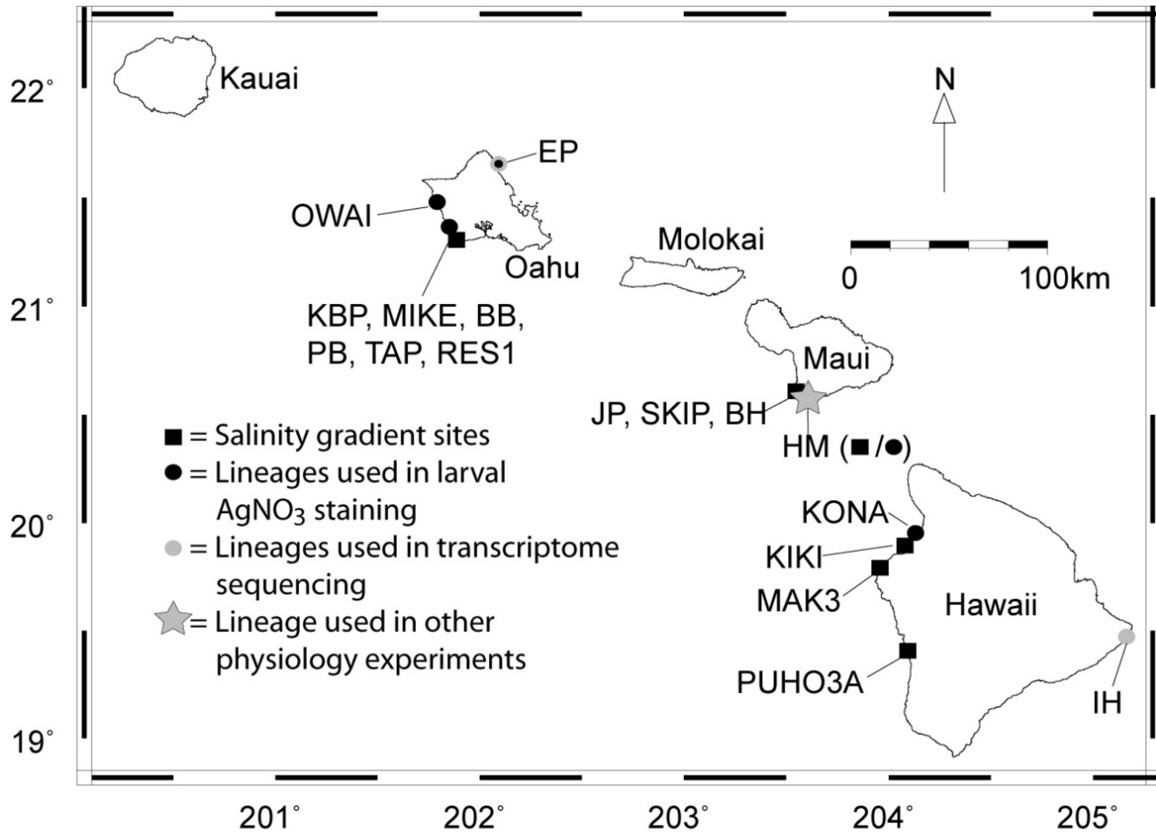

Fig. 1. Map depicting anchialine habitats and animal origins for this study. Black squares indicate habitats where vertical salinity gradients were measured, black circles indicate origins of *Halocaridina rubra* larvae used in silver nitrate ($AgNO_3$) staining, gray circles indicate origins of *H. rubra* lineages used in transcriptome sequencing, and the gray star indicates the origin of animals used in the other experiments. *Halocaridina rubra* were observed at all sites except for PUHO3A. Abbreviations: BB: Baby Bear; BH: Blue Hole; EP: Eric's pond; HM: Cape Hanamanioa; IH: Issac Hale; JP: Joe's Pond; KBP: Kalaeloa Unit; KIKI: Keawaiki Bay; KONA: Kona Coast; MAKA3: Makalawena 3; MIKE: Mike's Pond; OWAI: Waianae Boat Harbor; PB: Papa Bear; PUHO3A: Pu'uhonua o Hōnaunau National Historical Park 3A; RES1: Restoration 1; SKIP: Skippy's Pond; TAP: Tap's Pond.

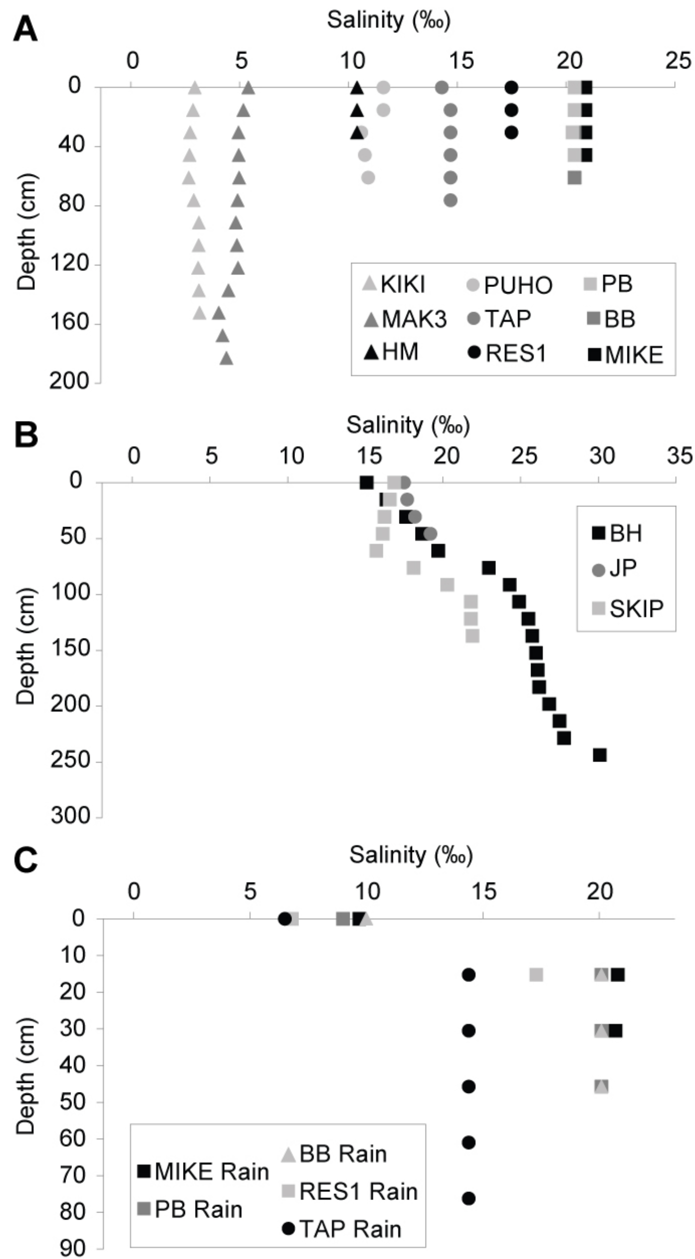

Fig. 2. Different vertical salinity gradient profiles identified from Hawaiian anchialine habitats. (A) Many habitats exhibited little salinity variation with depth. (B) Profiles from three habitats depicting appreciable vertical salinity gradients, with salinity increasing with depth (from 15‰ to 30‰ in the most extreme case). (C) Profiles from five habitats surveyed ~8h after a rainfall event, with evidence for lens of fresher water extending to a depth of 2–3 cm from the surface. Abbreviations follow Fig. 1.

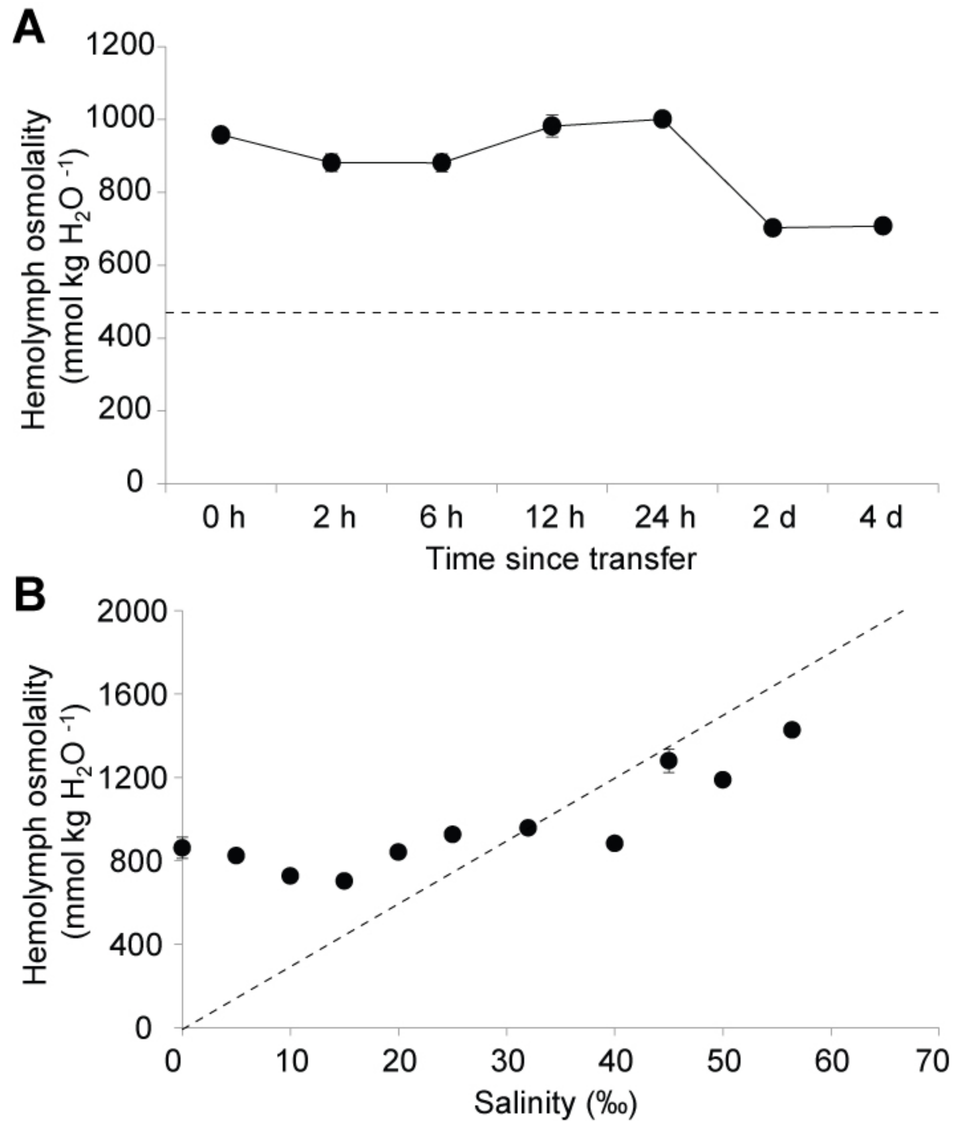

Fig. 3. Osmolality of *Halocaridina rubra* hemolymph as a function of salinity and acclimation time. (A) Hemolymph osmolality (sold line) for *H. rubra* chronically acclimated to 32‰ (time = 0 hours) then transferred to 15‰ for 4 days (dashed line represents 15‰). (B) Hemolymph osmolality for *H. rubra* acclimated to ~0‰, 5‰, 10‰, 15‰, 20‰, 25‰, 32‰, 40‰, 45‰, 50‰, and 56‰ for at least 2 days (dashed line represents the iso-osmotic line). Values are means ± s.e.m., n = 3–6 samples of 6 pooled animals per sample.

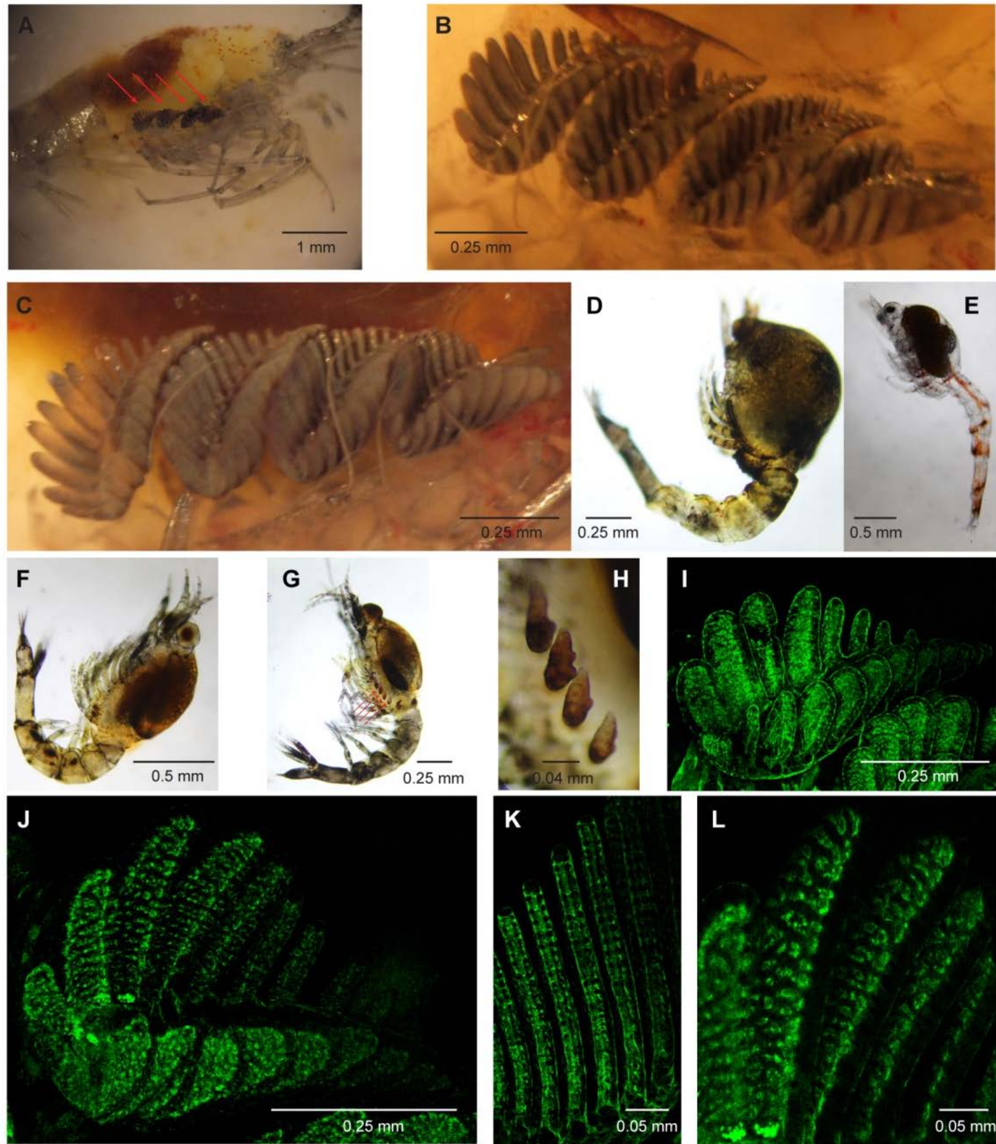

Fig. 4. Silver nitrate ($AgNO_3$) and vital staining of mitochondria-rich cells (MRCs) in the gills of *Halocaridina rubra*. (A) An adult chronically acclimated to 15‰ and treated with $AgNO_3$, showing gills (i.e., the pleurobranchs of the 5th–8th thoracomeres) as the primary sites of ion transport (indicated by red arrows). (B) Close-up of gills treated with $AgNO_3$ after chronic acclimation to 2‰. (C) Same as for (B), but after chronic acclimation to 32‰. Note lack of staining in some central lamellae of the posterior gill compared to (B). (D) Zoea$_1$ stage of *H. rubra* larva treated with $AgNO_3$ (from the KBP lineage; Fig. 1). (E)

Zoea$_2$ stage of *H. rubra* larva treated with $AgNO_3$ (from the KBP lineage; Fig. 1). (F) Zoea$_3$ stage of *H. rubra* larva treated with $AgNO_3$ (from the OWAI lineage; Fig. 1). (G) Zoea$_4$ stage of *H. rubra* larva treated with $AgNO_3$ (from the OWAI lineage; Fig. 1), with developing gills (indicated by red arrows). (H) Close-up of gills from (G). (I) Confocal laser scanning micrograph of adult *H. rubra* gill 4 (i.e., most posterior gill) acclimated to 2‰ and following *in vivo* staining of MRCs using DASPMI (positive cells are green). (J) Same as for (I), but from adult *H. rubra* acclimated to 32‰. (K) Transverse section of (I) under high magnification. Note afferent vessel epithelia near bottom of image and efferent vessel epithelia near top of image. (L) High magnification image of (J). Images (I) – (L) were created by taking multiple photos at 2–12 μM increments and splicing them into a single, three-dimensional image (i.e., a z-stack). All adult gills are in the same orientation as (A).

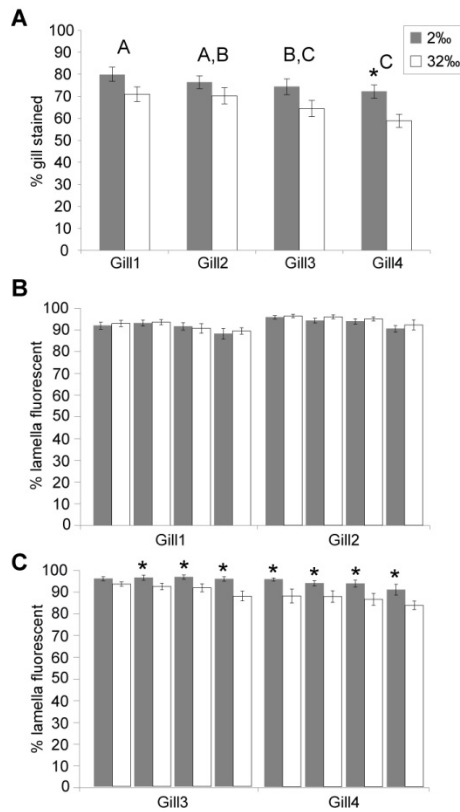

Fig. 5. Area fraction of *Halocaridina rubra* gills stained by silver nitrate ($AgNO_3$) or vital staining of mitochondria-rich cells (MRCs). (A) Percent of whole gills stained with $AgNO_3$ from *H. rubra* acclimated to 2‰ or 32‰ (± s.e.m., $n = 10$ animals/20 gills per salinity). Letters indicate statistically significant groupings among gills (linear fixed-effects model, $P < 0.01$). Although there was not an overall effect of salinity (linear fixed-effects model, $P = 0.13$), gill 4 (i.e., most posterior gill) did have a statistically significant higher proportion of $AgNO_3$ staining at 2‰ when considered separately (indicated by asterisk, Student's unpaired *t*-test, $P = 0.003$). (B) and (C) Percent of central four lamellae stained with DASPMI for vital MRCs from *H. rubra* acclimated to 2‰ or 32‰ (± s.e.m., $n = 10$ animals/20 gills per salinity). There was a statistically significant effect of salinity for lamellae (*t*-test, $P < 0.049$ for all comparisons) indicated with an asterisk.

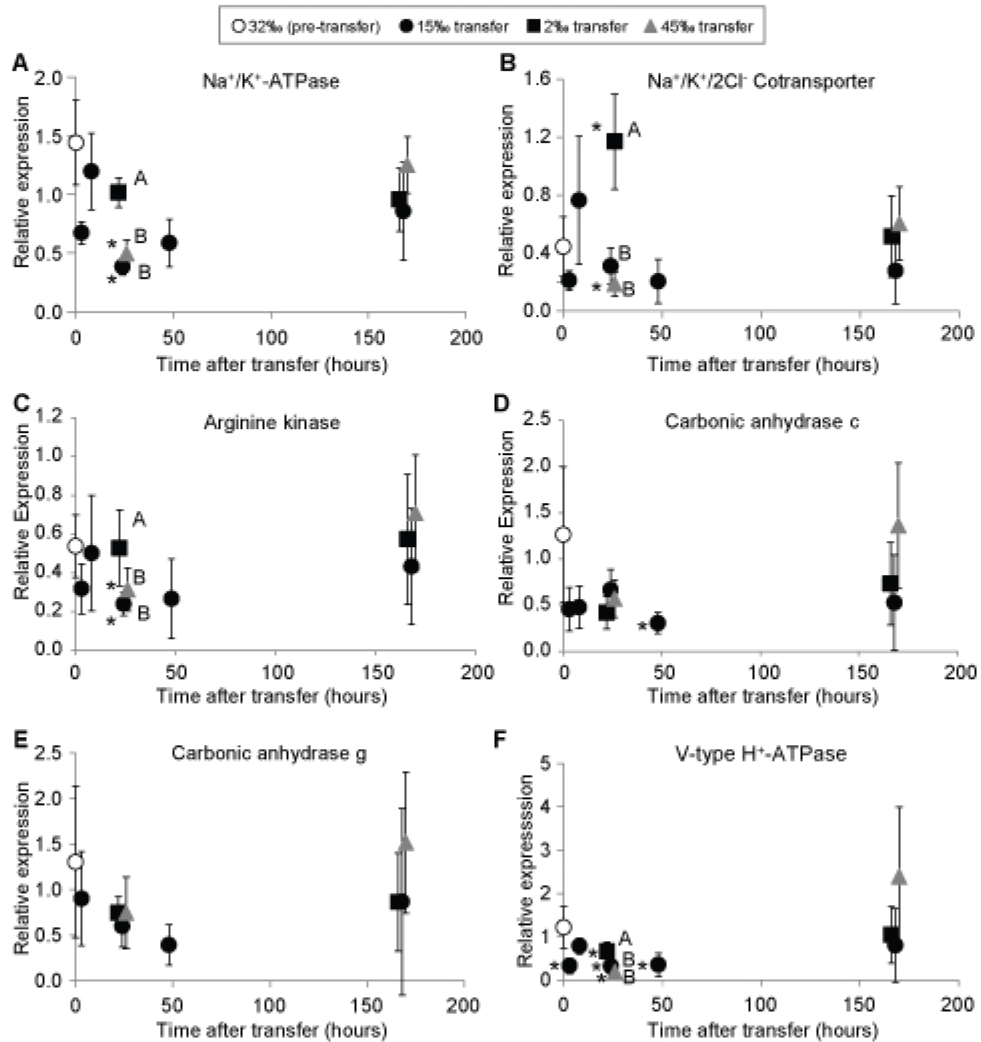

Fig. 6. Relative expression of ion transporters and supporting other osmoregulatory enzymes in the gills of *Halocaridina rubra* following salinity transfers. mRNA expression of (A) Na^+/K^+-ATPase (NKA), (B) $Na^+/K^+/2Cl^-$ co-transporter (NKCC), (C) arginine kinase (AK), (D) the cytoplasmic isoform of carbonic anhydrase (CAc), (E) the membrane-associated isoform of carbonic anhydrase (CAg), and (F) V-type H^+-ATPase (HAT) were quantified 0, 3, 8, 24, 48, and 168 h (7 d) after transfer from 32‰ to 15‰ (open and closed circles, respectively). Transfers from 32‰ to 2‰ and 45‰ (closed triangles and squares, respectively) were also examined 24h and 7d after transfer. Symbols represent mean values (relative to a single sample from the 2‰, 24 h treatment)

± s.e.m., n = 5–6 samples of 3–6 pooled "gill undercarriages", per treatment (see Materials and Methods). Values with asterisks to their left are statistically different from the pre-transfer measurements (Student's t-test, $P < 0.05$ for all). Letters at 24 h and 7 d time points represent statistically significant groupings among salinity treatments (two-way ANOVA with Tukey post-hoc tests, $P < 0.05$ for all).

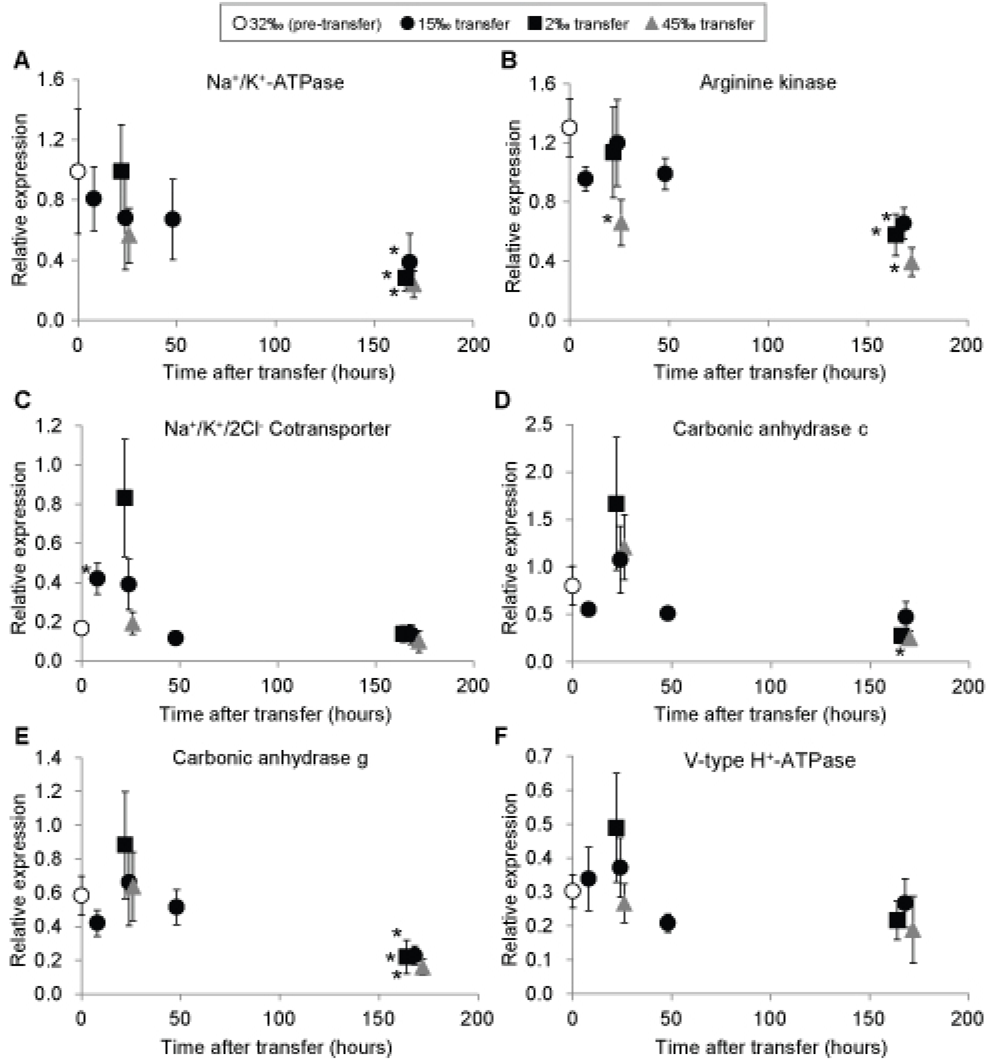

Fig. 7. Relative expression of ion transporters and supporting enzymes in controls (i.e., other tissues besides the “gill undercarriage”, see Materials and Methods) of *Halocaridina rubra* following salinity transfers. Genes, symbols, asterisks, letters, and statistical analyses are as in Fig. 6, with the exception that the 3 h, 15‰ treatment was not investigated for the control tissue samples.

Movie 1. Video depicting *Halocaridina rubra* moving regularly and freely through the water column, including across vertical salinity gradients in some habitats. This movie was taken at a deep (~1.5 m) crack adjacent to the Cape Hanamanioa habitat and similar behaviors were observed at the other anchialine habitats. Movie provided courtesy of Dr. David Weese. Movie can be found at http://youtu.be/EHHDFJ78Ryc

Chapter 5. Taking their breath away: Metabolic responses to low-oxygen levels in anchialine shrimps (Crustacea: Atyidae and Alpheidae)

5.1 Abstract

Crustaceans generally act as oxy-regulators, maintaining a constant metabolic rate (measured by oxygen uptake) as oxygen pressures decrease, until a critical low level is reached at which ventilation and aerobic metabolism shut down. Cave-adapted animals, including crustaceans, often show a reduced metabolic rate, owing to the hypoxic nature of such environments. However, metabolic profiles have not been explored thoroughly in crustaceans from anchialine habitats (coastal ponds and caves), despite the prevalence of anoxic layers in such habitats. Here we describe an unusual oxy-conforming pattern of oxygen uptake and other metabolic characteristics in the Hawaiian anchialine atyid *Halocaridina rubra*. We suggest that near-maximal gill ventilatory rates in normoxia are responsible for oxyconformity in hypoxia. This is likely because *H. rubra* gills appear almost entirely specialized for osmoregulation at the expense of gas exchange. Despite this trade-off, animals maintained in anoxic waters survived for seven days (the duration of the experiment) with no measureable oxygen uptake or noticeable change in behavior, suggesting a reliance on anaerobic metabolism. Supporting this, lactate dehydrogenase activity was high, even in normoxia, and oxygen debts were quickly repaid by an unusually extreme increase in oxygen uptake upon exposure to normoxia. However, other anchialine shrimps from the Ryukyus Islands of Japan with varying levels of relatedness to *H. rubra* had alternate properties more similar to previously studied crustaceans, including an oxy-regulating strategy and low tolerance to anoxia. To determine the roles

of environment and evolutionary history in shaping respiratory physiology, diversity among anchialine species should be explored further.

5.2 Introduction

The assimilation of energy from food sources for bodily maintenance and reproduction is a general principle uniting nearly all studies of physiology. Therefore, it is not surprising that metabolism can be altered due to environmental conditions to best match energetic demands. For example, many animal phyla enter a state of drastically reduced metabolism (sometimes to immeasurable levels) when environmental conditions become harsh (Guppy and Withers, 1999). Furthermore, colonizations of novel environments can result in lineages with radically different metabolic traits than ancestral populations. Such cases include marsupials adapted to desert habitats having reduced metabolic rates compared to those from temperate and tropical environments, likely due to lower food availability (Hurlbert and Dawson, 1974) and cod (*Gadus morhua*) inhabiting brackish waters exhibiting an elevated metabolic rate relative to their marine counterparts (Nelson et al., 1994), potentially due to increased osmoregulatory demands. These examples of altered metabolism in different environments exemplify that habitat selection results in physiological consequences, and ultimately has ecological ramifications (Huey, 1991).

Animals adapted to living in cave environments or in underground waters (i.e., hypogeal environments) often possess morphological, behavioral, and physiological adaptations to cope with the extreme conditions characteristic of subterranean systems, including darkness, depauperate food webs, and low, often fluctuating oxygen levels

(Hervant and Mallard, 2012). These adaptations include reduced eyes, little to no coloration, energy-efficient feeding strategies, and lowered metabolism (Culver and Poulson, 1971; Sket, 1996; Bishop et al., 2004). Of these, metabolic adaptation to a subterranean lifestyle may be a response to the low availability of food or decreased oxygen levels limiting ATP production (Spicer, 1998). Regardless of the reason, low rates of oxygen uptake when compared to surface-dwelling (i.e., epigeal) relatives have been documented for cave-dwelling fishes (Hüppop, 1986; Salin et al., 2010), amphibians (Hervant et al., 2000; Hervant et al., 2001), and crustaceans (Hervant and Mathieu, 1995; Gannon et al., 1999; Bishop et al., 2004; Mejía-Ortíz and López-Mejía, 2005; Issartel et al., 2005; Bishop and Iliffe, 2012). Moreover, cave-dwelling crustaceans possess elevated levels of enzymes involved in anaerobic metabolism (Bishop and Iliffe, 2012) and against reactive oxygen species (Lawniczak et al., 2013), allowing some species to survive in anoxic conditions for many hours, or even days (Hervant et al., 1995, 1999; Issartel et al., 2009).

Although aspects regarding the metabolic physiology of cave-dwelling crustaceans are fairly well-studied, those of crustaceans from the anchialine ecosystem are less thoroughly characterized. This ecosystem encompasses coastal caves, ponds, and pools with no surface connection to the open ocean, but with subterranean links to both marine and freshwater sources (Holthuis, 1973; Sket, 1996). While anchialine habitats are found worldwide, they are most concentrated in the Hawaiian Islands (Maciolek and Brock, 1974; Brock, 1987; Brock et al., 1987), where anchialine ponds far outnumber anchialine caves (Maciolek and Brock, 1974; Fig. 1A, B). Previous studies of crustaceans from anchialine caves outside Hawaii suggest they have a reduced metabolic rate and

enzyme levels poised for anaerobic metabolism (Bishop et al., 2004; Bishop and Iliffe, 2012), similar to other cave-dwelling crustaceans. However, it can be hypothesized that crustaceans from anchialine ponds exhibit intermediate responses since they spend time in both the below- and above-ground portions of the habitat, and species inhabiting anchialine caves may differ in metabolic physiology from species inhabiting anchialine ponds.

The shrimp *Halocaridina rubra* Holthuis, 1963 (Decapoda: Atyidae) is an endemic Hawaiian anchialine species representing an opportunity to extend previous studies of cave-dwelling anchialine crustaceans to one that regularly transverses the hypogeal and epigeal habitats (Bailey-Brock and Brock, 1992). Notably, anecdotal evidence suggests *H. rubra* may tolerate prolonged periods of hypoxia or anoxia since 1) an individual shrimp sealed in a polyethylene bag with ~100 mL of water survived for over one year without water changes or feeding (Maciolek, 1983) and 2) these shrimp are sold in hermetically sealed containers as the "perfect pet" because they require no special care (Weese and Santos, 2009). In this latter case, populations of *H. rubra* have persisted for > 10 years, often undergoing periodic bouts of reproduction (Maciolek, 1983; S.R. Santos, pers. obs.). While these observations suggest *H. rubra* possesses an interesting and potentially unusual metabolic physiology, no studies have measured metabolic rate or enzymatic activities in this species. Here, we compared the metabolic rate in *H. rubra* and four shrimp species from anchialine caves across the Ryukyus Islands, Japan to previously studied crustaceans. Along with this, we investigated 1) whether species repay an oxygen debt following a period of hypoxia; 2) if salinity transfer influences metabolic

rate; 3) if ventilation rate is influenced by hypoxia, and; 4) if levels of lactate and lactate dehydrogenase change under hypoxia or anoxia.

5.3 Materials and methods

5.3.1 Animals and holding conditions

Halocaridina rubra were collected from a single anchialine pond at Cape Hanamanioa, Maui (Fig. 1C) using hand nets and shipped to Auburn, AL within two days of collection in ~1 L jars containing filtered water from the habitat and plastic "Easter egg" grass as padding/substrate for the animals. Given that *H. rubra* is comprised of at least eight different genetic lineages across the Hawaiian Islands (Craft et al., 2008), it is important to note that all animals used in the following experiments were from a single lineage. In the laboratory, *H. rubra* were maintained in 38 L aquaria (~250 animals per aquarium) containing 15‰ water and baskets of porous volcanic rock for shelter. These holding tanks experienced no water changes, circulation, or feeding, and are favorable for the rearing of *H. rubra* since they have resulted in periodic reproduction for other aquaria in the laboratory throughout several years.

To complement the studies of *H. rubra*, four additional shrimp species were collected from anchialine caves in the Ryukyus Islands, Japan. These were: 1) *Caridina rubella* Fujino & Shokita, 1975 (Decapoda: Atyidae) collected from two closely separated caves (Yamato-ga and Butora-ga) on the island of Miyako-jima; 2) *Halocaridinides trigonophthalma* Fujino & Shokita, 1975 (Decapoda: Atyidae) from four anchialine caves (Shuga-ga, Fushyato-ga, Ama-ga, and Futatsu-ga) on the island of Tarama-jima; 3) *Antecaridina lauensis* Edmondson, 1935 (Decapoda: Atyidae) collected

from Shuga-ga on Tarama-jima, and; 4) *Metabetaeus minutus* Whitelegge, 1897 (Decapoda: Alpheidae) from Futatsu-ga on Tarama-jima (Fig. 1D). Each species also encompasses 2-3 genetic lineages in the Ryukyus (Weese et al., 2013), so this sampling scheme was designed to ensure only a single lineage (i.e., the one with the most widespread distribution) was examined per species. Within two days of collections, shrimp were shipped to the Sesoko Marine Station – Tropical Biosphere Research Center in Okinawa, Japan, using a similar approach as described above and maintained in the laboratory under conditions like those described for *H. rubra*.

5.3.2 Closed cell respirometry

Oxygen uptake (V_{O_2}) was measured for all species using a Strathkelvin 782 2-Channel Oxygen System (Strathkelvin Instruments Ltd., North Lanarkshire, Scotland). The experimental set-up (Fig. 2A) consisted of two 50 mL glass Erlenmeyer flasks, each with an attached SI130 microcathode electrode connected to the Oxygen System and sealed with parafilm, resting on a magnetic stir plate (after Szczebak et al., 2013). During each experiment, a single shrimp was placed in one flask while the other was left unoccupied as a control, with oxygen partial pressure (P_{O_2}) measured in each chamber every 20 s. Electrodes were calibrated prior to each experiment using both air-saturated water (for a fully air-saturated reading, typically 150 torr) and anoxic water treated with Na_2SO_3 (0 torr). Parallel measurements of P_{O_2} (Fig. 2B) in the control chambers were used to monitor potential electrode drift, with experiments where control levels fluctuated appreciably (i.e., > 10%) being discarded. Shrimp were weighed at the end of the experiment and the Data Analysis Module of the 782 Oxygen System was used to

calculate mass-specific V_{O_2} during various time intervals of the experiment. Initially, animals were intentionally starved for 24 hours prior to experiments by maintaining them in sterile chambers without rocks or substrate for grazing. However, metabolic rate was similar whether starvation was performed beforehand or if animals were taken directly from tanks. Given this subsequent experiments used animals maintained under both conditions.

V_{O_2} was calculated for *H. rubra* (*n* = 10), *C. rubella* (*n* = 10), *M. minutus* (*n* = 10), *H. trigonophthalma* (*n* = 5), and *A. lauensis* (*n* = 5) under "laboratory-acclimated" conditions (i.e., air-saturated water of the same salinity as the holding tanks) to record resting metabolic rate at room temperature (24 °C). Along with this, V_{O_2} was also calculated for *H. rubra*, *C. rubella*, and *M. minutus* (*n* = 10 for each) during salinity transfers. Prior to transfers, animals were maintained for at least one month in the initial salinity to ensure chronic acclimation before being moved to the new salinity (after Jillette et al., 2011). Also for these three species (*n* = 10 for each), oxygen debt was investigated by monitoring P_{O_2} values in the respiratory chambers until they were reduced to a low and constant level, indicating that V_{O_2} had ceased, and then transferring the shrimp to a fresh chamber with normoxic water to determine if there was a subsequent rapid and large increase in V_{O_2}. Lastly, tolerance to acute hypoxia was investigated in *H. rubra* (*n* = 10) by transferring shrimp directly into hypoxic water (~50 torr, prepared by N_2 bubbling) and comparing those V_{O_2} to "control" experiments where shrimp were transferred to air-saturated water.

Each experimental period persisted either until P_{O_2} approached 0 torr or stabilized in the animal chamber or when shrimp began showing signs of stress (e.g., not responding to physical stimuli or not righting itself while on their back). For each experiment, V_{O_2} was calculated against duration of the experiment (i.e., time) and P_{O_2}.

5.3.3 Ventilation measurements

To quantify ventilation during normoxia, hypoxia, anoxia, and hyperoxia, individual *H. rubra* (n = 10) were immobilized in glass chambers (i.e., tubes of ~2 mm diameter by 5 cm in length) that allowed simultaneous visualization of an individual's scaphognathite (i.e., the pumping organ used to ventilate gills, aka "gill bailers") and heart through the side of the body. Water (15‰) from a reservoir was pumped through chambers at a rate of 12.55 ± 0.77 mL min^{-1}, with shrimp being first acclimated to normoxia for 30 min by bubbling air into the reservoir (P_{O_2} = 150 torr) before switching over to N_2 for 1-2 minutes to lower P_{O_2} to ~125 torr. Following a 15 min acclimation to the new P_{O_2}, 2–3 minutes of video were recorded using a digital camera (Canon Rebel T2i DS126271, Canon Inc., Japan) attached to a light microscope (Model MX4300, Meiji Techno Co., LTD, Japan) at 4X magnification. The above process was then repeated for 100 torr, 75 torr, etc., until anoxia was achieved. In a separate set of experiments, the same individuals were exposed to hyperoxic conditions (P_{O_2}= 200, 250, 300, and 500 torr) via bubbling of pure O_2 in a similar experimental procedure. In all cases, P_{O_2} was recorded throughout each experiment using the Strathkelvin 782 2-Channel Oxygen System (Strathkelvin Instruments Ltd.) as detailed above. Frequencies of scaphognathite

pumping and heart beating were quantified from 30 s of video analyzed at either normal, half, or quarter speeds.

5.3.4 Lactate levels and lactate dehydrogenase (LDH) activity

To examine anaerobic metabolism during hypoxia and anoxia exposure, levels of lactate as well as lactate dehydrogenase (LDH) activity were quantified in *H. rubra* during similar experiments to those described above. Specifically, shrimp were transferred into air-saturated water, with either one or five individuals per chamber, and P_{O_2} measured oxygen uptake. Sampling for lactate and LDH was conducted at five points in the experiment: 1) before transfer to the chamber (i.e., control); 2) when P_{O_2} reached ~50 torr (i.e., hypoxia); 3) following exposure to anoxia for 1–3 hours (i.e., acute anoxia); 4) anoxia for ~24 hours, and; 5) anoxia for ~3 days (i.e., chronic anoxia).

Lactate levels were quantified using a Lactate Assay Kit (Cat. No. MAK064, Sigma-Aldrich, St. Louis, USA). Briefly, shrimp were wicked dry and weighed to the nearest 0.1 mg before being transferred to 1.5 mL tubes on ice containing 250 µL of assay buffer and ~20 0.5 mm diameter zirconia beads (Cat. No. 11079105z, BioSpec Products, Bartlesville, USA). Samples were then homogenized with a Mini-Beadbeater-96 (BioSpec Products), centrifuged for 10 minutes at 13,000 g, and the upper soluble fraction deproteinized via a 10 kDa MWCO spin filter (Sigma-Aldrich) for 20 minutes at 14,000 g. Approximately 35 µL of the collected filtrate was utilized in quantifying lactate levels following the manufacturer’s protocol.

Activity of LDH was assayed using a LDH Activity Kit (Cat. No. MAK066, Sigma-Aldrich). Shrimp were weighed and transferred to 500 µL of assay buffer prior to

being homogenized as described above, with LDH activity examined in 2 μL of homogenate following the manufacturer's protocol. Samples and standards were run in duplicate for all of the above assays.

5.3.5 LDH sequencing and phylogenetic analysis

To infer the phylogenetic placement of the lactate dehydrogenase (LDH) gene from *H. rubra*, sequences were mined from three (3) transcriptomes publically available as a searchable BLAST database (www.auburn.edu/~santosr/halo_blast.htm). Briefly, transcriptome data were generated from adults of the East Hawaii and Windward Oahu genetic lineages (EP and IH, see Craft et al., 2008) as well as $zoea_4$ larvae from EP, with 46,267 contigs having high (e.g., >100) bit scores following annotation via BLASTX (Altschul et al., 1990) against the NCBI nr database. One full-length LDH transcript was identified from each of the adult transcriptomes and their amino acid sequences aligned to available crustacean LDH sequences from GenBank using MUSCLE v 1.0 (Edgar, 2004), as implemented in SeaView v 4.0 (Gouy, et al., 2010). The resulting amino acid alignment matrix was then utilized in aligning the coding regions of the nucleotide sequences using the program tranalign in the EMBOSS v 6.4 suite (Rice et al., 2000). This final nucleotide alignment matrix was used to infer a maximum likelihood (ML) phylogeny of crustacean LDH sequences using PhyML v 3.0 (Guindon et al., 2009) with the GTR + I + Γ model (GTRGAMMAI) and 1000 bootstrap replicates. Additionally, a Bayesian inference (BI) phylogeny was generated using MrBayes v 3.2.1 (Ronquist and Huelsenbeck, 2003) using four chains, the same model of evolution as in the ML analysis and 5,000,000 generations (a static likelihood score was confirmed after this number of

generations). Three BI runs were performed to ensure the same topology was achieved, and a majority-rule consensus phylogeny was generated using a burnin of 20% of the sampled trees per run.

5.3.6 Statistical analyses

All statistics were performed in the R v 2.12.0 statistical software environment (code available on request; R Core Team, 2013).

5.4 Results

5.4.1 Oxygen uptake under different scenarios

Rates of mass-specific oxygen uptake (V_{O_2}) for *H. rubra* during isotonic transfers to fully oxygenated water (i.e., "control" conditions) were 0.733 ± 0.137 µL O_2 h^{-1} mg^{-1} and decreased with declining P_{O_2} as time progressed, with V_{O_2} reaching immeasurable levels (i.e., 0 µL O_2 h^{-1} mg^{-1}) at a P_{O_2} of 60–0 torr by ~36 h into the experiment (Fig. 3A). The same general trend was observed whether V_{O_2} was measured as a function of time since the experiment began or ambient P_{O_2} in the chamber (Fig. 3B); given this, V_{O_2} is presented only as a function of time throughout (data based on ambient P_{O_2} available upon request). Although all ambient O_2 was consumed by *H. rubra* in many experiments, P_{O_2} leveled off before reaching anoxia in 7 of 20 (35%) experiments, ranging from ~10 torr (6.5% oxygenated) to ~50 torr (30% oxygenated) at the end of the experimental period. Initial experiments were continued for ~3 days before cessation, with all individuals (*n* = 10) surviving. Additional experiments with *H. rubra* (*n* = 10) conducted

for seven days resulted in similar decreasing of V_{O_2} with time (Fig. 3C). All individuals survived the six days without any measureable V_{O_2} as well.

Under "laboratory acclimated" conditions, the four other anchialine shrimp species showed a constant V_{O_2} throughout the experiment (Fig. 3D) and consumed all oxygen in the chamber (i.e., P_{O_2} was ~0 torr at the end of every experiment). Notably, individuals of these species died if left in anoxia for more than ~1 hour (with the exception of a single *A. lauensis* that survived for ~20 hours) and signs of stress/damage were often obvious (e.g., not responding to physical stimuli, not righting, or constantly swimming near the top of the chamber) near the end of the experimental period. Therefore, experiments were terminated when P_{O_2} approached anoxic values for *C. rubella*, *H. trigonophthalma*, *A. lauensis* and *M. minutus*.

Individuals of *H. rubra*, *C. rubella*, and *M. minutus* were also transferred to air-saturated water of a different salinity to determine if hypo- or hyper-tonic transfers impacted V_{O_2}. Experiments with *H. rubra* continued for 24 h while those for the other two species proceeded until P_{O_2} approached anoxic values. For *H. rubra*, both hyper- and hypo-tonic transfers (15‰ to 32‰ and 32‰ to 15‰, respectively) yielded an average V_{O_2} that was not statistically different from isotonic transfers (P = 0.053–0.895), except for a single time interval where isotonic transfer had a higher V_{O_2} (P = 0.040, one-way ANOVA with Tukey post-hoc analysis; Fig. 4A). For *C. rubella*, those animals undergoing hyper-tonic transfers (0‰ to 32‰) had an average V_{O_2} statistically similar to values for trials in which the same individuals underwent isotonic transfers at every time interval (P > 0.137, paired Student's *t*-test; Fig. 4B). Lastly, although *M. minutus* had a

significantly higher V_{O_2} during isotonic than hypo-tonic transfer (32‰ to 0‰) for a single time interval (1–2 hours after transfer, $P = 0.045$, paired Student's *t*-test; Fig. 4C), no significant differences were found for other time intervals or for the average during the experiment ($P > 0.150$, paired Student's *t*-test; Fig. 4C).

To determine if shrimp repaid an oxygen debt after experiencing hypoxic/anoxic conditions, *H. rubra*, *C. rubella*, and *M. minutus* were transferred from anoxic water (i.e., water in which all oxygen had been consumed during the initial experiment; in the case of *H. rubra*, transfer was after two days even though in some cases oxygen remained detectable, see above) to air-saturated water. For *H. rubra*, V_{O_2} post-transfer was significantly higher than at any time interval during initial experiments with, on average, a 17.2 fold increase in V_{O_2} during the first 15 minutes after transfer to a fresh chamber ($P = 0.002$ between initial two hours of experiment and first 15 minutes after transfer, paired Student's *t*-test; Fig. 5A). However, by 45 minutes post-transfer, V_{O_2} returned to initial levels ($P = 0.165$, paired Student's *t*-test) and remained as such until the experiment was terminated one day after transfer. In contrast, V_{O_2} did not significantly increase after transfer to fresh chambers ($P > 0.520$ for all comparisons, paired Student's *t*-test) for *C. rubella* (Fig. 5B) and *M. minutus* (Fig. 5C).

For *H. rubra*, individuals ($n = 10$) transferred directly to hypoxic water (~50 torr) had a decreased V_{O_2} compared to those under "laboratory-acclimated" conditions (Fig. 6), both during the first two hours of the experiment as well as averaged across the entire 24 hours ($P = 0.007$ and 0.019, respectively, unpaired Student's *t*-test; Fig. 6). Additionally, *H. rubra* transferred directly to hypoxic water often died (50% mortality) quickly, or shortly thereafter, P_{O_2} approached 0 torr. Finally, unlike transfers to air-

saturated water, *H. rubra* always consumed all oxygen in the chamber when transferred to hypoxic water.

5.4.2 Ventilation response to hypoxia and hyperoxia

Both rates of scaphognathite pumping and heart beating were fairly constant in normoxic and hypoxic conditions, as analyzing either 15 s or 2 min of video produced statistically indistinguishable results (P = 0.274–0.862, Poisson regression, z = -1.09–0.147, df = 1, 19). However, with increasing hyperoxia, both scaphognathite pumping and heart beating became less consistent, with up to 10 s passing without any scaphognathite pumping for some individuals. This is shown in Movie 1, which tracks the same individual under normoxic, anoxic, and hyperoxic conditions. Ventilation rate via the scaphognathite was 307.2 ± 22.6 beats min^{-1} in normoxia and increased as P_{O_2} decreased ($P < 0.001$, Poisson regression, z = -28.7, df = 1, 109), with pumping rising 15.2% in anoxia and falling 35.5% in 500 torr as compared to normoxia (Fig. 7, Movie 1). Heart rate followed a similar trend ($P < 0.001$, Poisson regression, z = -28.4, df = 1, 109), initially being 224.1 ± 12.2 beats min^{-1} and increasing by 7.1% in anoxia while decreasing by 45.0% in 500 torr (Fig. 7, Movie 1). Ventilatory shutdown was not observed in anoxia, as scaphognathite pumping continued at a high rate (averaging 354 beats min^{-1}) throughout the entire experimental period (totaling ~20 min).

5.4.3 Lactate levels and LDH activity during exposure to hypoxia and anoxia

Levels of lactate in normoxia were 67.7 ± 11.9 ng mg^{-1} tissue and generally increased during anoxia (five *H. rubra* were included per chamber to ensure all oxygen

was consumed), with exposure for one and three days resulting in 8.6- and 4.7-fold higher lactate levels, respectively ($P < 0.05$, one-way ANOVA with Tukey post-hoc analysis; Fig. 8A). However, LDH activity did not change significantly among treatments ($P = 0.336$, one-way ANOVA; Fig. 8B). In these experiments, obvious stress and mortality (up to 80%) were observed in some experiments with 5 animals after 1–3 days exposure to anoxia.

5.4.4 Phylogenetic analyses of crustacean LDH sequences

Two unique and full-length LDH transcripts were recovered from the available *H. rubra* transcriptome data (GenBank #KF650071 and KF650072), with nucleotide sequences of the coding region being 99% similar (989/999 positions). Translation into amino acid residues found both to be identical, indicating all sequence differences between the two LDH gene copies to be synonymous. The amino acid sequence of LDH from *H. rubra* shared 91.7% sequence identity with LDH isoform 1 of *Litopenaeus vannamei* (GenBank #AFI47929).

Along with the *H. rubra* sequences, 20 additional crustacean LDH sequences, representing different isoforms or species, were acquired from GenBank for phylogenetic analyses using ML and BI methods (Fig. 9). The resulting topologies confirmed a strongly supported split between L- and D- LDH isoforms, which was used to root the tree (i.e., Cristescu et al., 2008). For the L-LDH isoform, one clade consisting of two different *Daphnia* specific sequences was recovered sister to a Decapoda clade consisting of sequences from the shrimps *H. rubra* and *L. vannamei* and the crabs *Ocypode quadrata* and *Uca thayeri*, with some topological differences between the ML and BI

analyses. Members of the D-LDH isoform were less represented, with only two sequences from the copepod *Lepeophtheirus salmonis* comprising the D-LDH clade.

5.5 Discussion

Aquatic and marine environments present animals with a broad range of oxygen partial pressures (P_{O_2}), and animals from anchialine habitats often encounter deoxygenated strata (Sket, 1996; Seymour et al., 2007; Pohlman, 2011). Presented here is the first study comparing oxygen uptake (V_{O_2}) under differing physiological conditions for five anchialine shrimp species from across the Pacific basin. Although many aquatic and marine invertebrate species cannot be strictly placed into one category, oxygen uptake can be broadly classified as being either oxygen-dependent/oxy-conformity, in which V_{O_2} is dependent on P_{O_2}, or oxygen-independent/oxy-regulation, in which V_{O_2} is constant over a wide environmental P_{O_2} range (Magnum and van Winkle, 1973; Grieshaber et al., 1994). Here, V_{O_2} decreased with declining P_{O_2}(or with length of experimental period) for the Hawaiian anchialine atyid *Halocaridina rubra*, consistent with an oxygen-dependent/oxy-conformer status (Fig. 3A–C). This response is similar to that of the anchialine shrimp *Barbouria cubensis* which had a lower V_{O_2} when collected from a hypoxic environment compared to a normoxic one (Bishop and Iliffe, 2012). In contrast, the anchialine species *Antecaridina lauensis*, *Halocaridinides trigonophthalma*, *Caridina rubella*, and *Metabetaeus minutus* from the Ryukyu Islands were near-perfect oxy-regulators, as V_{O_2} did not vary significantly over P_{O_2} ranging from normoxia to anoxia (Fig. 3D). Notably, most crustaceans undergo oxyregulation at P_{O_2} of 50–150 torr

before switching to oxyconformity when oxygen levels reach hypoxic (i.e., < 50 torr) ranges (Magnum and van Winkle, 1973; Burke, 1979; Bridges and Brand, 1980a; Wheatly and Taylor, 1981; Taylor, 1982; Bradford and Taylor, 1982; Burd, 1985; Innes, 1985; Morris and Taylor, 1985; Henry et al., 1990; Das and Stickle, 1993; McAllen et al., 1999; González-Ortegón et al., 2012). Thus, the oxygen uptake characteristics of *H. rubra* and *B. cubensis* are more similar to that of cnidarians and annelids (Magnum and van Winkle, 1973; Stickle et al., 1989), which generally show a greater metabolic depression during hypoxia, while the other anchialine species are more typical of crustaceans.

To maintain a constant V_{O_2} at lower P_{O_2} (i.e., 50-150 torr), crustaceans usually increase pumping of the scaphognathite and the overall ventilation rate of their gills (McDonald et al., 1980; Wheatly and Taylor, 1981; Bradford and Taylor, 1982; Massabuau and Burtin, 1984; Morris and Taylor, 1985; Swain et al., 1987; Henry et al., 1990). Although ventilation rate did increase 15.2% for *H. rubra* in anoxia, an increase of 200–300% relative to that of normoxic values is typical for other species (Wheatly and Taylor, 1981; Bradford and Taylor, 1982; Massabuau and Burtin, 1984; Morris and Taylor, 1985; Swain et al., 1987; Henry et al., 1990; Zainal et al., 1992; Aireiess and McMahon, 1994). Furthermore, ventilation rates at normoxia were ~3.5-fold higher for *H. rubra* than for previously studied species (see above references). A similar trend to scaphognathite pumping frequency was observed for heart rate in *H. rubra*, although heart rate decreases slightly or remains constant during hypoxia for other species. Moreover, in other crustacean species (see above references) including shrimps (Wu et al., 2002; Guadagnoli et al., 2005), scaphognathite pumping and heart beating often cease

during exposure to anoxia. In contrast, ventilation rates for *H. rubra* remained constant over the ~20 minutes of anoxia, and no mortality was observed. Lastly, while ventilation rates were not measured for the anchialine species from the Ryukyu Islands, their apparent oxygen-independent/oxy-regulator nature suggests they increase ventilation rates at lower P_{O_2}, as in other crustaceans.

Why does *H. rubra* act as an oxyconformer, have an elevated ventilation rate, and not undergo ventilatory shutdown in anoxia, which is the typical response for crustaceans in general? One possible explanation is that respiratory gas-exchange in the gills of *H. rubra* is compromised since gills are primarily specialized for osmoregulation. Generally, the gills of oxy-independent crustaceans are numerous (i.e., 8-9) and highly complex, with many (i.e., ~300) lamellae and a relatively large surface area. In contrast, *H. rubra* has fewer (i.e., 4) gills characterized by comparatively thick, finger-like lamellae that are also few in number (i.e., 10-16), all of which implies a lower overall gill surface area (Havird et al., in review/companion ms). Furthermore, most crustacean species have either specialized respiratory gills (e.g., portunids such as the blue crab *Callinectes sapidus* and green crab *Carcinus maenas*) or specialized respiratory areas on each gill (e.g., crayfish) (Copeland and Fitzjarrell, 1968; Neufeld et. al., 1980; Dickson et al., 1991). All gills of *H. rubra,* however, appear to be involved in osmoregulation, with the near entirety (i.e., ~80%) of each lamella staining for ion transport ability regardless of salinity (Havird et al., in review/companion ms). Along with this, respiratory epithelia are comprised of primarily thin (1 to 2 μm) undifferentiated cells whereas osmoregulatory epithelia are characterized by thick (i.e., 10 to 20 μm) mitochondria-rich cells (e.g., Taylor and Taylor, 1992; Freire et al., 2008). While epithelial cell thickness was not

directly measured in *H. rubra*, a typical thick ion transporting epithelium is likely given the dominance of vital mitochondrial and silver staining in the gills (Havird et al., in review/companion ms). Taken together, it is hypothesized that this combination of low surface area and large diffusion distance leads to the need for high ventilatory rates towards maintaining a sufficient P_{O_2} gradient across the gills as a means of driving O_2 uptake. As a result, ventilation rates are already at near-maximum frequencies under normoxic conditions and cannot decrease to the same extent as in other species in hyperoxia. As P_{O_2} decreases, ventilation cannot increase meaningfully, resulting in lower oxygen uptake in hypoxia and an overall pattern of oxyconformity for *H. rubra*.

Based on the above scenario, one would expect *H. rubra* to have little tolerance for hypoxia/anoxia and rapidly undergo ventilatory/aerobic shutdown at low P_{O_2}. However, ventilation never ceased during anoxia, which is especially well-tolerated by *H. rubra*. This is likely accomplished by switching at low P_{O_2} from aerobic to anaerobic metabolic pathways. Levels of lactate increased in *H. rubra* during exposure to anoxia as in other crustaceans (Taylor et al., 1977; Zou et al., 1996; Maciel et al., 2008). On the other hand lactate levels did not increase under hypoxia as is typical for crustaceans (Zou et al., 1996). Interestingly, levels of lactate leveled or decreased following three days of anoxia, suggesting *H. rubra* may somehow be avoiding an excessive accumulation of lactate. Unexpectedly, lactate dehydrogenase (LDH) activity did not increase during hypoxia (e.g., Soñanez-Organis et al., 2012) and instead remained at constant, high levels when compared to previously studied crustaceans (e.g., at least ~12-fold higher than values reported in Walsh and Henry, 1990; Yaikin et al., 2002; Oliveira et al., 2004; Bishop and Iliffe, 2012; Lauer et al., 2012; Soñanez-Organis et al., 2012; Rodrigues et

al., 2013; Fig. 8). High LDH activity as well as high LDH:citrate synthase ratios have also been described for hypogean relative to epigean crustaceans (Hervant, 1996; Bishop and Iliffe, 2012). Taken together, this suggests *H. rubra* (along with other select anchialine and hypogean species) are consistently "poised" towards switching to anaerobic pathways when P_{O_2} becomes low. The expressed LDH transcript of *H. rubra* is well conserved to that of isoform-1 from *Litopenaeus vannamei* (91.7% identical amino acid sequence), including identical residues at all substrate-binding sites (Soñanez-Organis et al., 2012). This implies that LDH activity in *H. rubra* is not because of unique enzyme characteristics, but instead likely due to high expression levels of LDH mRNA. Such a strategy may play a role in preparing shrimp for periodic bouts of hypoxia in anchialine habitats, similar to how terrestrial isopods with correspondingly high LDH activities (though those of *H. rubra* are still ~4-fold higher) deal with the hypoxia occasionally encountered in their sand burrows (Wright and Ting, 2006).

Another physiological mechanism by which *H. rubra* apparently tolerates low P_{O_2} is the quick repayment of an oxygen debt. While crustaceans typically repay an O_2 debt after prolonged exposure to hypoxia or anoxia by increasing V_{O_2} (Bridges and Brand, 1980b; Taylor, 1982; Zou et al., 1996), the magnitude of this increase as well as its duration for *H. rubra* are atypical. Specifically, V_{O_2} generally increases ~2–4 fold for several hours during recovery from hypoxia (e.g., Bridges and Brand, 1980b; Zou et al., 1996). In contrast, an average 17.2 fold increase was observed (ranging from 3.7–72.0 fold) for *H. rubra*, with V_{O_2} returning to normal by 45 min post normoxia (it is unknown if lactate levels also decline by this time). This implies *H. rubra* can adequately recover from extended periods of hypoxia or anoxia after only relatively brief contact with

normoxic waters. Taken together, the rapid repayment of an oxygen debt, along with a high activity of LDH, reinforces the idea of a switch to anaerobic metabolism in low P_{O_2} for *H. rubra*. In the case of the other four anchialine shrimp species, although it is unusual that *M. minutus* and *C. rubella* did not repay an O_2 debt after hypoxia (Fig. 5B, C), this has been reported for other crustaceans (e.g., Spoek, 1974; Stickle et al., 1989) and would be expected if anaerobic end-products do not accumulate during hypoxia (Herreid, 1980) or there is a switch to facultative air-breathing (Taylor et al., 1973; Taylor, 1982). In support of the latter possibility, these species were observed to continuously swim towards the top of the chamber during severe hypoxia, possibly to seek out surface waters, which are generally more oxygenated in anchialine habitats (Sket, 1996).

For three of the species examined here, the fact that salinity transfer did not alter V_{O_2} was unexpected. Most crustaceans act as passive osmoconformers in seawater, switching to an active osmoregulatory strategy in dilute waters (reviewed in Henry, 2001; Henry et al., 2012), and it has been demonstrated that *H. rubra* also follows these general trends (Havird et al., in review/companion ms). With osmoregulation being energetically expensive, excised gill preparations as well as whole animals often (but see Piller et al., 1995) show increased oxygen uptake in dilute waters relative to seawater (Flemister and Flemister, 1951; King, 1965; Laird and Haefner, 1976; Jury et al., 1994; McAllen and Taylor, 2001; McGaw, 2006). Given this, it is unusual that V_{O_2} did not change for *H. rubra*, *C. rubella* or *M. minutus* during similar salinity transfers (Fig. 4). In the case of *H. rubra*, osmoregulatory mechanisms such as up-regulation of relevant genes and high mitochondria-rich cell populations in the gills are already activated in seawater (Havird et

al., in review/companion ms), apparently alleviating an increase in V_{O_2} due to the need for transcriptional up-regulation following salinity transfer. Increased V_{O_2} in dilute waters may also be difficult for *H. rubra* due to near-maximal ventilation rates in seawater (see above). Further studies of osmoregulation in *A. lauensis*, *H. trigonophthalma*, *C. rubella*, and *M. minutus* may shed light on why V_{O_2} remains constant in these species during salinity transfers as well.

The results presented here suggest *H. rubra* copes with changing salinities at the expense of respiration and aerobic metabolism. Interestingly, the other four anchialine shrimp species did not exhibit similar patterns and it is tempting to consider evolutionary history as a possible explanation for this difference. Phylogenies of the Atyidae inferred from *16S*, *28S*, and *H3* DNA sequence data place *A. lauensis*, *H. rubra* and *H. trigonophthalma* in the "anchialine clade" of atyids (von Rintelen et al., 2012). Furthermore, a phylogeny based on *16S* sequences from *C. rubella* excludes this species from the anchialine clade, instead placing it within the "*Caridina*-like" clade of atyids (unpublished phylogeny) as hypothesized by von Rintelen et al. (2012). Lastly, *M. minutus* (and the previously studied *B. cubensis*) fall outside of Atyidae in the family Alpheidae. While studies regarding osmoregulation in *A. lauensis*, *H. trigonophthalma*, *C. rubella*, and *M. minutus* are on-going, preliminary data suggest the gills of *C. rubella* and *M. minutus* are unlike those of *H. rubra*, instead possessing specialized regions for respiration as in other crustaceans (unpublished data). Taken together, the physiological characteristics of *H. rubra* do not appear to be shared by members of the anchialine clade as a whole or those atyids and alpheids examined to date from the anchialine ecosystem.

Instead, the osmoregulation/respiration trade-off described for *H. rubra* may be confined to this specific evolutionary lineage.

Although *H. rubra* may be an unusual (and potentially unique) case among anchialine shrimp species when it comes to its physiology, it has been suggested that troglobitic and/or anchialine species in general have lower metabolic rates than comparable species from other habitats (Bishop et al., 2004; Bishop and Iliffe, 2012). However, our data do not support this conclusion, as V_{O_2} from the five anchialine species examined here ranged from 0.146–0.827 µL O_2 hr^{-1} mg^{-1}, which is not significantly different from pelagic species of similar sizes (0.217–0.371 µL O_2 hr^{-1} mg^{-1}, $P = 0.135$, Student's unpaired *t*-test, data for pelagic species taken from Table 2 of Bishop et al., 2012, originally presented in Cowles et al., 1991; Donnelly and Torres, 1988). This implies anchialine species may be more variable in metabolic rate than previously thought and that the degree to which V_{O_2} is suppressed may depend on the particular anchialine taxa or habitat context under examination.

In summary, the data presented here suggest the atyid *H. rubra* of the Hawaiian anchialine ecosystem possesses distinctive metabolic responses during low oxygen conditions relative to other crustaceans. These include: 1) an inability to significantly alter resting ventilatory rates; 2) a pattern of oxyconformity, due to near-maximal ventilator rates regardless of P_{O_2}; 3) high levels of LDH activity, which are constitutively maintained even under normoxic conditions, and; 4) an unusually rapid repayment of oxygen debts. These characteristics are likely due to a trade-off in gill physiology and ultrastructure between osmoregulation and respiration. The result is that while *H. rubra* is well suited for the salinity fluctuations characteristic of anchialine habitats, it must rely

on anaerobic pathways when low oxygen conditions are encountered. In spite of this trade-off, *H. rubra* tolerates anoxia remarkably well, possibly due to modified anaerobic pathways. Notably, other anchialine shrimps so far examined do not possess similar metabolic responses, as four species from the Ryukyus Islands of Japan were more typical of previously studied crustaceans. Future studies should explore the diversity in metabolic response to hypoxia among anchialine species.

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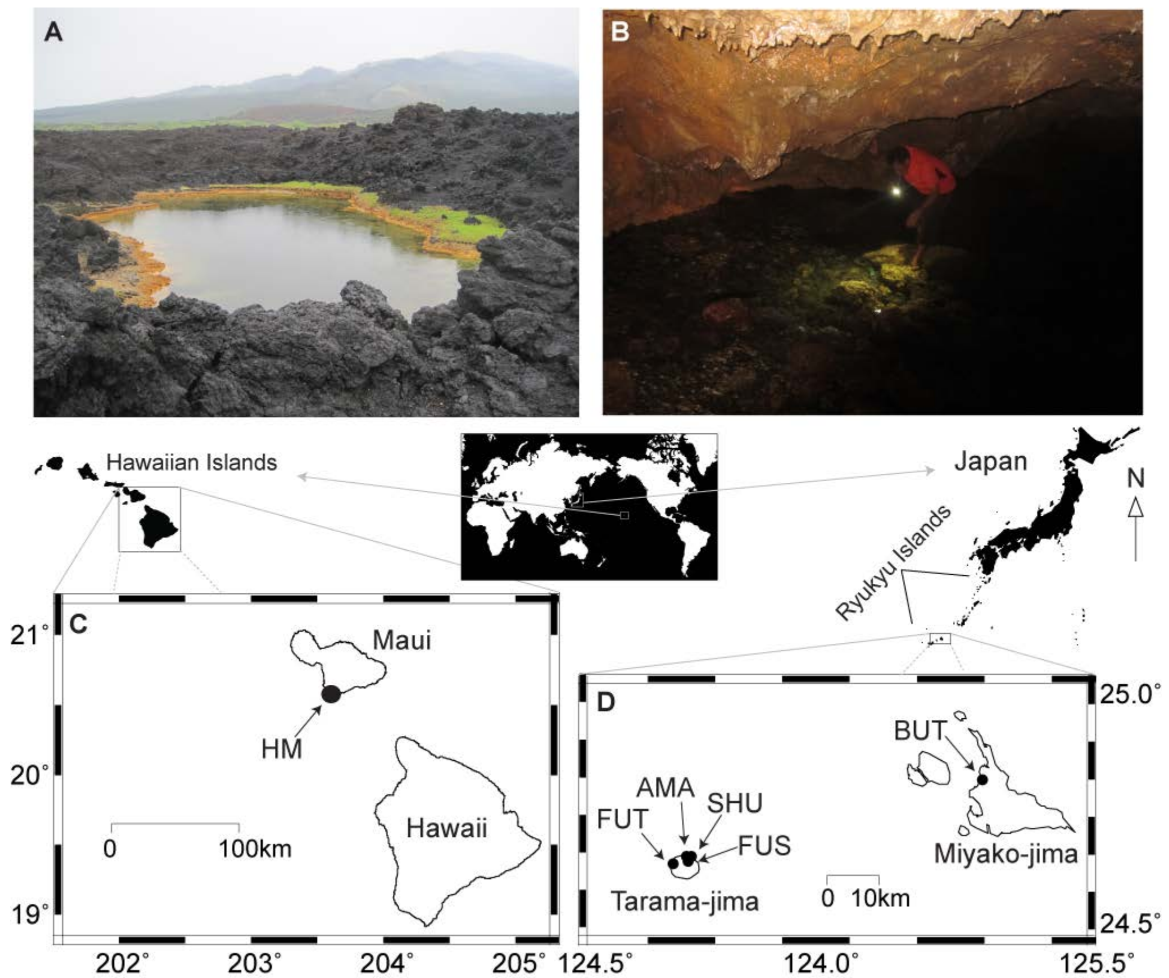

Fig. 1. Sampling sites in this study. (A) An anchialine pond characteristic of those found in Hawaii (Skippy's Pond; see Craft et al., 2008). (B) An anchialine cave characteristic of those found in the Ryukyus Islands (Kikya-ga; see Weese et al., 2013). (C) and (D) Sites sampled in this study in Hawaii and Japan. Abbreviations: AMA: Ama-ga; BUT: Butori-ga/Yamato-ga; FUS: Fushyato-ga; FUT: Futatsu-ga; HM: Cape Hanamanioa; SHU: Shuga-ga.

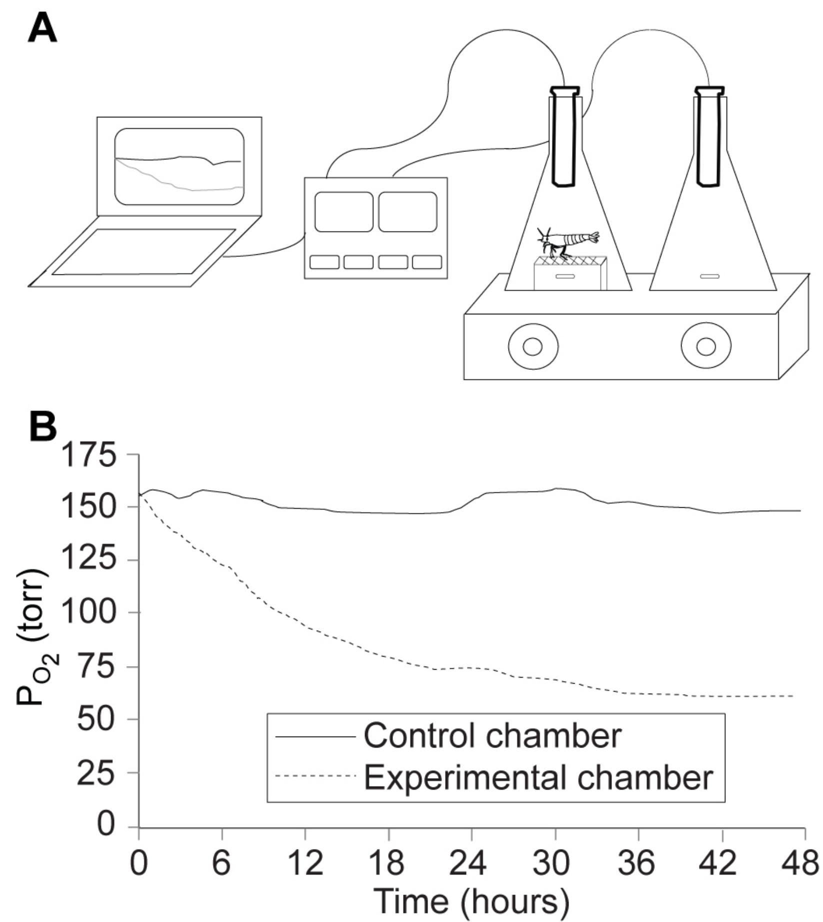

Fig. 2. The experimental set-up and representative oxygen partial pressure (P_{O_2}) traces. (A) Oxygen electrodes connected to a Strathkelvin 782 2-Channel Oxygen System (Strathkelvin Instruments Ltd., North Lanarkshire, Scotland) were placed in 50 mL Erlenmeyer flasks, with one flask containing an animal and the other acting as a control. Water in the flasks was kept mixed using magnetic stir bars. In the experimental chamber, a wire mesh was used to prevent the stir bar from striking the shrimp. (B) Oxygen uptake (V_{O_2}) was only calculated for experiments where the control chamber reading was stable, as depicted here. For *H. rubra*, aerobic shutdown often occurred before all oxygen was consumed in the chamber, as depicted here.

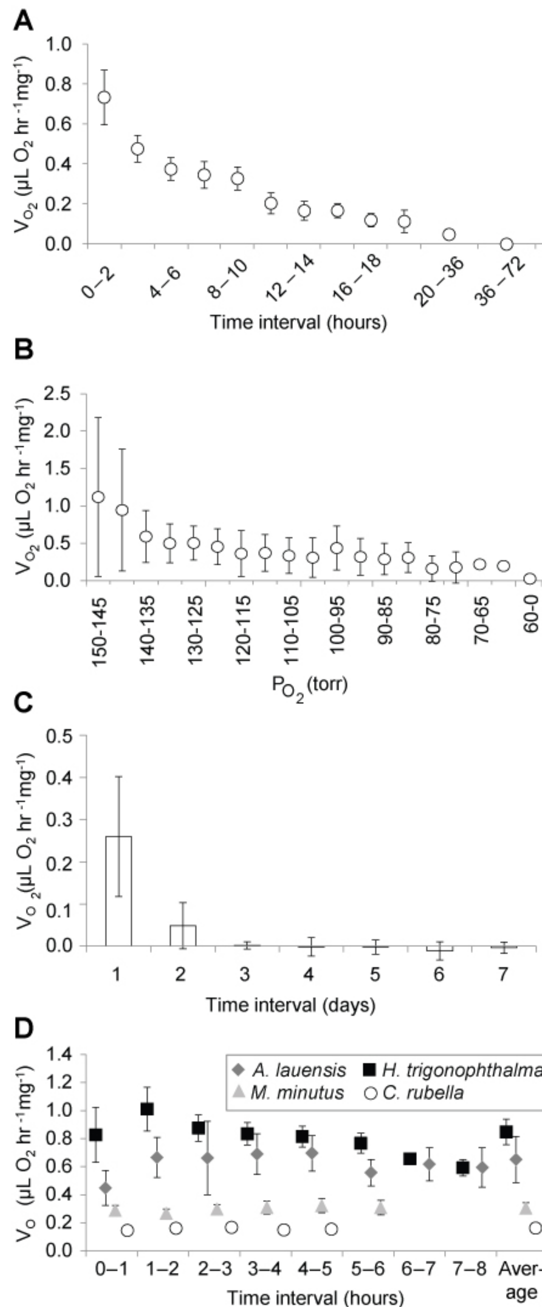

Fig. 3. Effect of experimental duration/oxygen partial pressure (P_{O_2}) on oxygen uptake (V_{O_2}) in anchialine shrimps. (A) For *Halocaridina rubra,* V_{O_2} decreased as duration of the experiment increased, which (B) was a reflection of ambient P_{O_2}. (C) For *H. rubra*, V_{O_2} remained at a low, near zero value for several days during long-term experiments. All animals survived these experiments. (D) Anchialine shrimp species from the Ryukyus

Islands had a constant V_{O_2} throughout the experiment (and based on P_{O_2}). Experiments were shorter than for *H. rubra*, because these species could not survive in anoxia for more than ~1 hour. Error bars show ± S.E.M. and n = 10 except for *Antecaridina lauensis* (n = 5) and *Halocaridinides trigonophthalma* (n = 5).

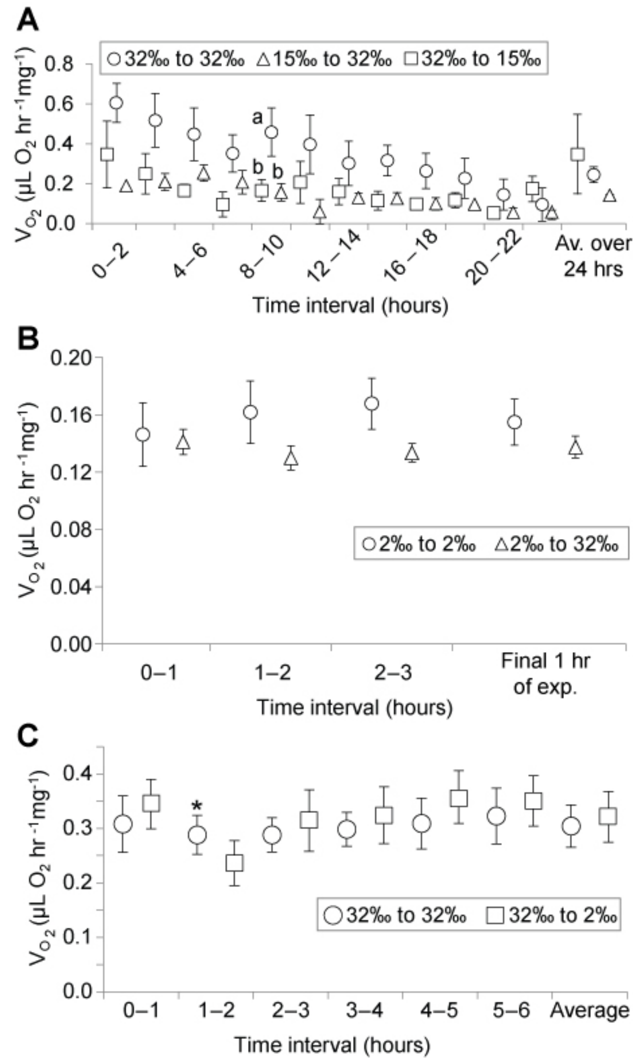

Fig. 4. Oxygen uptake (V_{O_2}) during salinity transfers in anchialine shrimps. (A) V_{O_2} in *Halocaridina rubra* during three different salinity transfers. Letters indicate significant groupings among transfers within a time interval based on ANOVA with Tukey post-hoc analyses ($P = 0.040$). (B) V_{O_2} in *Caridina rubella* during isotonic and hypertonic salinity transfers. (C) V_{O_2} in *Metabetaeus minutus* during isotonic and hypotonic salinity transfers. Asterisks indicate significant differences between salinity transfers within a time interval based on Student's paired *t*-test ($P = 0.045$). Error bars show ± S.E.M. and $n = 10$.

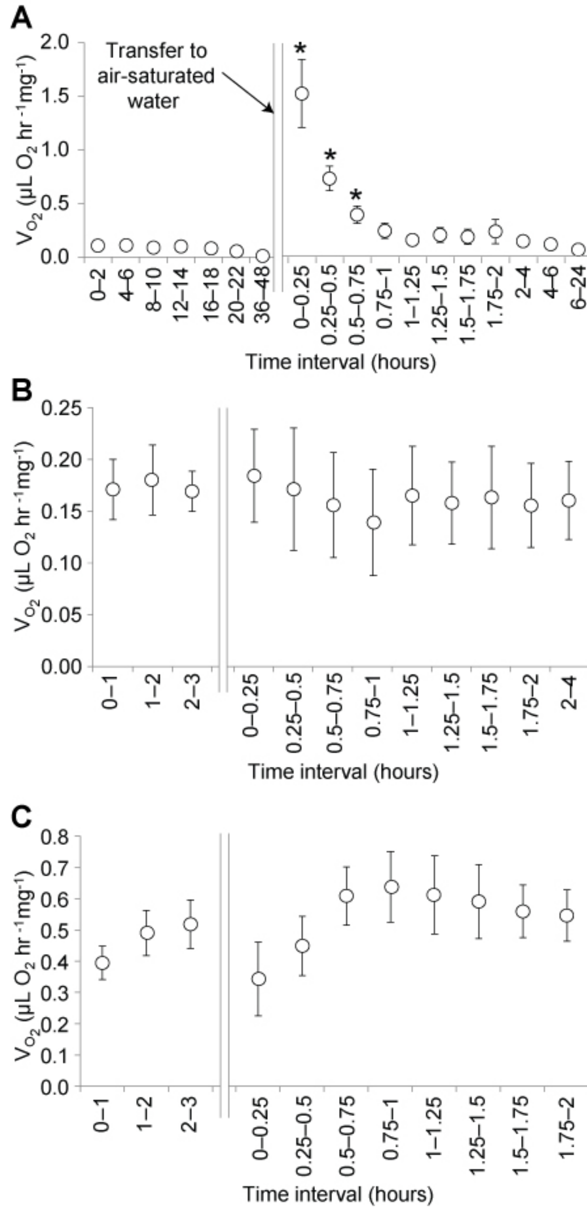

Fig. 5. Oxygen debt in anchialine shrimps, with double lines indicating transfers to fully air-saturated water after prolonged hypoxia or anoxia. (A) Oxygen intake (V_{O_2}) in *Halocaridina rubra* before and after transfer. Asterisks show significant differences compared to the initial rate (the rate at 0–2 hours; Student's paired t-test, $P < 0.030$). V_{O_2} in (B) *Caridina rubella* and (C) *Metabetaeus minutus* before and after transfer. Error bars show ± S.E.M. and $n = 10$.

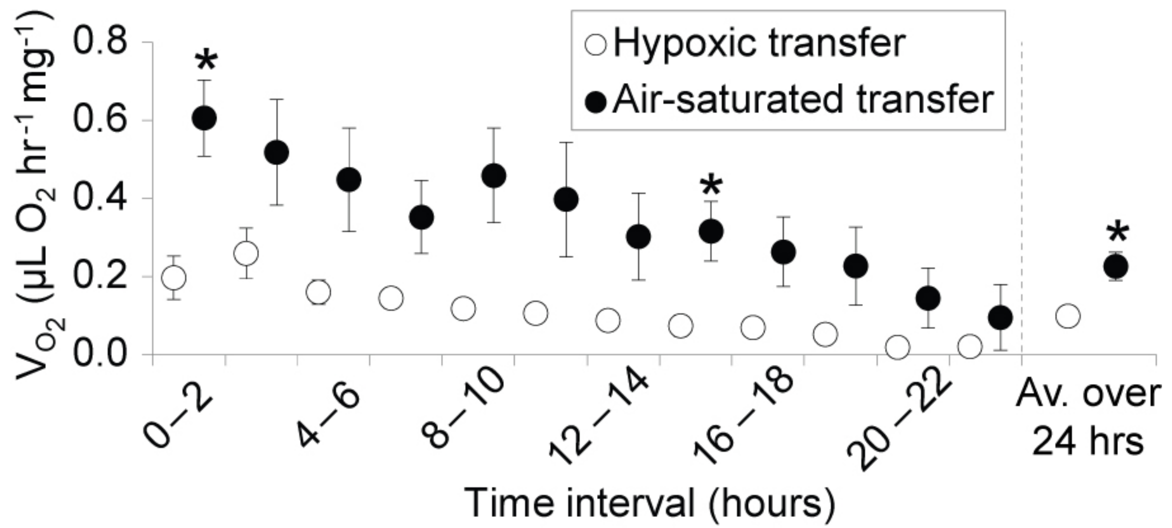

Fig. 6. Oxygen uptake (V_{O_2}) in *Halocaridina rubra* during transfers to either air-saturated water (i.e., ~150 torr, control conditions) or hypoxic water (~50 torr). Asterisks indicate differences between transfers within each time interval or on average throughout the experiment based on a Student's unpaired *t*-test ($P < 0.020$). Error bars show ± S.E.M. and $n = 10$.

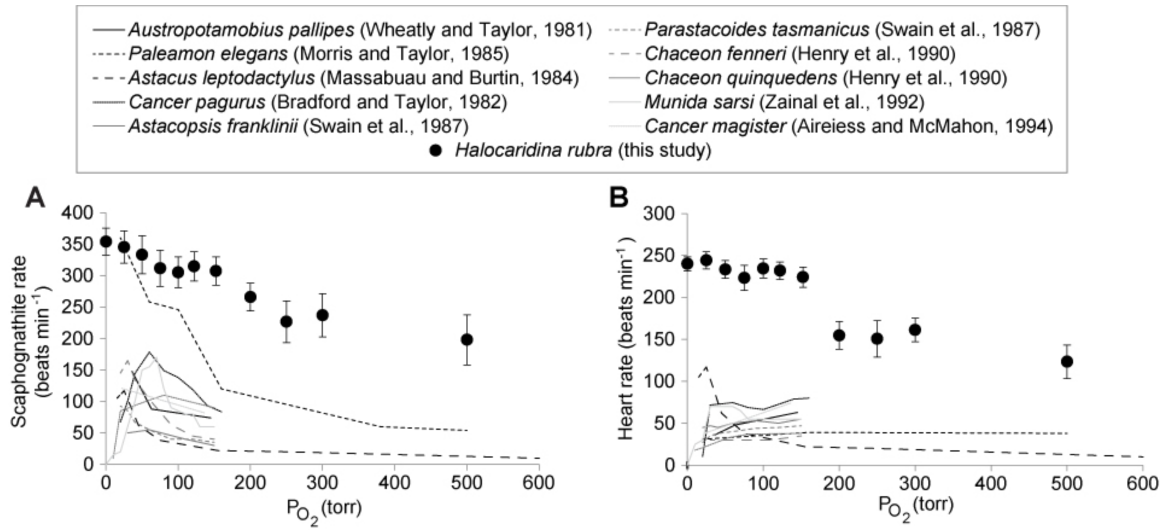

Fig. 7. Ventilatory rate of gills and heart rate in *Halocaridina rubra* during normoxia, hypoxia, anoxia, and hyperoxia compared to 10 previously studied crustaceans. Ventilatory rates were measured by counting the number of scaphognathite beats per minute and rates were measured for the same individuals under different P_{O_2} created by bubbling water with air, N_2, or O_2 (n = 10 per P_{O_2}). Error bars show ± S.E.M.

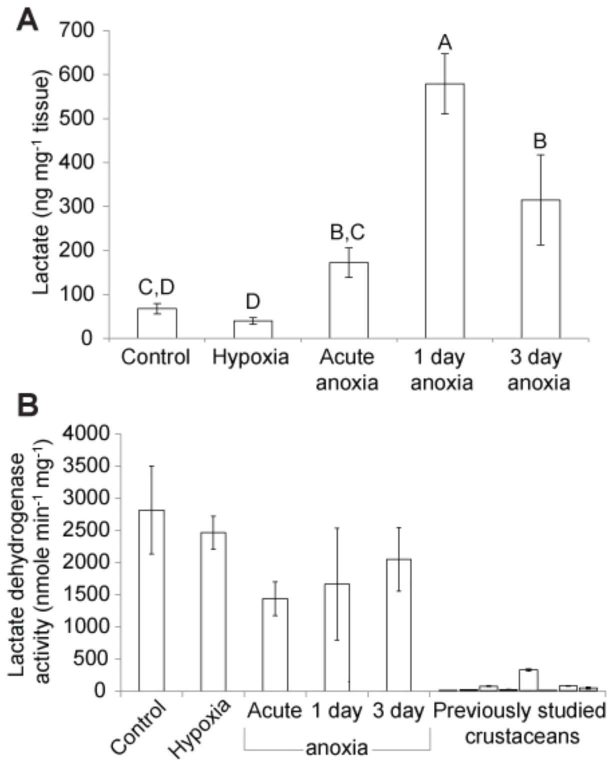

Fig. 8. Lactate and lactate dehydrogenase (LDH) activity during hypoxia/anoxia in *Halocaridina rubra*. (A) Lactate concentration during control (n = 10), hypoxia (~30% oxygenated, n = 14), acute anoxia (~1 hour, n = 9), and chronic anoxia (one day and three days, n = 4 and 5, respectively) treatments. Letters indicate significant groupings among treatments based on ANOVA with Tukey post-hoc analyses ($P < 0.05$). (B) LDH activity during the same treatments (control: n = 9; hypoxia: n = 10; acute anoxia: n= 10; one day anoxia: n= 6; three days anoxia: n = 7). Also presented for comparisons are data from eight previously studied crustaceans (From left to right: *Chaceon fenneri*, *Chaceon quinquedens*, and *Callinectes sapidus*: Walsh and Henry, 1990; *Petrolisthes laevigatus*: Yaikin et al., 2002; *Alloniscus perconvexus*: Wright and Ting, 2006; *Barbouria cubensis*: Bishop and Iliffe, 2012; *Neohelice granulata*: Lauer et al., 2012; *Carcinus maenas*: Rodrigues et al., 2013). Error bars show ± S.E.M.

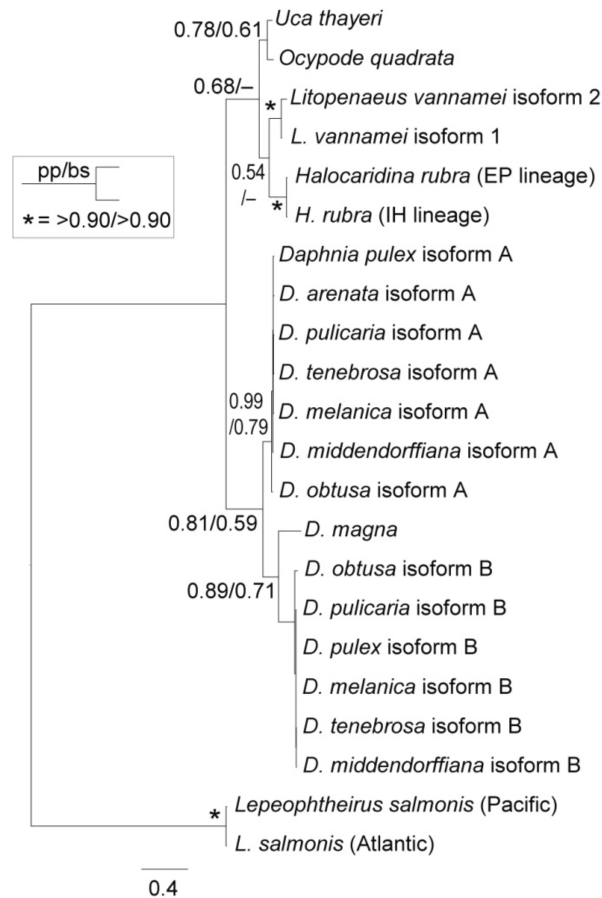

Fig. 9. Bayesian inference (BI) phylogeny of crustacean lactate dehydrogenase (LDH) nucleotide sequences. The phylogeny was rooted at the well characterized split between L- and D-LDH (Cristescu et al., 2008). Recently sequenced transcriptomes from two *Halocaridina rubra* lineages (EP and IH; see Craft et al., 2008) were used to infer novel LDH sequences, which were analyzed with all available crustacean LDH sequences from GenBank. Evolutionary relationships were inferred using both BI and maximum likelihood (ML) methods. Numbers at nodes represent support values (left values are posterior probabilities of the BI analysis based on 5 million generations and right values are based on 1000 bootstrap replicates from the ML analysis). Dashes to the right of BI support values indicate relationships that were not supported in the ML analysis. The scale bar indicates 0.4 replacements per site.

Movie 1. *Halocaridina rubra*, showing ventilation of gills via scaphognathite beating and heart rate for the same individual under normoxic (155 torr), anoxic (0 torr), and hyperoxic (500 torr) conditions. Rates are constant in normoxic and anoxic conditions, but become erratic and irregular in extreme hyperoxia. Movies are shown at half speed in order to easily view scaphognathite beating (as was done when recording rates). Movie can be viewed at https://www.youtube.com/watch?v=bKZ3tJITS1I&feature=youtu.be

Chapter 6. Conclusions and future directions

6.1 General summary

Riedl (1966) first referenced anchialine habitats as "Randhoehlen" (marginal caves) and since Holthuis (1973) termed the phrase "anchialine pool" to describe the habitat of several species of unusual shrimps, interest in these coastal ponds, pools, cracks, lakes, and caves has been growing. These habitats have unusual geological, biological, and physical properties, which have sparked the interest of scientists as well as the general public. However, because these habitats have only recently been described, in-depth studies of their fauna are severely lacking. Most previous studies have focused on describing new species from these habitats, and a recent study describing a new genus of crab from Hawaiian anchialine ponds (Ng, 2011) demonstrates that the diversity of anchialine organisms from even well-studied areas may still be underestimated. Studies from the Santos Laboratory using molecular techniques to identify cryptic diversity in anchialine species have supported this hypothesis, as *Halocaridina rubra* was shown to encompass at least eight distinct evolutionary lineages (Santos, 2006; Craft et al., 2008) and Japanese species from the Ryukyu Islands were found to contain a similar level of cryptic diversity (Weese et al., 2012; Weese et al., 2013). Unfortunately, these habitats and their unique/unknown fauna are being destroyed at an alarming rate (Brock et al., 1987). It is therefore critical to address the lack of knowledge on the biology of anchialine organisms to aid in their conservation.

In the preceding chapters, studies have been conducted to examine the ecology and physiology of anchialine organisms. Taken together, the results presented in these

chapters suggest that in Hawaiian anchialine habitats the endemic shrimp *H. rubra* is largely able to avoid predation by invasive fishes by adopting a novel diel migration strategy. This species is also able to cope with the natural fluctuations in salinity characteristic of anchialine habitats by exhibiting a novel osmoregulatory strategy in which osmoregulatory processes such as dense mitochondria-rich cell populations in the gills are constitutively activated. This includes constant, high expression of ion transporters, which are normally only up-regulated during salinity transfer based on a meta-analysis of previous studies. However, this osmoregulatory capability has arisen at the expense of respiration, another critical function of crustacean gills. This species must therefore ventilate its gills at near-maximum frequencies even under normoxia, which results in decreased oxygen uptake under even moderate hypoxia. Despite this trade-off, *H. rubra* can tolerate extreme periods of hypoxia/anoxia, likely due to high levels of lactate dehydrogenase and rapid repayment of oxygen debts. Importantly, studies of anchialine shrimp species from the Ryukyus Islands suggest that the mechanisms described here for *H. rubra* cannot be applied indiscriminately to all anchialine shrimps.

6.2 Effects of invasive fishes on the anchialine ecosystem

Since the completion of Chapter 2 (Havird et al., 2013a), additional studies from the Hairston Laboratory at Cornell University have attempted to shed light on the ecological consequences of invasive fishes in the Hawaiian anchialine ecosystem (Carey et al., 2011; Dalton et al., 2012). These studies also found that anchialine shrimps in fish-invaded ponds adopt a diel migration strategy in which shrimp retreat underground during the day and emerge into the epigeal component of the habitat at night, when fishes

undergo behavioral sleep and do not represent a predation threat. However, these studies as well as those presented in Havird et al. (2013a) have only described this for a small fraction of anchialine habitats across Hawaii. Unpublished results of a diel survey conducted during this dissertation research suggest that this pattern is consistent with fish-invaded habitats across Hawaii, with fish-invaded habitats only possessing appreciable numbers of shrimps in the surface waters at night (Fig. 1). However, surveys of fishless ponds did not agree with the results of Carey et al. (2011) and other studies (Capps et al., 2009; Sakihara, 2012), which suggested that shrimp are most dense during the day in fishless ponds. While this was true of fishless habitats on the Kona Coast of Hawaii (where the other studies were performed), at Hanamanioa on Maui shrimp were denser at night than during the day. Also, two fish-invaded Kona habitats did not possess appreciable shrimp populations either during the day or at night (although individuals were sporadically observed at KIKI). Finally, one fish-invaded Kona habitat (KAHO 54) possessed noticeable, although low, numbers of both fish and shrimp during the day. These results suggest that future studies should focus on understanding the natural patterns of diel migration in fishless habitats as well as documenting any progression during fish invasion (e.g., from both present during the day, to shrimp only present at night, and finally to shrimp completely extirpated).

These additional studies have also shed light on the chemical properties of fish-invaded vs. fishless habitats. Dalton et al. (2012) found that fish-invaded habitats had higher nutrient concentrations, increased epilithon biomass, and productivity than fishless habitats. These results led to the conclusion that top-down effects from fish-invasion and behavioral alteration of *H. rubra* have profound consequences for the entire anchialine

ecosystem. Moreover, nutrient input into fishless habitats also resulted in bottom-up effects such as increased productivity, although top-down effects exerted a more extreme change in ecosystem productivity (Dalton et al., 2013). Results from these recent studies suggest that the entire food web of an anchialine habitat may be affected during fish-invasion. However, these results are based on field observations, which may be more applicable to real-world situations, but often lack the control and replication characteristic of laboratory experiments (Kimball and Levin, 1985; Eberhardt and Thomas, 1991; Edmondson, 1993; Schmitdz, 2005).

In an attempt to determine if *H. rubra* can also alter primary productivity in a replicated experiment, mesocosms were constructed consisting of single 2 L tanks with nine tiles each that contained a cultured community of algae originally taken from a pond at the North Auburn Fisheries Unit. Each tank had a variable number of *H. rubra* that was determined based on the field survey described above (Fig. 1). These treatments (n = 6 replicates per treatment) included 1300, 500, 200, and 0 individuals m^{-2} (corresponding to 25, 15, 5, and 0 individuals per tank), which corresponded to four ecological states: 1) the highest density observed for a habitat in the survey; 2) the average density during the day in a fishless habitat; 3) average density at night in a fish-invaded habitat, and; 4) average density in a fish-invaded habitat during the day. As a measure of productivity, chlorophyll *a* (Chl *a*) was quantified before shrimp were added to the mesocosms and at eight time points after shrimp were added (1–52 days) by removing the growth from a tile (following procedures similar to Sarnelle et al., 1993) and subjecting it to fluorometry (following Sartory and Grobbelaar, 1986). Shrimp densities did not significantly influence [Chl *a*] until 30 days of grazing pressure (Fig. 2), when the treatment with no

shrimp had significantly higher [Chl *a*] than treatments with moderate or high numbers of shrimp (one-way ANOVA, $P < 0.001$). This trend was also found at the end of the 52 day experiment.

The results of this experiment indicate that even in an artificial community dominated mostly by algae, grazing by *H. rubra* can exert significant influence on algal abundance. One possibility is that in fish-invaded habitats lack of *H. rubra* grazing results in an alternate ecological state in which a low-diversity community primarily composed of filamentous algae dominates the natural, high diversity community of an orange cyanobacterial-bacterial mat characteristic of some Hawaiian anchialine habitats (Bailey-Brock and Brock, 1993). Although the results of Chapter 2 suggest that predation of *H. rubra* by invasive fishes is low, the altered behavior induced by invasive fishes may result in a lower overall grazing pressure by *H. rubra*, which could lead to the proposed alternate ecological state. This would mean that *H. rubra* actively maintains the microbial communities characteristic of the orange cyanobacterial-bacterial mat by grazing, and suggests that *H. rubra* selectively grazes on particular members of this community.

In an ongoing collaboration with the Hairston Laboratory, we are testing this hypothesis using a combination of field-based and laboratory mesocosm experiments. Generally, these experiments seek to shed light on selective grazing by *H. rubra* and microbial community structure among habitats by using next-generation sequencing to profile microscopic eukaryotes and prokaryotes based on ribotype analyses. Briefly, microbial communities in the digestive tract and feces of *H. rubra* will be compared to crust and water column communities from their habitats of origin. Additionally, laboratory experiments using algae-coated tiles similar to those described above will be

used to determine the community structure of environmental, digestive tract, and fecal samples after varying numbers of days of *H. rubra* grazing. Preliminary results using denaturing gradient gel electrophoresis for both *16S* and the cyanobacterial specific gene *nifH* indicate that communities in *H. rubra* digestive tracts are very different than those in environmental samples from their habitat of origin (Fig. 3), and preliminary analyses based on 16S rRNA gene sequencing support this conclusion (Fig. 4). One possible implication of these preliminary results is that *H. rubra* does selectively graze on anchialine microbial communities. Ongoing collaborations will continue to shed light on the ecological consequences of fish invasions in anchialine habitats, which will have strong implications for conservation efforts.

6.3 Use of meta-analysis to interpret results and shape future studies

The meta-analysis presented in Chapter 3 (Havird et al., 2013b) confirms previous qualitative reviews (e.g., Evans et al., 2005; Henry et al., 2012; McNamara and Faria, 2012) indicating that genes encoding ion transport proteins and accessory enzymes generally increase in expression following salinity transfer. However, the statistical techniques employed in meta-analyses allows for quantitative assessment of this up-regulation under different scenarios. This revealed that for crustaceans specifically, the most extreme up-regulation of osmoregulatory genes occurred 24 hours after salinity transfer during transfers from high to low salinities encompassing the most dissimilar salinities. These results will allow future studies to be designed in such a way that any changes in gene expression can be observed using the minimal number of time points/samples when funds are limited or the technique is particularly expensive. For

example, ongoing studies investigating gene expression in the entire gill transcriptome during salinity transfer in anchialine shrimps using RNA-Seq (e.g. after Meyer et al., 2011) will make use of this result by investigating this time point for multiple species.

The results highlighted in Chapter 3 also indicated that there was a lack of salinity-mediated gene expression studies in non-brachyuran crustaceans. The gene expression experiments in Chapter 4 are an important first step towards addressing this lack of knowledge. The results of these experiments suggest that typically studied crustacean osmoregulatory genes altered expression in an atypical manner in salinity-transferred *H. rubra*. The results of this meta-analysis allowed this apparent pattern to be quantified. For the Na^+/K^+-ATPase (NKA), the most well studied crustacean osmoregulatory gene, when the raw relative expression values obtained in Chapter 4 were converted to ln *RR* scores like those presented in Chapter 3 and compared with those results, it was apparent that the results for *H. rubra* fall outside of 95% confidence intervals based on previously studied crustaceans (Fig. 5).

In addition to identifying non-brachyuran crustaceans as understudied targets for future salinity-induced gene expression analyses, Chapter 3 also identified other areas for future studies that are lacking in the current literature. This includes examining differential expression of isoforms in non-teleosts. Results presented in Chapter 4 offer a possible opportunity to address this, as transcriptome sequencing of *H. rubra* identified multiple isoforms of NKA and other osmoregulatory genes that could be targeted in future studies. Ongoing RNA-Seq experiments with anchialine shrimps may identify other isoforms that have differential functions in salinity acclimation. Chapter 3 also identified a major lack of field-based studies in salinity induced gene expression studies.

Fiddler crabs (*Uca* sps.) from tidal creeks and estuaries may represent a good system to address these studies in crustaceans, as they are found throughout a salinity gradient, could be easily transported and maintained in mesocosms in large numbers, and have large gills that are easily studied.

6.4 A functional trade-off in the gills of *Halocaridina rubra*

The results presented in Chapter 3 indicate that *H. rubra* acts as a strong osmoregulator by constantly activating osmoregulatory mechanisms in the gills including proliferation of large mitochondria-rich cell (MRC) populations and up-regulation of relevant osmoregulatory genes. Interestingly, all gills in *H. rubra* appear to be specialized for osmoregulation, which is in contrast to most previously studied crustaceans which tend to only have the posterior gills specialized for osmoregulation (Copeland and Fitzjarrell, 1968; Neufeld et. al., 1980; Dickson et al., 1991). In these species, the anterior gills are specialized for respiration, and although all gills act as osmoregulatory in the crayfish, specialized portions of each gill (the outer portions of lamellae) are specialized for respiration (Dickson et al., 1991). However, no significant portion of *H. rubra* gills appears specialized for respiration.

This may represent an evolutionary trade-off in the gills of *H. rubra* whereby advanced osmoregulation has been achieved at the expense of respiration and aerobic metabolism. This scenario is supported by the results of Chapter 5, which showed *H. rubra* to have a high resting ventilation rate compared to other crustaceans. If all or most of the gill epithelia of *H. rubra* are specialized for osmoregulation (as indicated by silver nitrate and vital MRC staining results presented in Chapter 4), this would indicate a thick

epithelia (~10 μM vs. ~1 μM in respiratory epithelia) (Taylor and Taylor, 1992; Freire et al., 2008), which would pose problems for diffusive oxygen transport from the water across the gill epithelia and into the hemolymph. To overcome this large diffusion distance, a large gradient in oxygen partial pressure would need to be maintained across the gills. One way to achieve this gradient would be with the high ventilation rate observed in Chapter 5.

The atyids have a decisively freshwater ancestry, as no extant marine atyids are known (Huxley, 1880; Fryer, 1977), most adults cannot tolerate seawater (Smith and Williams, 1981), and cretaceous freshwater deposits of atyids suggest a long freshwater ancestry (Buerlen, 1950; Glaessner, 1969). Therefore, the early evolutionary lineage that eventually gave way to *H. rubra* likely invaded anchialine habitats from freshwater streams (freshwater atyids still exist in Hawaiian streams today). Osmoregulatory adaptations may have been selected for over respiratory adaptations initially because unlike stream habitats, anchialine habitats are relatively oxygen poor and have nutrient poor food webs, making them low energy habitats. Therefore, the high energy associated with aerobic metabolic pathways may not have been necessary in anchialine habitats, allowing the gills to specialize for osmoregulation and not respiration. Alternatively, atyids may have initially been confined to portions of the anchialine ecosystem with little fluctuations in salinity (e.g., epigeal portions of inland habitats). As shrimp began to gradually expand into other habitats/portions of the ecosystem, osmoregulatory adaptations in the gills may have been selected for while respiratory function was lost or diminished.

Also supported by the results of Chapter 5, invasion of anchialine habitats may

have resulted in or selected for alternative versions or modifications of anaerobic metabolic pathways. Use of anaerobic pathways may have been selected for in anchialine habitats because of the existence of hypoxic and anoxic areas in many habitats (Sket, 1996). In *H. rubra*, these anaerobic modifications could have included the high levels of lactate dehydrogenase observed in Chapter 5.

6.5 Conservation of extreme physiology across anchialine shrimp species

Most anchialine shrimps are found in the "anchialine clade" of atyids based on a molecular phylogeny of atyid shrimps (von Rintelen et al., 2012). However, as hypothesized by von Rintelen et al. (2012), the Japanese species *Caridina rubella* falls outside of this clade based on a phylogeny using unpublished *16S* sequences (Fig. 6). This means that *C. rubella* likely invaded habitats independently from members of the anchialine clade. Likewise, *Metabetaeus minutus* falls outside of the Atyidae in the Alpheidae and represents a third invasion of anchialine habitats, likely from seawater/marine habitats. Therefore, it is possible that each independent invasion resulted in different physiological adaptations, especially osmoregulatory adaptations in *M. minutus*, as this species likely invaded from seawater habitats, whereas all the other invasions were likely from freshwater habitats. Future studies examining osmoregulation in the Japanese species investigated in Chapter 5 will shed light on whether the unusual osmoregulatory adaptations observed for *H. rubra* in Chapter 4 are confined solely to this species, exclusively to members of the anchialine clade, to all anchialine atyids, or to all shrimps from anchialine habitats.

Of particular interest is whether the osmoregulatory vs. respiratory trade-off

described for *H. rubra* gills is present in other anchialine species/lineages. In Chapter 5, only *H. rubra* was shown to employ an oxy-conforming strategy of oxygen uptake, quickly repay oxygen debts, and survive indefinitely in anoxia, suggesting the trade-off may only exist in this species and respiration/aerobic metabolism may not be impeded in the Japanese species. Supporting this, preliminary results of silver nitrate staining in *C. rubella* and *M. minutus* suggest that portions of gills in these species are specialized for respiration, not osmoregulation (Fig. 7), unlike *H. rubra*. Therefore, the unique osmoregulatory properties and respiratory trade-off described for *H. rubra* may be confined to the anchialine clade of atyids or a unique feature of *H. rubra*. Ongoing studies investigating gene expression during salinity transfer in the Japanese species will further shed light on their osmoregulatory adaptations.

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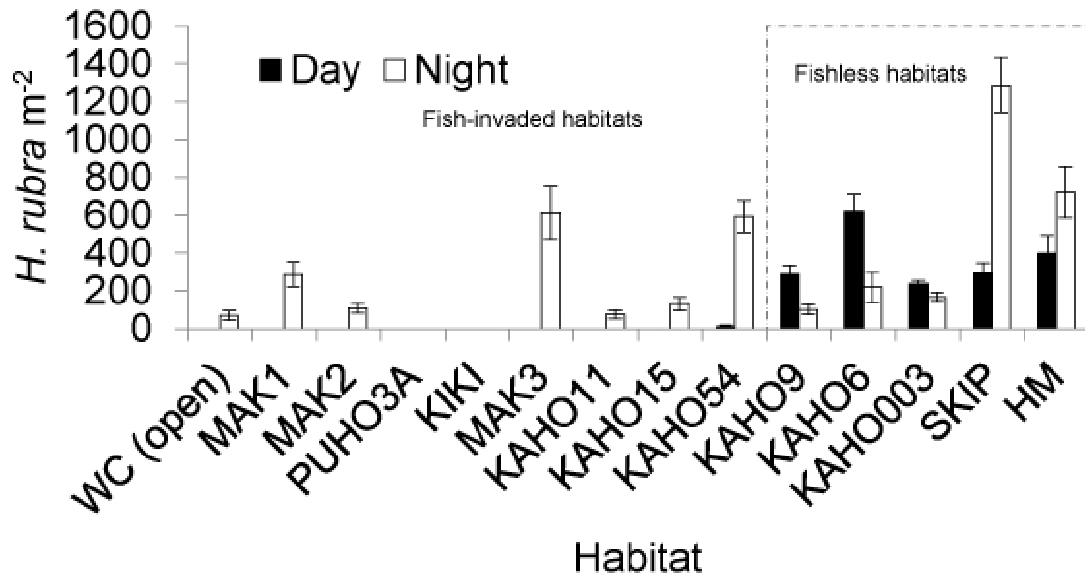

Fig. 1. Diel migration patterns for anchialine shrimp (*Halocaridina rubra*) from fish-invaded and fishless anchialine habitats in Hawaii. Shrimp densities (n = 11 per sampling) were quantified using the methods outlined in Chapter 2. Abbreviations: WC: Waianapanapa Cave, Maui; MAK: Makalawena Beach, Hawaii; PUHO: Pu'uhonua O Hōnaunau National Historical Park, Hawaii; KIKI: Kieawe Iki Beach, Hawaii; KAHO: Kaloko-Honokōhau National Historical Park, Hawaii; SKIP: Skippy's Pond, Maui; HM: Hanamanioa, Maui. Error bars = 95% C.I.

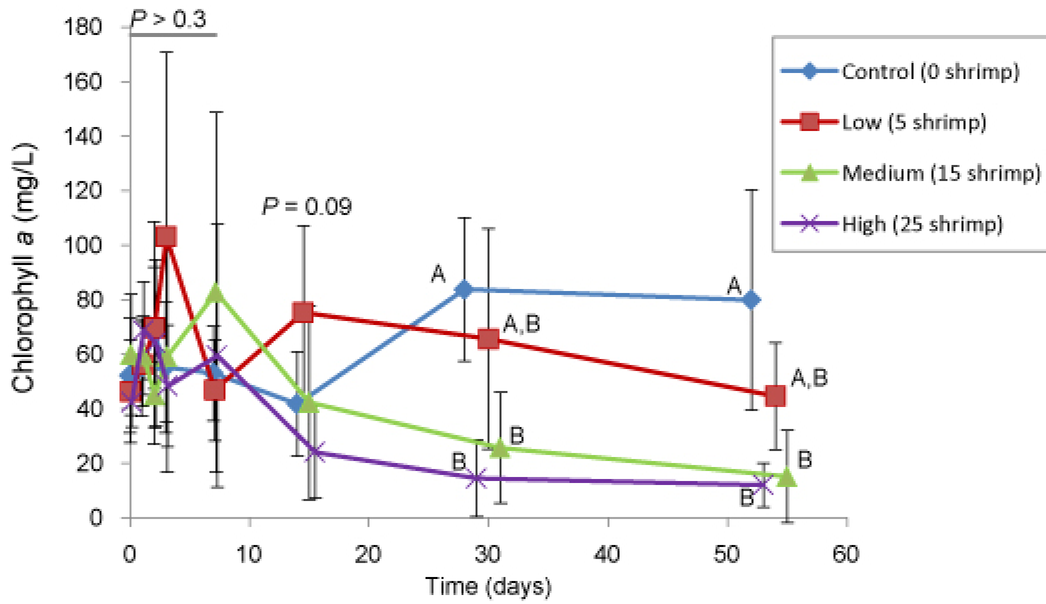

Fig. 2. Chlorophyll *a* concentration on experimental tiles over two months of grazing pressure by varying densities of *Halocaridina rubra*. Densities of *H. rubra* were based on environmental densities described in Fig. 1 and correspond to four ecological states: 1) fish-invaded during the day (0 shrimp); 2) average fish-invaded at night (5 shrimp); 3) average fishless habitat during the day (15 shrimp), and; 4) highest density observed (at SKIP during the night, 25 shrimp). [Chl *a*] (n = 6 tiles per sample) was recorded after 1, 2, 3, 7, 14, 28, and 52 days of grazing pressure. Letters indicate significant groupings among treatments at each time point (one-way ANOVA with Tukey post-hoc analysis). Error bars = 95% C.I.

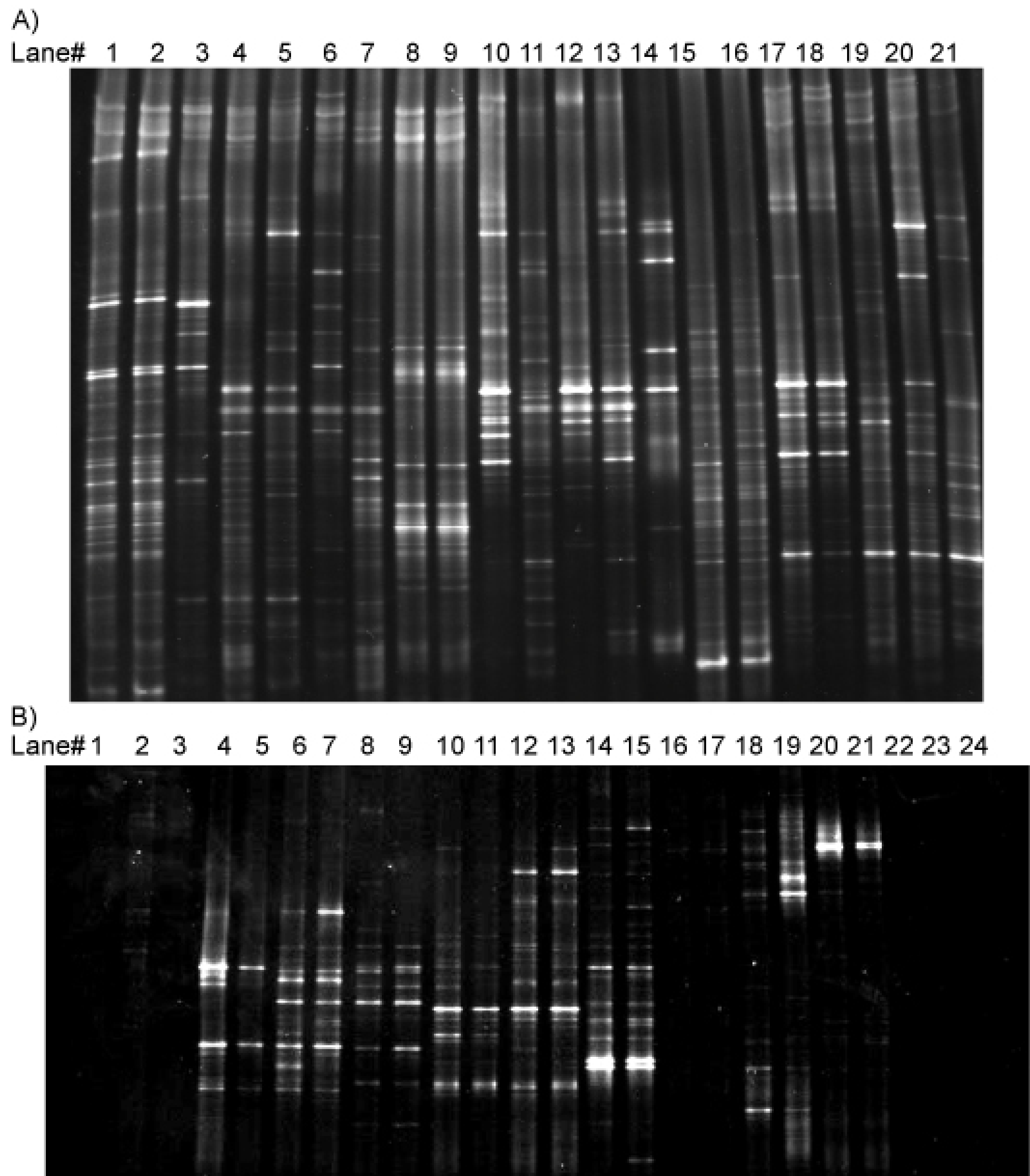

Fig. 3. Denaturing gradient gel electrophoresis (DGGE) analysis of environmental and shrimp digestive tract samples. A) Using the universal bacterial marker *16S*. Lanes: 1-2) Benthic orange crust samples (two replicates) from Pohue Bay, Hawaii; 3-7) Shrimp digestive tracts from five individuals taken from Pohue Bay; 8-9) Benthic orange crust samples (two replicates) from Skippy's Pond, Maui; 10-14) Shrimp digestive tracts from five individuals taken from Skippy's Pond; 15-16) Benthic orange crust samples (two

replicates) from Hanamanioa, Maui, and; 17-21) Shrimp digestive tracts from five individuals taken from Hanamanioa. B) Using the cyano-bacterial specific marker *nifH*. Lanes: 1-3) Benthic environmental samples from three anchialine caves; 4-15) Benthic environmental samples from 12 anchialine habitats with the distinctive orange cyanobacterial-bacterial crust; 16-21) Benthic environmental samples from six anchialine habitats with sediment/soil substrate, and; 22-24) Shrimp digestive tracts from three individuals taken from habitats with the distinctive orange cyanobacterial-bacterial crust.

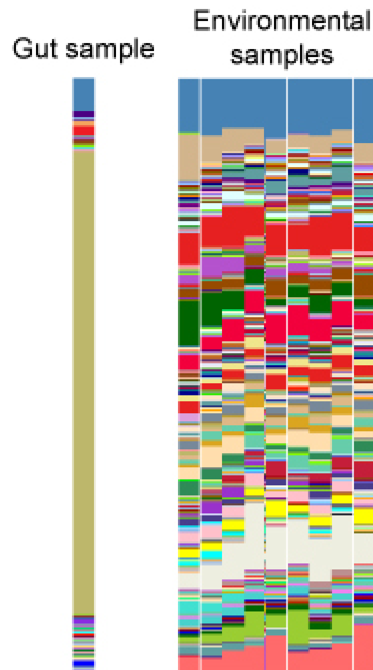

Fig. 4. Meta-genetic structure of bacterial communities in a single *Halocaridina rubra* digestive tract and nine benthic environmental samples from Skippy's Pond, Maui. Each column represents a single sample and each colored bar in the column represents a single operational taxonomic unit (OTU), which generally corresponds to a unique bacterial species. The height of each colored bar represents the relative proportion of that OTU in the sample. OTUs were clustered based on 97% similarity in the V6 region of *16S*, with 187,842 ± 53,430 (S.E.M.) reads per sample. OTUs were annotated using NBCI BLAST and blue bars at the top of each sample represent reads that could not be annotated. The large brown-green bar that makes up 79% of the reads in the digestive tract sample was annotated as a *Massilia* sp. in the β-proteobacteria.

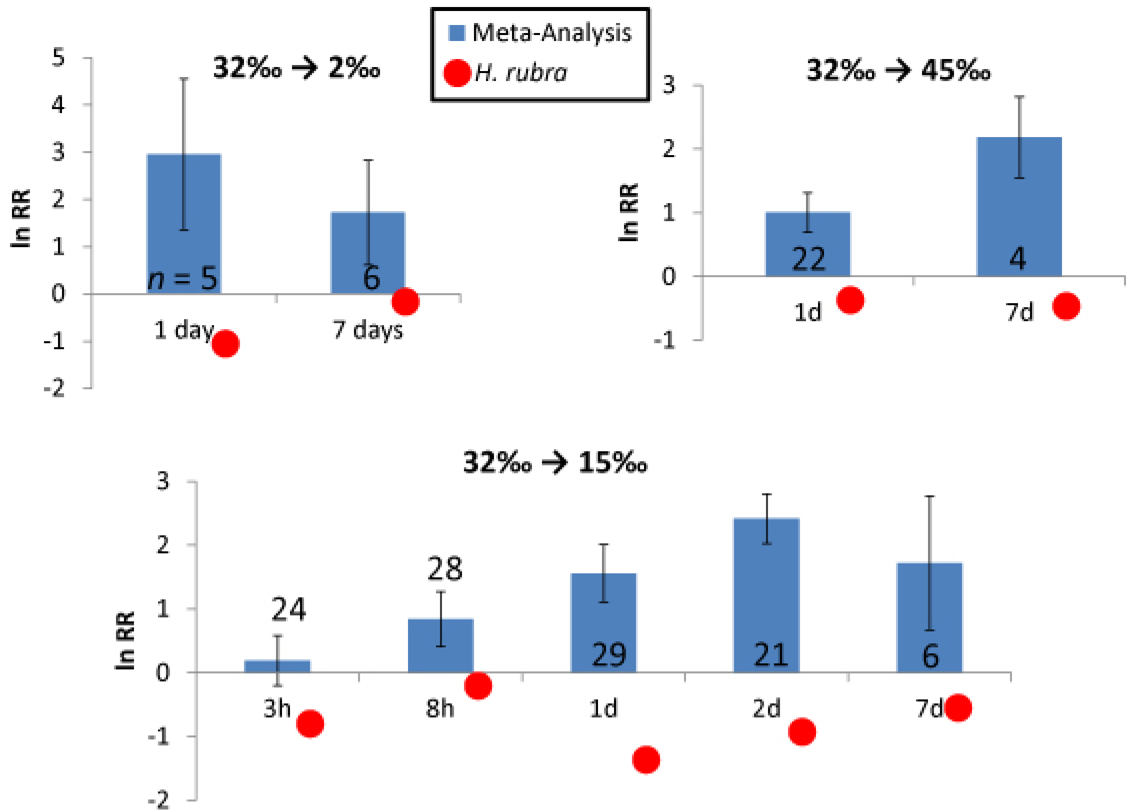

Fig. 5. Na^+/K^+-ATPase (NKA) expression in the gills of *Halocaridina rubra* during three commonly studied salinity transfers as compared to other crustaceans based on the meta-analysis presented in Chapter 3. Sample sizes are based on the number of effect sizes used to calculate the average and 95% C.I. (error bars) for the meta-analysis. Ln *RR* is a common effect size metric and, in this case, values above zero represent up-regulation of NKA and values below zero represent down-regulation of NKA. Values from the meta-analysis are based on the results from Chapter 3 and values for *H. rubra* are from Chapter 4.

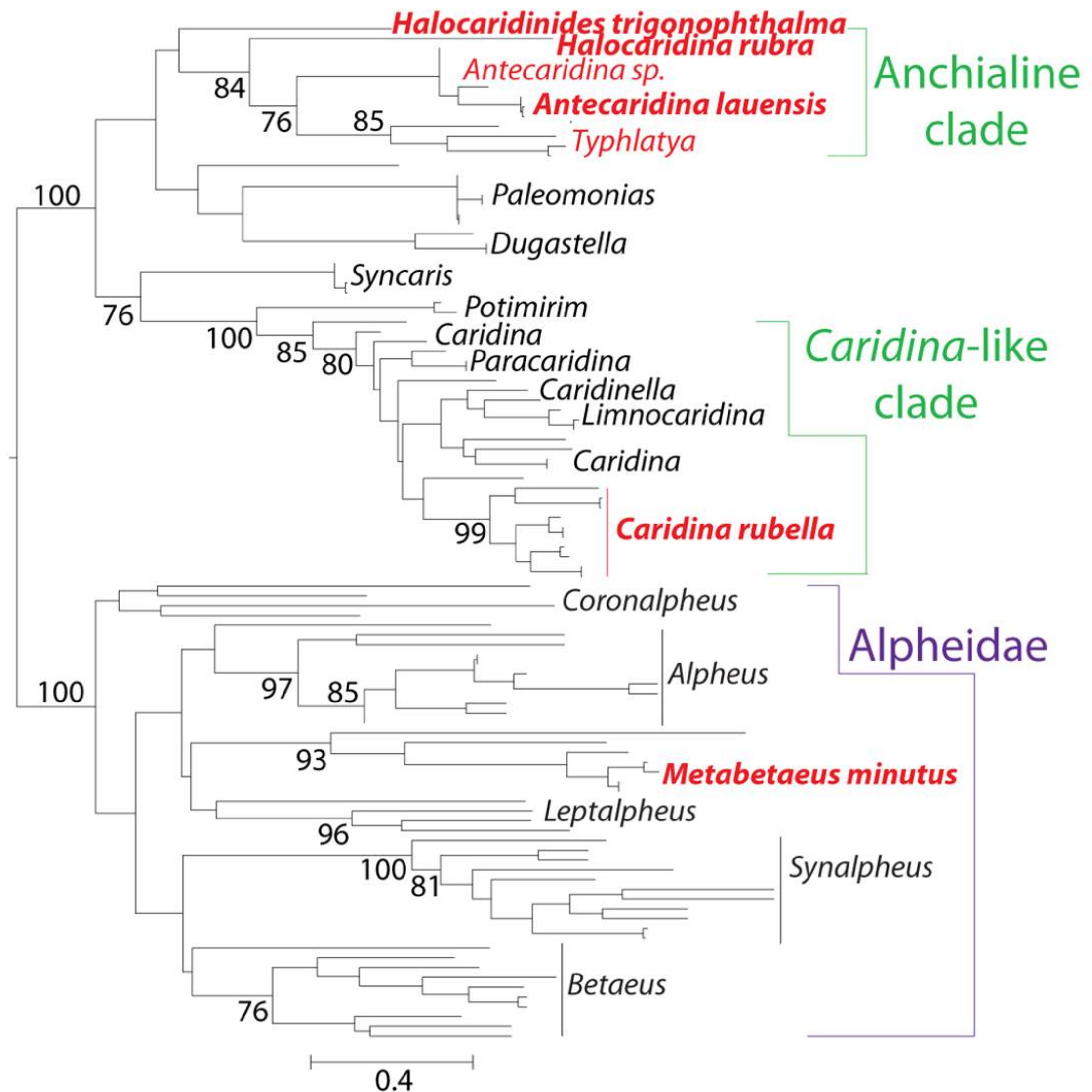

Fig. 6. Maximum likelihood phylogeny of atyid and alpheid shrimp based on publically available and unpublished (for *C. rubella*, provided by Dr. David Weese) *16S* sequences. Anchialine taxa are presented in red, taxa used in dissertation research are bolded. “Anchialine” and “*Caridina*-like” clades are named based on von Rintelen et al. (2012). Bootstrap support values > 75 are presented based on 1000 rapid bootstrap replicates. The scale bar indicates 0.4 nucleotide substitutions per site.

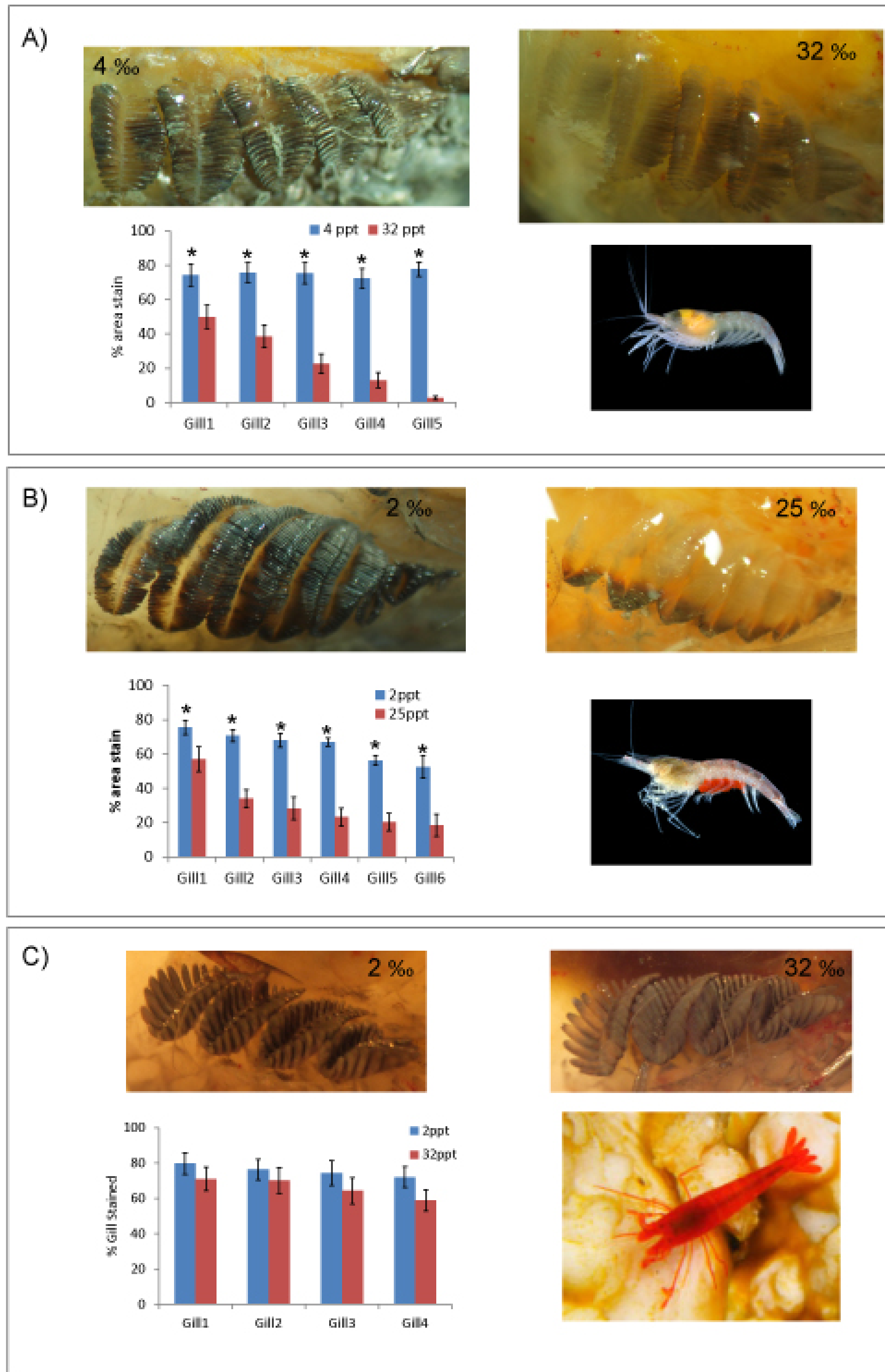

Fig. 7. Silver nitrate ($AgNO_3$) staining in the gills of anchialine shrimp species. A) *Metabetaeus minutus* (n = 6), B) *Caridina rubella* (n = 10), and C) *Halocaridina rubra* (n = 10). Methods and results follow those presented for *H. rubra* in Chapter 4.