

RESEARCH ARTICLE

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

Justin C. Havird^{1,2,*}, Scott R. Santos^{1,2} and Raymond P. Henry¹**ABSTRACT**

Studies of euryhaline crustaceans have identified conserved osmoregulatory adaptations allowing hyper-osmoregulation in dilute waters. However, previous studies have mainly examined decapod brachyurans with marine ancestries inhabiting estuaries or 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 freshwater 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 of ~868 mOsm kg⁻¹ H₂O in freshwater. 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, expression of ion transporters and supporting enzymes that typically undergo upregulation during salinity transfer in osmoregulatory gills (i.e. Na⁺/K⁺-ATPase, carbonic anhydrase, Na⁺/K⁺/2Cl⁻ cotransporter, V-type H⁺-ATPase and arginine kinase) were generally unaltered 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 that are encountered in anchialine habitats. Thus, anchialine taxa represent an interesting avenue for future physiological research.

KEY WORDS: Arginine kinase, Carbonic anhydrase, Crustacean, Euryhaline, Gene expression, Gill, Na⁺/K⁺-ATPase, Na⁺/K⁺/2Cl⁻ co-transporter, qPCR, Salt/ion transport, Transcriptome, V-type H⁺-ATPase

INTRODUCTION

Euryhaline crustaceans can function as strong osmoregulators that maintain internal osmotic concentrations above those of the external

environment when in dilute seawater (SW) (reviewed by Mantel and Farmer, 1983) through active salt transport across the gills (reviewed by Péqueux, 1995; Charmantier et al., 2009; Henry et al., 2012; McNamara and Faria, 2012). In SW, 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 transfer to water 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 16–18‰ (Kinne, 1971; Hsueh et al., 1993). Thus, only osmotic/ionic regulators are capable of traversing wide salinity ranges like those spanning from SW (35‰) to freshwater (FW; near 0‰).

Osmoregulation in brachyuran crabs takes place in the mitochondria-rich cells (MRCs) of the posterior gills, which are characterized by a thick (10–20 μm) osmoregulatory epithelium, 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). However, all gills in crayfishes and shrimps possess MRCs and are involved in osmoregulation, although some areas of individual gills remain specialized for respiration (Wheatly and Henry, 1987; Dickson et al., 1991; McNamara and Lima, 1997; Ordiano et al., 2005; Huong et al., 2010). Active salt absorption in the MRCs is accomplished via a suite of ion transporters and supporting enzymes (Evans et al., 2005; 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⁻/HCO₃⁻ 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 HCO₃⁻ needed to support Na⁺/H⁺ and Cl⁻/HCO₃⁻ 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 versus stenohaline crustaceans (Henry, 1984; Harris and Bayliss, 1988) (but see Piller et al., 1995), (2) in gills versus other tissues (e.g. Henry, 2001; Lucu and Flik, 1999), (3) in osmoregulatory versus respiratory gills (Henry, 1984;

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List of symbols and abbreviations

AK	arginine kinase
BB	Baby Bear
BH	Blue Hole
CA(c/g)	carbonic anhydrase (c or g isoform)
C _t	threshold cycle
DASPMI	4-[4-(dimethylamino)styryl]-N-methylpyridinium iodide
EP	Eric's Pond
FW	freshwater
HAT	V-type H ⁺ -ATPase
HM	Cape Hanamanioa
ICP-OES	inductively coupled plasma optical emission spectrometry
IH	Issac Hale
JP	Joe's Pond
KBP	Kalaeloa Unit
KIKI	Keawaiki Bay
KONA	Kona Coast
MAKA3	Makalawena 3
MIKE	Mike's Pond
MRCs	mitochondria-rich cells
NKA	Na ⁺ /K ⁺ -ATPase
NKCC	Na ⁺ /K ⁺ /2Cl ⁻ cotransporter
OWAI	Waianae Boat Harbor
PB	Papa Bear
PUHO3A	Pu'uhonua o Hōnaunau National Historical Park 3A
RES1	Restoration 1
SKIP	Skippy's Pond
SW	seawater
TAP	Tap's Pond

Holliday, 1985; Böttcher 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; Henry et al., 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 usually increases in the osmoregulatory gills following salinity transfer, with transfers from higher to lower salinities over 1–3 days inducing the greatest increases in expression (Havird et al., 2013). For example, *Chasmagnathus granulatus* (subsequently renamed *Neohelice granulata*) (Sakai et al., 2006) increased NKA expression 33- to 55-fold (Luquet et al., 2005) when transferred from 30‰ to 2‰. Similarly, cytoplasmic CA (CAc; the osmoregulatory isoform of CA) underwent a 100-fold increase in expression during high to low salinity 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 FW species also exist with regard 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, FW species typically have low conductance ('tight') epithelia, which hinders ion loss (reviewed in Henry et al., 2012). NKA activity is also uniformly distributed across all gills in the euryhaline FW 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). FW 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 with ~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 SW or FW. Given this, extending such studies to other taxonomic or ecological groups could provide further insight into the evolution of osmoregulatory mechanisms. For example, studies utilizing the FW caridean genus *Macrobrachium* have revealed mechanisms that contrast with those in brachyuran crabs (McNamara and Lima, 1997; Ordiano et al., 2005; Huong et al., 2010). Crustaceans from the anchialine ecosystem represent another 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 SW and FW through underground connections (Holthuis, 1973; Sket, 1996). Accordingly, organisms from these habitats can experience daily fluctuations in salinity comparable to those in estuaries due to tides (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 FW ancestry as: (1) FW 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; Hunte, 1979b), most adult atyids outside the 'anchialine clade' are intolerant of SW (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 across multiple organizational levels.

RESULTS**Haloclines in Hawaiian anchialine habitats**

Measurements of vertical salinity gradients from 12 Hawaiian anchialine habitats revealed that while variation existed between habitats (e.g. spanning 2.8–20.9‰), most lacked a detectable vertical gradient from the surface to the bottom (i.e. they possessed the same salinity across depth; Fig. 1A). For the three habitats with vertical gradients, salinity increased measurably with depth (Fig. 1B). 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 2–3 cm below the surface were measured in some habitats following rainfall (Fig. 1C). 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 (supplementary material Movie 1).

Osmotic gradients during salinity transfer

Hemolymph osmolality of *H. rubra* chronically acclimated to SW (32‰; 960±1.6 mOsm kg⁻¹ H₂O) reflected that of an osmoconformer (i.e. 958±30.6 mOsm kg⁻¹ H₂O). Therefore,

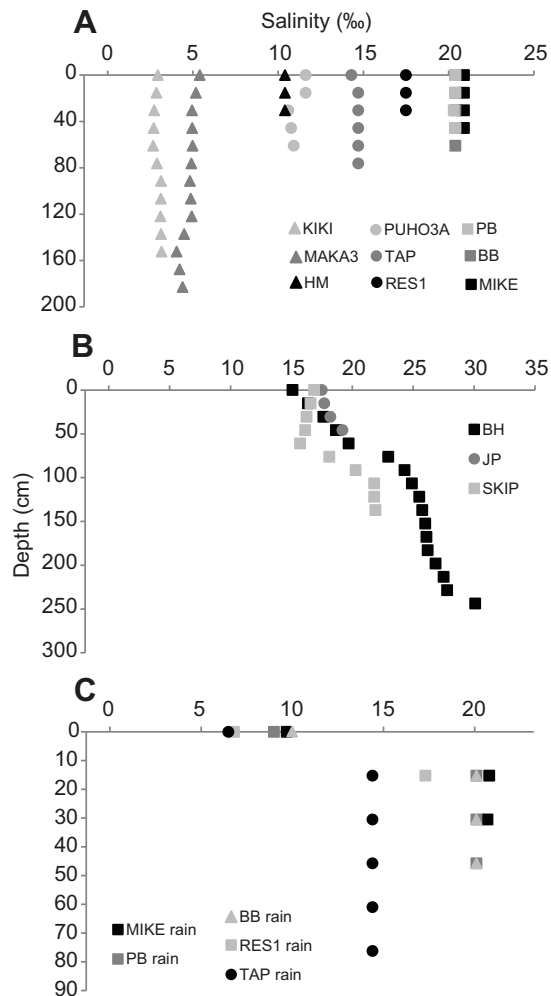


Fig. 1. Vertical salinity gradient profiles identified from Hawaiian anchialine habitats. (A) Many habitats exhibited little salinity variation with depth. (B) Profiles from three habitats depicting appreciable vertical salinity gradients, with salinity increasing with depth (from 15‰ to 30‰ in the most extreme case). (C) Profiles from five habitats surveyed ~8 h after a rainfall event, with evidence for a lens of fresher water extending 2–3 cm below the surface. For abbreviations, see Fig. 7.

chronically SW-acclimated animals were used as ‘controls’ for salinity transfer experiments, as is common in crustaceans (Henry et al., 2012). After transfer to 15‰ (450 mOsm kg⁻¹ H₂O), hemolymph osmolality remained elevated (1000±10.8 mOsm kg⁻¹ H₂O) 24 h post-transfer before dropping to ~700 mOsm kg⁻¹ H₂O by 2 days post-transfer and remaining at that level until the end of the 4 day experiment (Fig. 2A). Because hemolymph osmolality stabilized by 2 days 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 versus ~30 mOsm kg⁻¹ H₂O in FW; 825 versus 153 mOsm kg⁻¹ H₂O at 5‰; 727 versus 317 mOsm kg⁻¹ H₂O at 10‰; 703 versus 443 mOsm kg⁻¹ H₂O at 15‰; 860 versus 585 mOsm kg⁻¹ H₂O at 20‰; 927 versus 742 mOsm kg⁻¹ H₂O at 25‰), reflecting a strong hyper-osmoregulatory response (Fig. 2B). In FW, hemolymph osmolality was ~868±60.3 mOsm kg⁻¹ H₂O higher than that of the ambient medium. For transfers to hyper-saline conditions (i.e. 40–56‰), hemolymph osmolality remained below ambient

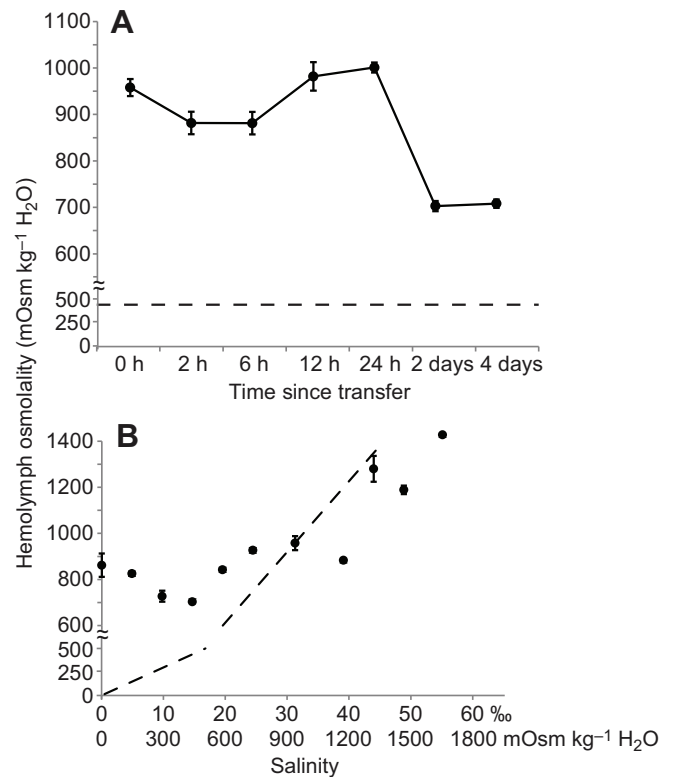


Fig. 2. Osmolality of *Halocaridina rubra* hemolymph as a function of salinity and acclimation time. (A) Hemolymph osmolality (solid line) for *H. rubra* chronically acclimated to 32‰ (time=0 h) then transferred to 15‰ for 4 days (dashed line represents 15‰). (B) Hemolymph osmolality for *H. rubra* acclimated 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 six pooled animals per sample.

(Fig. 2B). Notably, hypo-osmoregulation was still detected at even the highest examined salinity (1428±9.5 mOsm kg⁻¹ H₂O hemolymph versus 1692 mOsm kg⁻¹ H₂O medium). Although molt stage was not monitored during measurements of hemolymph osmolality, the lack of any observed molts during the experimental period as well as the relatively constant hemolymph osmolalities among salinities suggests all animals were in the intermolt stage.

Ultrastructure, ion transport staining and MRCs in gills

Silver nitrate (AgNO₃) 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. 3A), each with 10–16 pairs of lamellae pointing anteriorly and dorsally. The gills are phyllobranchiate, which is typical of caridean shrimps, with plate- or leaf-like lamellae extending from both sides of the flattened, central gill shaft (Freire et al., 2008). There were no apparent visual differences in morphology or AgNO₃ staining between the anterior versus posterior gills or between shrimp acclimated to 2‰ (Fig. 3B) and 32‰ (Fig. 3C). Support for this latter point comes from no significant difference being detected in the area fraction of each gill stained between the 2‰ and 32‰ treatments (linear fixed-effects model, $P=0.13$; Fig. 4A). However, posterior gills tended to have a smaller fraction of area stained than more anterior gills (linear fixed-effects model, $P<0.01$; Fig. 4A). Lastly, there was no significant interaction between salinity and gill number effects (F -drop test, $P=0.24$), suggesting all gills (i.e. anterior versus posterior) responded similarly to salinity.

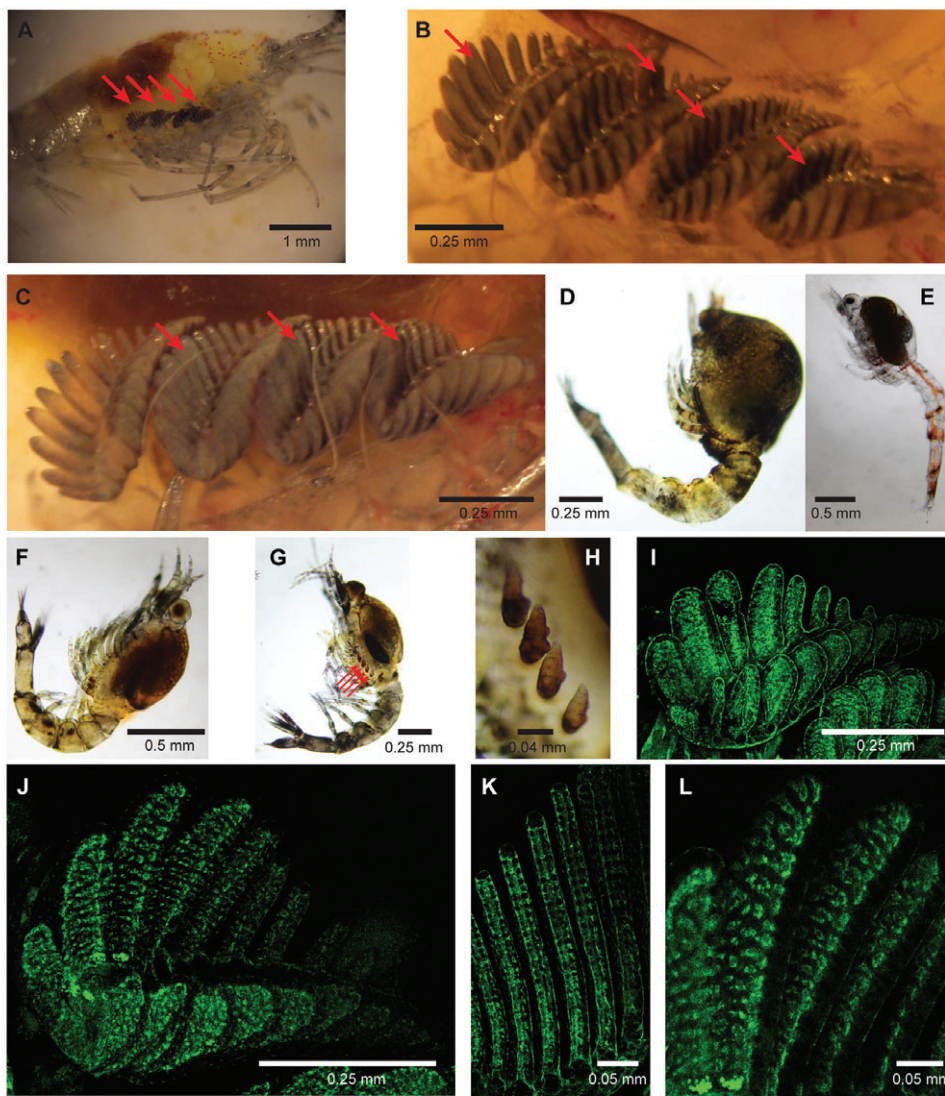


Fig. 3. Silver nitrate (AgNO_3) and vital staining of mitochondria-rich cells (MRCs) in the gills of *H. rubra*. (A) An adult chronically acclimated to 15‰ and treated with AgNO_3 , showing gills (i.e. the pleurobranches of the 5th–8th thoracomeres) as the primary site of ion transport (indicated by red arrows). (B) Close-up of gills treated with AgNO_3 after chronic acclimation to 2‰ (darkly stained lamellae indicated by red arrows). (C) Same as for B, but after chronic acclimation to 32‰. Note the lack of staining in some central lamellae of the posterior gill compared with B. (D) Zoea₁ stage of *H. rubra* larva treated with AgNO_3 (from the KBP lineage; Fig. 7). (E) Zoea₂ stage of *H. rubra* larva treated with AgNO_3 (from the KBP lineage; Fig. 7). (F) Zoea₃ stage of *H. rubra* larva treated with AgNO_3 (from the OWAI lineage; Fig. 7). (G) Zoea₄ stage of *H. rubra* larva treated with AgNO_3 (from the OWAI lineage; Fig. 7), 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/lacunae epithelia near the bottom of the image and efferent vessel/lacunae epithelia near the top of the 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 in A.

AgNO_3 staining of Zoea₁–Zoea₄ larvae from *H. rubra* identified low levels of ubiquitous staining in the exoskeleton of early zoeal stages (Fig. 3D–F). Clearly stained gills were not observed until the Zoea₄ stage, suggesting either that gills do not develop until this stage or that previous stages possess gills that do not undergo ion transport (Fig. 3G). Relative to adult gills, Zoea₄ stage gills were undeveloped, with only buds of a few lamellae detected per gill (Fig. 3H). However, gills were the only structures with consistent and heavy staining in Zoea₄.

Vital staining using 4-[4-(dimethylamino)styryl]-N-methylpyridinium iodide (DASPMI) revealed an abundance of MRCs in the gills when shrimp were acclimated to both 2‰ (Fig. 3I) and 32‰ (Fig. 3J). Furthermore, populations of MRCs were identified in gill lamellae under both salinities (Fig. 3K,L), with vessels/lacunae within the lamellae readily visible (Fig. 3K). MRCs were distributed evenly throughout each lamella, absent around their perimeters or outer marginal canals (after McNamara and Lima, 1997) (Fig. 3L), and were not confined to particular gills. Instead, a large fraction (>80%) of all gills and lamellae fluoresced under both salinities (Fig. 4B,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. 4B). 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‰ versus 32‰ (*t*-test, $P < 0.046$ for all comparisons; Fig. 4C). Lastly, all lamellae had a significantly larger area fraction that fluoresced in 2‰ versus 32‰ for gill 4 (*t*-test, $P < 0.049$ for all comparisons, Fig. 4C).

Gene expression during salinity transfer

Homologs of NKA, CAC, CA g isoform (CAG), NKCC, HAT and arginine kinase (AK) were identified from the *H. rubra* transcriptomic 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 amino acid insertion near the C-terminus, were identified. The smaller isoform (similar to the NKA of *Panaeus 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 quantitative real-time (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 base pair, 22% of total length) CAG transcript was identified from the East Hawaii genetic lineage. Full-length

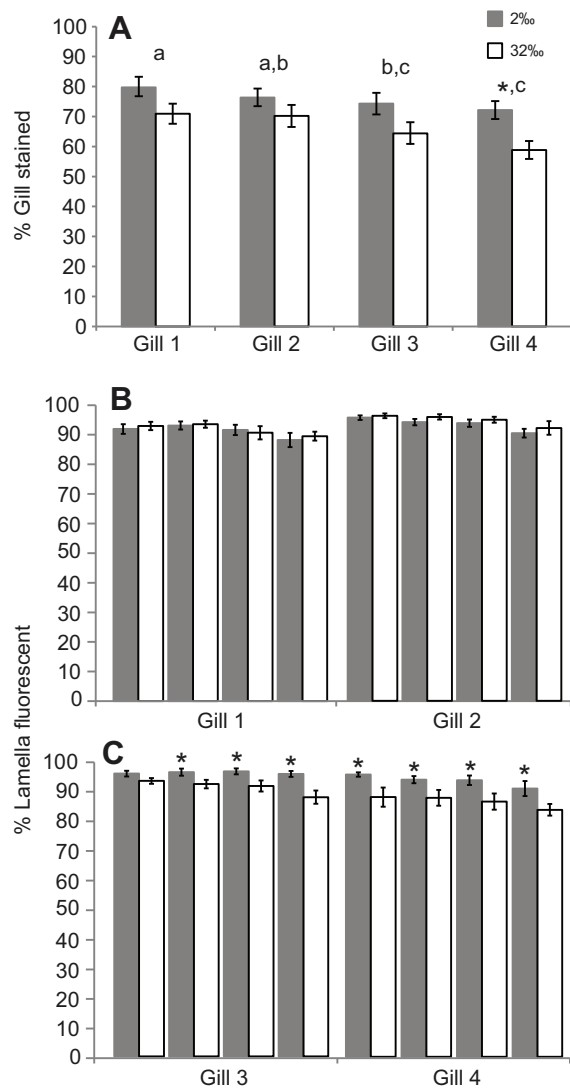


Fig. 4. Area fraction of *H. rubra* gills stained by AgNO_3 or vital staining of MRCs. (A) Percentage of whole gills stained with AgNO_3 from *H. rubra* acclimated to 2‰ or 32‰ (\pm s.e.m., $N=10$ animals/20 gills per salinity). Letters indicate statistically significant groupings among gills (linear 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. the most posterior gill) did have a statistically significant higher proportion of AgNO_3 staining at 2‰ when considered separately (indicated by an asterisk, Student's unpaired t -test, $P=0.003$). (B, C) Percentage of central four lamellae stained with DASPMI for vital MRCs from *H. rubra* acclimated to 2‰ or 32‰ (\pm 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.

transcripts of NKCC were obtained from both genetic lineages as well as a partial transcript of a second NKCC isoform (76% identical in amino acid 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.

Salinity transfers did not alter the expression of ion transporters and supporting enzymes in the gills of *H. rubra*. For example, NKA expression in the gill 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 days post-transfer (remaining as such until the end of the experiment 7 days post-transfer; Fig. 5A). 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 days after transfer. At 24 h, NKA levels were significantly higher (~2-fold) in the gills of animals transferred to 2‰ than of 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. 5B,C), with NKCC expression increasing ~2.6-fold compared with 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 days post-transfer, except for a 76% reduction of the initial level 48 h after transfer to 15‰ (t -test, $P=0.03$; Fig. 5D). Overall, no changes in gill CAG expression were detected during any treatment (Fig. 5E). Lastly, HAT expression decreased significantly in all transfers between 3 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. 5F). 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. 5F). By 7 days post-transfer, HAT expression levels returned to their initial level and were not significantly different between salinity treatments (Fig. 5F).

In most cases, gene expression was also not significantly altered during salinity transfers in the control tissue (i.e. the remainder of the shrimp body excluding the 'gill undercarriage'). Specifically, expression of NKA in the control tissue was not significantly different from the initial level in any transfer until 7 days post-transfer, when levels decreased in all three salinity treatments (t -test, $P<0.03$ for all; Fig. 6A). A similar trend was noted for AK (Fig. 6C). 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. 6B). Likewise, CAC expression in the control tissue showed a single significant change relative to the initial level, decreasing ~30% 7 days after transfer to 45‰ (t -test, $P=0.04$, Fig. 6D) while CAG decreased ~50% by 7 days post-transfer in all transfers (t -test, $P<0.02$ for all, Fig. 6E). Finally, HAT expression in the control tissue did not change significantly with any treatment (Fig. 6F). Overall, there were no differences in expression levels in the control tissue between salinity treatments at either 24 h or 7 days post-transfer for any of the six genes (Fig. 6).

DISCUSSION

Understanding the 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 (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‰

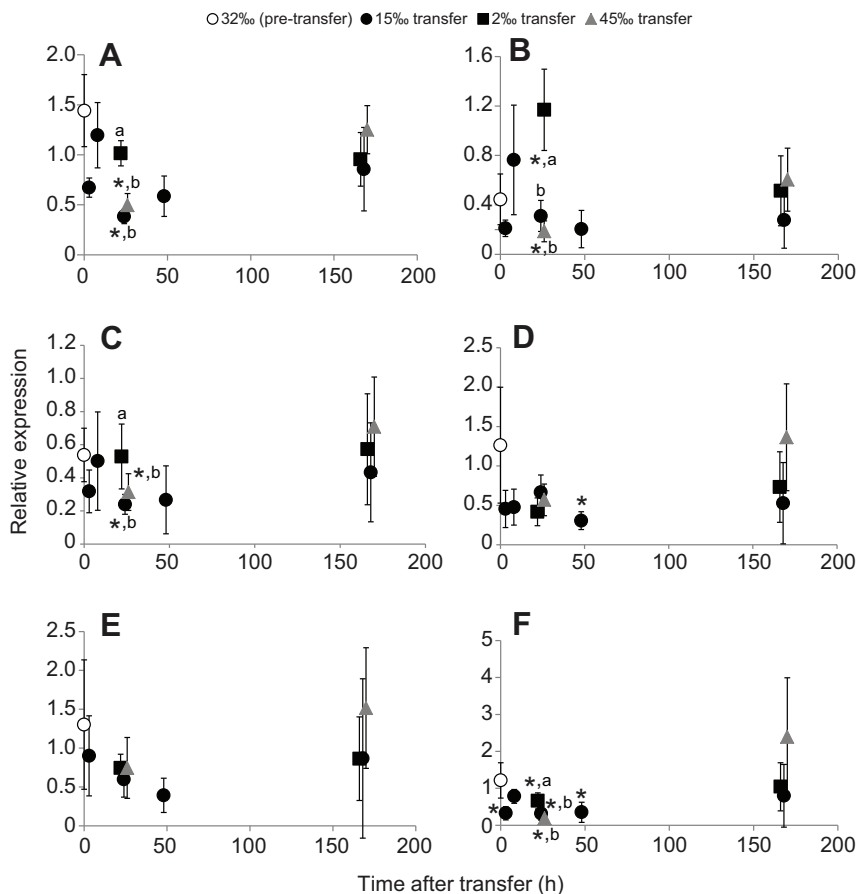


Fig. 5. Relative expression of ion transporters and supporting enzymes in the gills of *H. rubra* following salinity transfers. mRNA expression of (A) Na⁺/K⁺-ATPase (NKA), (B) Na⁺/K⁺/2Cl⁻ co-transporter (NKCC), (C) arginine kinase (AK), (D) the cytoplasmic isoform of carbonic anhydrase (CAc), (E) the membrane-associated isoform of carbonic anhydrase (CAg) and (F) V-type H⁺-ATPase (HAT) was quantified 0, 3, 8, 24 and 48 h, and 7 days (168 h) after transfer from 32‰ to 15‰ (open and filled circles, respectively). Transfers from 32‰ to 2‰ and 45‰ (filled triangles and squares, respectively) were also examined 24 h and 7 days 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). Asterisks indicate a significant difference from the pre-transfer measurements (Student's *t*-test, $P<0.05$ for all). Letters at 24 h and 7 day time points represent statistically significant groupings among salinity treatments (two-way ANOVA with Tukey *post hoc* tests, $P<0.05$ for all).

reported here for deeper habitats from the same island and geographic region (Fig. 1B). Furthermore, high salinity waters (i.e. SW) likely occur in the fissures leading to the hypogean environment of the habitats and surface FW lenses were apparent following a rainfall event (Fig. 1C). Thus, while many of the habitats exhibited no vertical salinity stratification (Fig. 1A), 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 range of 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 marine 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 with a marine ancestry such as the blue crab (*Callinectes sapidus*) maintain an osmotic gradient between the external medium and their hemolymph of ~600 mOsm kg⁻¹ H₂O (Cameron, 1978; Henry, 2001). In contrast, the finding that *H. rubra* maintains a gradient of ~868 mOsm kg⁻¹ H₂O when transferred to FW (Fig. 2B) 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). Also atypical for crustaceans, hyper-osmoregulating capacity appeared to decrease for *H. rubra* between 25‰ and 15‰ before increasing again between 15‰ and 0‰ (Fig. 2B). Interestingly, other crustaceans with a similar FW ancestry, such as crayfish, maintain a gradient of 370–450 mOsm kg⁻¹ H₂O and are considered 'weak' osmoregulators

(Mantel and Farmer, 1983). Previously studied FW atyids are also classified as 'weak' osmoregulators and maintain osmotic gradients of 335–400 mOsm kg⁻¹ H₂O (Born, 1968; Dhaouadi-Hassen et al., 2004). An exception to this (besides *H. rubra*) may be the Chinese mitten crab (*Eriocheir sinensis*), which spends most of its adult life in FW habitats and maintains an osmotic gradient of 550–700 mOsm kg⁻¹ H₂O (Onken, 1999). However, this species likely represents a secondary FW invasion by a member of a marine family, while *H. rubra* represents an invasion of a euryhaline habitat by a member of a FW-adapted family. This suggests *H. rubra*, and potentially other anchialine atyid shrimp, may be unique in maintaining strong osmotic gradients because of the selection pressure of fluctuating salinities in anchialine habitats, despite having an evolutionary history tied to FW environments.

Unlike *H. rubra*, most euryhaline crustaceans rapidly decrease hemolymph osmolality to new levels following transfer from SW 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 SW until 2 days post-transfer (Fig. 2A). 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

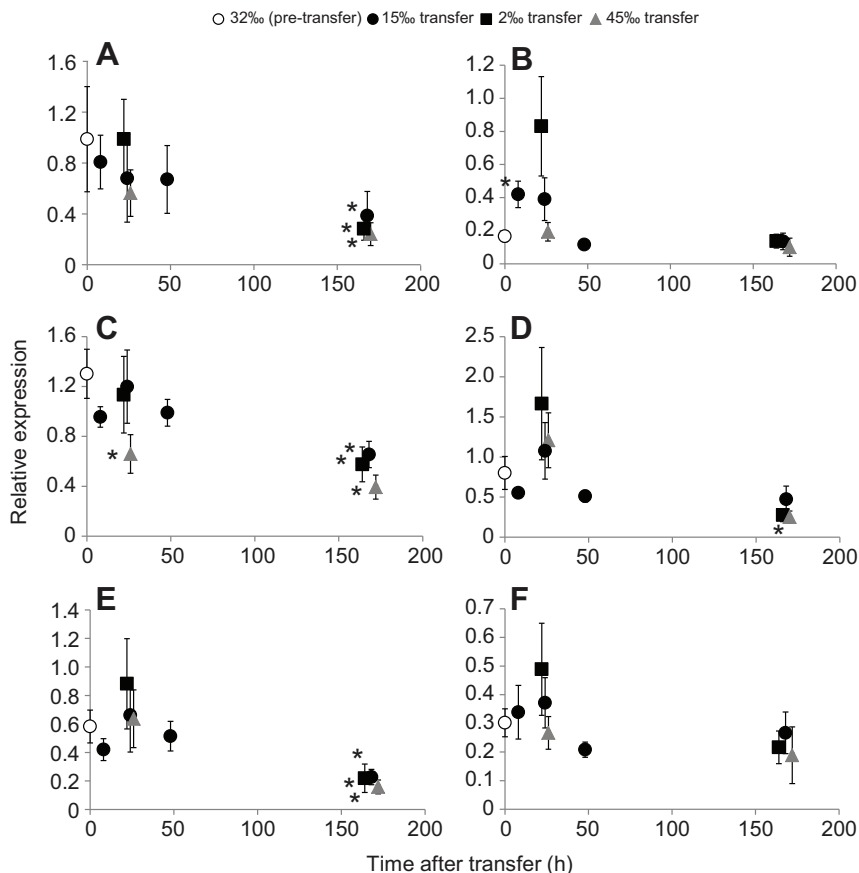


Fig. 6. Relative expression of ion transporters and supporting enzymes in control tissues of *H. rubra* following salinity transfers. Control tissues are tissues other than the gill undercarriage (see Materials and methods). Genes, symbols, asterisks, letters and statistical analyses are as in Fig. 5, with the exception that the 3 h, 15‰ treatment was not investigated for the control tissue samples.

or hours, may be energetically expensive and inefficient, and thus has been selected against. Rather, this species appears to maintain constantly active osmoregulatory mechanisms for coping with low salinities even when at high salinities as in nature low salinities are regularly encountered. This is supported by FW crustaceans typically having ‘tight’ gill epithelia (reviewed in Henry et al., 2012), which would reduce diffusive ion loss and contribute to maintaining the high osmotic gradient seen in *H. rubra*.

AgNO₃ staining revealed the gills as the primary site of ion transport in *H. rubra*, as is typical of crustaceans and fishes (Evans et al., 2005; Henry et al., 2012), with the gills of *H. rubra* being morphologically similar to those of the anchialine atyid *Typhlatya arfae* (Jaume and Bréhier, 2005). Generally, the gills of *H. rubra* appear less complex than those of other euryhaline crustaceans, with fewer gills and fewer lamellae per gill [e.g. four versus eight gills and 10–16 versus ~300 lamellae per gill compared with *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 AgNO₃ staining did not change significantly with transfer to low salinity, although there was a trend towards greater staining in 2‰ versus 32‰ (linear fixed-effects model, $P=0.13$, Fig. 4A). Furthermore, when considered individually, the most posterior gill (i.e. gill 4; Fig. 3B,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, AgNO₃ staining never exceeds more than 30% of the total lamellar surface area for the moderate osmoregulator *C. maenas* (Compere et al., 1989), and for strong osmoregulators, AgNO₃ staining went from 35% of the lamellae in the osmoregulating gills to 52% during transfer from SW 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 59–71% AgNO₃ staining in all gills when acclimated to 32‰ 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 as brachyuran gills are typically either respiratory or osmoregulatory in nature, with AgNO₃ 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 some lamellae in all gills show staining in FW crayfish, it is localized to the central filaments of each gill (Dickson et al., 1991; Khodabandeh et al., 2005).

AgNO₃ staining of larval *H. rubra* revealed that ion-transporting gills develop at late zoeal stages (i.e. Zoea₄), which corresponds to ~15 days post-hatch (Courlet 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), *C. granulatus* (Charmantier et al., 2002) and *C. 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 versus adults. For

example, larvae of many estuarine and riverine crustaceans are 'exported' into the ocean where they undergo development before returning as juveniles (Anger, 2001). Furthermore, many atyids also export larvae to the ocean, and SW is necessary for development in some species (Hunte, 1979a; Hunte, 1979b). 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 important for developing larvae. Interestingly, larvae of *H. rubra* can undergo successful development in a wide range of salinities, from potentially full strength SW 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 (Couret and Wong, 1978); 20‰ in Iwai (Iwai, 2005)]. Therefore, while it is possible the ontogenetic shift in osmoregulation hypothesized for *H. rubra* is due to larvae acting as osmoconformers in SW, 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 AgNO₃ 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 AgNO₃ 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 change during salinity transfer (Fig. 5), with similar results obtained from control tissue (Fig. 6). 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 *C. granulatus* increased 25- to 55-fold after transfer from SW to 45‰ and 2‰ (Luquet et al., 2005), with similar results for *Scylla paramamosain* (Chung and Lin, 2006), *Pachygrapsus marmoratus* (Jayasundara et al., 2007), *C. sapidus* (Serrano et al., 2007), *C. 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 24 h after transfer to 2‰. However, this upregulation is small compared with previous reports.

Why are well-characterized osmoregulatory genes not upregulated 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 SW. Support for this hypothesis comes from AgNO₃ and MRC staining,

which indicate elevated cellular mechanisms of osmoregulation in the gills of *H. rubra* regardless of salinity. Although it is unclear whether this hypothesized chronic upregulation of osmoregulatory processes is an adaptation to anchialine habitats, such a strategy may allow *H. rubra* to cope with the rapid and continuous salinity fluctuations 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 ~868 mOsm kg⁻¹ H₂O in FW. 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 whether selection, evolutionary history or a combination of the two is responsible for this deviation from previous models. Future studies should continue to explore the physiological responses of anchialine organisms to salinity as a means of developing a further understanding of osmoregulation in general.

MATERIALS AND METHODS

Haloclines in Hawaiian anchialine habitats

To determine the magnitude of the 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. 7). Field measurements were taken every

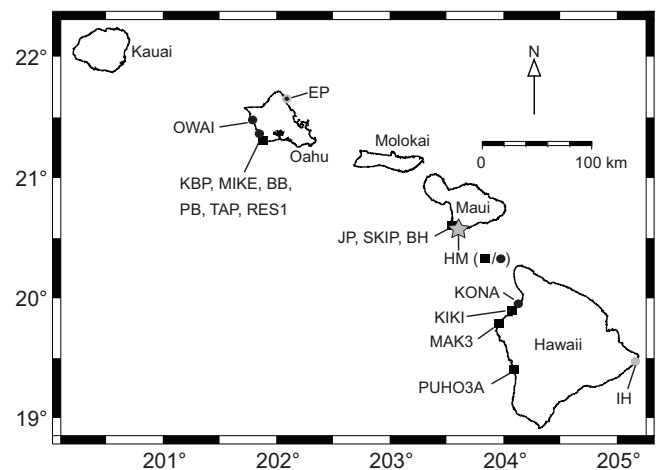


Fig. 7. Map depicting anchialine habitats and animal origins for this study. Black squares indicate habitats where vertical salinity gradients were measured, black circles indicate origins of *H. rubra* larvae used in AgNO₃ staining, gray circles indicate origins of *H. rubra* lineages used in transcriptome 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. 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; MAK3, Makalawena 3; MIKE, Mike's Pond; OWAI, Waianae Boat Harbor; PB, Papa Bear; PUHO3A, Pu'uhoonua o Hōnaunau National Historical Park 3A; RES1, Restoration 1; SKIP, Skippy's Pond; TAP, Tap's Pond.

15 cm from the habitats' surface to bottom with a handheld YSI conductivity meter (Yellow Springs Instruments, Yellow Springs, OH, USA). 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 h following rainfall.

Animals

Individuals of *H. rubra* were collected from Cape Hanamanioa (HM), Maui using hand nets and shipped to Auburn University, AL, USA, within ~2 days of collection during 2011. 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 and algal growth occurring on the porous volcanic rock present in the aquaria. This husbandry technique is ideal for *H. rubra*, yielding continuous, year-round reproduction in the laboratory.

Osmolality during salinity transfer

Prior to experiments, *H. rubra* individuals were acclimated to 32‰ in 4 l aquaria for at least 1 month. At this salinity marine crustaceans are osmoconformers, and this condition serves as the baseline for comparing changes when animals are exposed to altered salinities. Furthermore, the activity and expression of transport proteins and supporting enzymes (see below) are at low, baseline levels in crustaceans acclimated to high salinity; thus, those values have also served as a control, starting point for studies of induction/up-regulation in studies involving transfers to low salinity (reviewed by Henry et al., 2012). Shrimp were then transferred to 15‰ and sampled at 0, 2, 6, 12, 24, 48 and 96 h post-transfer. Hemolymph was extracted by anesthetizing shrimp on ice, wicking off any surface water using Kimwipes (Sigma-Aldrich, St Louis, MO, USA), and then lacerating the outer dorsal portions of the thoracic and abdominal 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 in tightly sealed tubes at -80°C to prevent evaporation 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 h 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 h. 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 $[\text{K}^+]$. On average, $[\text{K}^+]$ was $33.4 \pm 0.52 \text{ mmol l}^{-1}$, which is higher than previously reported for *Litopenaeus vannamei* hemolymph (16 mmol l^{-1}) (Sowers et al., 2006), but much lower than typical intracellular $[\text{K}^+]$ ($120-150 \text{ mmol l}^{-1}$). 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.

AgNO₃ 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% AgNO₃ while shaking for 20 min, and again rinsed three times with deionized water. This was followed by incubation for 1 h in saturated Kodak D-76 developer (Eastman Kodak Inc., Rochester, NY, USA) while shaking,

followed by a single rinse with deionized water. AgNO₃ staining blackens transport epithelia, which are permeable to silver and/or chloride ions, through the production of AgCl (Croghan, 1958; Holliday et al., 1990; Kikuchi and Shiraiishi, 1997). Animals were photographed using an S8 APO Stereo Microscope (Leica Microsystems, Wetzlar, Germany) at 1–8× magnification. To determine whether salinity influenced AgNO₃ staining, animals were chronically acclimated to either 2‰ or 32‰ for at least 1 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 as both left and right gills were measured per individual (20 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 AgNO₃ using the above protocol. Larvae came from five of the eight genetic lineages found across the Hawaiian Islands (Fig. 7) and were collected from distinct colonies maintained in the laboratory for ~7 years. The developmental stage of each larva (Zoea₁–Zoea₄) was scored based on morphological features described elsewhere (Courret and Wong, 1978; Iwai, 2005).

To more closely investigate changes in the gills of *H. rubra* during salinity transfer, MRC density was quantified using DASPMI (Molecular Probes, Invitrogen, Carlsbad, CA, USA) staining and confocal microscopy (Heijden et al., 1997; Choe et al., 1999). Shrimp were acclimated to either 2‰ or 32‰ ($N=10$ per salinity) for 1 month, anesthetized on ice and their gill undercarriage dissected and rinsed in a 700 mmol l⁻¹ NaCl solution (i.e. a shrimp Ringer's solution consistent with *H. rubra* hemolymph during hyperosmoregulation). This gill undercarriage consisted of the gills and a minimum amount of supporting musculature/exoskeletal material. This approach was utilized because individual gills were too small and delicate to be dissected separately. Following rinsing, gill undercarriages were incubated in shrimp Ringer's solution containing 25 µmol l⁻¹ DASPMI for 1 h while shaking at room temperature to label MRCs prior to being rinsed with shrimp Ringer's solution lacking DASPMI (Karnaky et al., 1984). Gill undercarriages were then placed on a glass slide with a concavity in a single drop of shrimp Ringer's solution 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.

Expression of osmoregulatory genes during salinity transfer

To quantify gene expression in *H. rubra* during salinity transfer, shrimp were chronically acclimated to 32‰ for 1 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 3, 8, 24 and 48 h and 7 days 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 24 h and 7 days post-transfer. Shrimp were anesthetized on ice and gill undercarriages (see above) dissected into tubes containing ice-cold denaturing solution from the RNeasy 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). 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 RNeasy Total RNA Isolation System (Promega) substituted with phenol–chloroform–isoamyl alcohol (P2069,

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

Target gene	Primer	Sequence (5' to 3')	Amplicon length (nucleotides)
NKA	NKAF2679	TGGCTTCCTCCTCCCAAACCTTT	175
	NKAR2854	TCAAATCGGCCCACTGGACAATCA	
CAc	CACf444	TTTGGGTGTGTTTCTGACCGTTGG	169
	CACr613	TCAGCGAACCAGGGTAGGTGAAAT	
CAg	CAGf373	AGCCTGGGGCTCAGAACACACTATT	189
	CAGr562	TTAGGGCACTGACCAAAGGAGTGA	
NKCC	NKCCF1826	ATCGTCCACAGCTCTTGGTGCTAA	194
	NKCCR2020	AGGCACGAATCTTATGACGAGCGA	
HAT	HATf800	AGTGCGAGAAGCACGTCCTCATTA	194
	HATr994	TGGGAATCTGGGTGATGGAACCTT	
AK	AKF821	ACCTGGGTACCACTGTTCTGTTCTT	180
	AKR1001	GTCAGGCCATACGACGTTTGTTA	

NKA, Na⁺/K⁺-ATPase α -subunit; CAc and CAg, carbonic anhydrase cytoplasmic and membrane-associated isoforms; NKCC, Na⁺/K⁺/2Cl⁻ co-transporter; HAT, H⁺-ATPase; AK, arginine kinase.

Sigma). 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 μ g 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 PCR prior to being subjected to 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), specific primers were designed for *H. rubra* targeting six genes: 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⁻ co-transporter (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. 7) 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 gene 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 μ l) were run in triplicate and consisted of 12.5 μ l iQ SYBR Green mix (2 \times), 10.9 μ l nuclease-free water, 0.3 μ l forward and reverse primers (25 μ mol l⁻¹; Table 1) and 1 μ l template cDNA, using the following cycle parameters: initial denaturing for 3 min at 95°C, followed by 40 cycles of 10 s 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 to 95°C with a heating rate of 0.5°C every 5 s) 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 versus log₁₀ cDNA volume) were

generated by serial dilution of a single sample from the 2‰ 24 h 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).

Statistical analyses

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

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.C.H., S.R.S. and R.P.H. designed the experiments. J.C.H. performed the experiments, analyzed the data and wrote the manuscript. J.C.H., S.R.S. and R.P.H. revised the manuscript.

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Supplementary material

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