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Reproduction and Development in *Halocaridina rubra* Holthuis, 1963 (Crustacea: Atyidae) Clarifies Larval Ecology in the Hawaiian Anchialine Ecosystem

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Abstract. Larvae in aquatic habitats often develop in environments different from those they inhabit as adults. Shrimp in the Atyidae exemplify this trend, as larvae of many species require salt or brackish water for development, while adults are freshwater-adapted. An exception within the Atyidae family is the "anchialine clade," which are euryhaline as adults and endemic to habitats with subterranean fresh and marine water influences. Although the Hawaiian anchialine atyid *Halocaridina rubra* is a strong osmoregulator, its larvae have never been observed in nature. Moreover, larval development in anchialine species is poorly studied. Here, reproductive trends in laboratory colonies over a 5-y period are presented from seven genetic lineages and one mixed population of *H. rubra*; larval survivorship under varying salinities is also discussed. The presence and number of larvae differed significantly among lineages, with the mixed population being the most prolific. Statistical differences in reproduction attributable to seasonality also were identified. Larval survivorship was lowest (12% settlement rate) at a salinity approaching fresh water and significantly higher in brackish and seawater (88% and 72%, respectively). Correlated with this finding, identifiable gills capable of ion transport did not develop until metamorphosis into juveniles. Thus, early life stages of *H. rubra* are apparently excluded from surface waters, which are characterized by lower and fluctuating salinities. Instead, these stages are restricted to the subterranean (where there is higher and more stable salinity) portion of Hawaii's anchialine habitats due to their inability to tolerate low salinities. Taken together, these data contribute to the understudied area of larval ecology in the anchialine ecosystem.

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Abbreviations: COI, cytochrome oxidase I; DASPMI, 4-[4-(dimethylamino)styryl]-N methylpyridinium iodide; *Halocaridina rubra* colonies: EP, Eric's Pond; EWA, Ewa Beach; HILO, Hilo, Hawaii; HM, Cape Hanamanioa; KBP, Kalaeloa Unit; KONA, Kona Coast, Hawaii; OWAI, Waianae Boat Harbor; WC, Waianapanapa Cave; MRC, mitochondrion-rich cell; Z_1–Z_4, zoeal stages 1–4.

Introduction

Larvae in aquatic systems often undergo a prolonged developmental stage, when they are potentially exposed to a range of environmental conditions over a period of days to months. Furthermore, during development, they may be exported to a different niche or ecosystem from adults. For example, larvae of numerous coral reef fish species are often found in mangrove and seagrass "nurseries" (Nagelkerken *et al.*, 2000). However, while larvae, particularly planktonic forms, may travel considerable distances (Scheltema, 1986), they often have a limited ability to circumnavigate undesirable environments (Fisher *et al.*, 2000). Therefore, larvae at these early developmental stages must possess physiological mechanisms to tolerate a wide range of conditions, or else remain confined to a relatively stable environment until later in their development.

Many shrimp in the Atyidae exemplify how larvae can experience substantially different environmental conditions from adults of the same species. Specifically, nearly all atyids are freshwater stream specialists, where they can occur more than 1000 km upstream of any marine influence,

and at an elevation of up to 925 m (Fryer, 1977; Hunte, 1979a; Hobbs and Hart, 1982). Atyids as adults are for the most part intolerant of seawater (Smith and Williams, 1981; von Rintelen *et al.*, 2012), and have no known or extant marine representatives (Fryer, 1977). The family has a well-documented freshwater ancestry, with freshwater deposits of atyids dating to the Cretaceous period (Glaessner, 1969; Smith and Williams, 1981). Despite being freshwater-adapted, many species exhibit an amphidromous life-history strategy, where larvae require brackish or salt water for development before returning to fresh water as juveniles (Hunte, 1979a, b). This strategy can result in the export of larvae hundreds of km downstream to marine environments like estuaries, with the subsequent and spectacular return of thousands of juveniles swimming or crawling upstream along stream banks (Bauer, 2013).

One particular group within the Atyidae, recognized as the "anchialine clade" (von Rintelen *et al.*, 2012), represents an apparent exception to the more or less strictly freshwater existence and amphidromous life-history strategy of the family. With adults consistently inhabiting brackish, salt, and (in some cases) hypersaline waters, members of this monophyletic clade are endemic to the anchialine ecosystem, which is composed of coastal ponds, pools, and caves that are fed by both fresh groundwater and marine seawater (Holthuis, 1973; Sket, 1996). A well-studied species of this clade is *Halocaridina rubra* Holthuis, 1963 from the Hawaiian Islands (Bailey-Brock and Brock, 1993). Adults of *H. rubra* can survive salinities ranging from fresh water (~0‰) to hypersaline (56‰) (Holthuis, 1973) *via* osmoregulatory mechanisms in the gills that are atypical among euryhaline crustaceans (Havird *et al.*, 2014a). Interestingly, larvae of *H. rubra* do not appear to develop identifiable gills until the late developmental stages (Havird *et al.*, 2014a), raising the question of how the larvae of anchialine species such as *H. rubra* cope with the varying salinity regimes encountered in this ecosystem.

The reproductive trends and larval biology of anchialine species such as *H. rubra* have not been extensively studied. Notably, over 50 years of field surveys have failed to report gravid, egg-bearing females or larvae of *H. rubra* in the epigeal (*i.e.*, surface) waters of Hawaii's anchialine habitats (Holthuis, 1963; Maciolek, 1983; Bailey-Brock and Brock, 1993). This implies that the species undergoes reproduction and development in the hypogeal, *i.e.*, underground, portion of the ecosystem (Maciolek, 1983; Craft *et al.*, 2008). In contrast to field surveys, laboratory studies have documented the presence of gravid females and development of *H. rubra* larvae in brackish waters of a constant salinity (*i.e.,* 15‰–20‰) (Couret and Wong, 1978; Iwai, 2005) as well as continuous reproductive output from *H. rubra* colonies over a 5-y period (this study). One possible explanation for the discrepancy between reproduction in nature and the laboratory is that anchialine habitats in the Hawaiian Islands and elsewhere can possess appreciable haloclines, in which waters nearing the surface are closer to 0‰ and those toward the bottom approach salinities of 20‰ to 36‰ (Holthuis, 1973; Maciolek, 1983; Havird *et al.*, 2014a). Given this halocline, the larvae of *H. rubra* may develop in the hypogeal component of the ecosystem because it has higher and more constant salinities. To test this hypothesis, we assessed survivorship of *H. rubra* larvae under laboratory conditions mimicking the vertical (*i.e.*, depth) range of salinities naturally encountered in the Hawaiian anchialine ecosystem. We supplemented this assessment with a description of gill development across larval stages of the species.

Materials and Methods

Animals, holding conditions, and 5-y monitoring of laboratory reproduction

Individuals (*i.e.*, 200–250) of *Halocaridina rubra* were collected using hand nets from each of eight different anchialine habitats in the Hawaiian Islands during June and July 2006, then shipped to Auburn University, AL, within about 2 d of collections. In Hawaii, *H. rubra* encompasses (at least) eight genetically divergent lineages with highly restricted geographic ranges (Santos, 2006; Craft *et al.*, 2008). These collections represented seven of the eight lineages, with the last coming from a population (*i.e.*, Kalaeloa Unit colony (KBP)) that is a natural mix of two lineages (South (EWA) and West Oahu (OWAI); abbreviations follow Craft *et al.*, 2008; Vaught *et al.*, 2014)). Colonies were maintained in 38-l aquaria and in darkness except for 12-h light/dark cycles for one week every month. Additional details on maintenance of the *H. rubra* colonies can be found in Vaught *et al.* (2014).

Reproduction in each *H. rubra* colony was visually monitored, and both the number of gravid females and free-swimming lecithotrophic (*i.e.*, yolk-bearing) larvae (up to a maximum of 100 individuals) were counted during a 10-min period approximately once per week across a 5-y period (Summer 2007–Summer 2012), for a total of 185 sampling dates. Adult females of *H. rubra* (~12 mm long) carry about 14–18 large (1 mm) eggs on their abdomens for approximately 27 d, then release larvae over 1 to 5 d (Iwai, 2005). Larvae develop in the water column for about 29 d, progressing through four zoeal stages and one mysis stage before metamorphosis and settlement (Couret and Wong, 1978; Bailey-Brock and Brock, 1993; Iwai, 2005). Therefore, visual monitoring once per week for a 10-min interval was considered sufficient to observe the majority of larvae present within a colony at that sampling date. Population sizes were maintained at about 200 individuals per colony by periodically removing larvae or adults and transferring them to new aquaria.

Salinity-mediated survivorship of larvae of Halocaridina rubra

In Summer 2014, approximately 20 gravid females of *H. rubra* of the OWAI colony (Craft *et al.*, 2008) released larvae within a few days of each other, providing a large supply of larvae that were about the same age and developmental stage. To minimize effects due to potential inter-lineage variation, only larvae from the OWAI lineage were utilized in the experiments described below. Initially, about 100 larvae were removed from the colony using a hand net, then kept together for 2 d in a 500-ml glass beaker containing 15‰ salinity water obtained from the same aquarium that housed the colony. During this period, mortality was about 10%, most likely due to handling stress. Twenty-five larvae were then haphazardly selected and inspected *via* light microscopy to confirm that they were of early developmental stages. Because females of *H. rubra* do not release all larvae simultaneously, it was not possible to obtain a sufficient number of Z_1 larvae for experimentation; rather, Z_1 or early Z_2 (according to morphological characters; Iwai, 2005) were utilized.

After the 2-d holding period, batches of 25 larvae each were transferred to one of three salinities: 2‰, 15‰, or 32‰. Treatments were created by mixing filtered water from the OWAI colony with either deionized or filtered hypersaline (50‰) water to the desired salinity. The 25 larvae allotted to each salinity treatment were distributed among five 100-ml beakers to avoid pseudoreplication. Survivorship was monitored every 1 to 2 d by counting the number of larvae that remained alive and removing any dead individuals from each beaker. Monitoring was done for 28 d, by which time all surviving larvae had settled and metamorphosed into benthic juveniles regardless of salinity treatment. Given the lecithotrophic nature of *H. rubra* larvae, no feeding was needed or done during the experimental period.

Staining of larval osmoregulatory tissues in Halocaridina rubra

To examine changes in functional, ion-transporting tissues during larval development in *H. rubra*, mitochondrion-rich cells (MRCs) were visualized through vital staining with 4-[4-(dimethylamino)styryl]-N methylpyridinium iodide (DASPMI) and confocal microscopy. In crustaceans, osmoregulation is energetically expensive; thus, cells in the gills responsible for ion transport are densely packed with mitochondria and termed MRCs (Taylor and Taylor, 1992; Freire *et al.*, 2008). It was shown previously that all gills in adults of *H. rubra* have dense MRC populations regardless of salinity (Havird *et al.*, 2014a), in contrast to other crustaceans that undergo MRC proliferation and gill remodeling in response to decreased salinity (Neufeld *et al.*, 1980; Genovese *et al.*, 2000). Here, the same protocol was followed to visualize MRCs in larvae of *H. rubra* at different developmental stages. Briefly, five larvae from each zoeal stage (Z_1–Z_4) of the OWAI colony maintained at 15‰ were stained with DASPMI by rinsing individuals in a 700 mmol l^{-1} NaCl solution, incubating them in the same solution containing 25 μmol l^{-1} DASPMI for 1 h while shaking at room temperature, then rinsing them again in the solution without DASPMI. Whole larvae were then placed on a microscope slide with a concavity and cover slip in a single drop of solution, without DASPMI, for confocal microscopy. Stained MRCs in the entire individual were visualized using a Nikon A1 confocal laser scanning microscope (Nikon Instruments, Inc., Melville, NY) by setting the excitation and emission filter for fluorescein isothiocyanate and creating a three-dimensional "z-stack" image from multiple photos taken at 2–12 μm increments at 4× or 10× magnification. In addition to the zoeal stages, five newly settled (within 2 d) juveniles of *H. rubra* from the 15‰ treatment of the survivorship experiment were also stained and examined for MRCs using the same protocol.

Statistical analyses

As described previously, counts of both gravid females and larvae from *H. rubra* colonies were conducted approximately once per week for the 5-y period from Summer 2007 to 2012. However, gravid females were often missed in these counts, as *H. rubra* individuals often hide among volcanic rock substrate used in the aquaria. Given the unreliability of the counts of gravid females, only larval counts were statistically analyzed. Furthermore, since larval presence was recorded as counts, but there were many instances of "zero" count data, a hurdle regression model was used to analyze the data (Cameron and Trivedi, 1998). Hurdle regression models utilize two components to evaluate such data: a truncated count component that evaluates positive count data and produces statistics for the number of events (*e.g.*, number of larvae in a colony), and a hurdle component that assesses zero count data and produces statistics regarding the likelihood of an event occurring (*e.g.*, probability of any larvae being present in a colony). Hurdle regressions were performed in the R v2.12.0 statistical software environment (R Core Team, 2014), using the "pscl" package (Zeileis *et al.*, 2008), with larval counts modeled as a function of genetic lineage/colony of origin and either month or season (but not both simultaneously, since these factors are correlated). Seasons were defined roughly to coincide with their start and end dates in Hawaii, and in an effort to equally distribute the data: Spring: March 19th–June 19th; Summer: June 20th–September 14th; Fall: September 15th–December 17th; and Winter: December 18th–March 18th.

Larval survivorship at the three investigated salinities was examined with the "survival" package in R (Therneau, 2014). Briefly, all individuals from the same salinity treatment were grouped together for analyses, and survival distribution functions and cumulative hazard rate functions were calculated on

a per-day basis using Kaplan-Meier (Kaplan and Meier, 1958) and Nelson-Aalen (Nelson, 1969; Aalen, 1978) non-parametric analyses, respectively. To determine if there were significant differences in survival probability between treatments, Cox proportional hazard regression models (Cox, 1972) were calculated between salinities, as implemented in the "survival" package. Moreover, numbers of larvae per container were compared between treatments for each time point using a Poisson regression. All R code and result files are available (Santos, 2015).

Results

Reproductive trends in Halocaridina rubra *laboratory colonies*

All colonies of *H. rubra* maintained under standardized laboratory conditions experienced at least some reproduction, although there was variation attributable to genetic lineage and time of year (Fig. 1). For example, hurdle regression models indicated differences between genetic lineages, both in regard to how many larvae were produced and whether larvae were present in a colony (Fig. 1A). Specifically, the KBP and WC colonies produced the most and the fewest larvae, respectively. Comparing these two extremes, KBP had 3.56 times as many larvae per week as WC (95% C.L. = 3.26–3.88, $z = 29.398$, $P < 0.001$, count component of hurdle regression with Poisson error structure), and was 4.20 times as likely to have larvae as WC (95% C.L. = 2.67–6.61, $z = 6.333$, $P < 0.001$, zero component of hurdle regression with binomial error structure). While there were many statistically significant differences between seasons, most of the effect sizes were small. The largest effect size was 2.11 times as many larvae present in Winter as in Summer (95% C.L. = 2.23–2.00, $z = 28.722$, $P < 0.001$, Fig. 1B). When the data were analyzed by month (Fig. 1C), a stronger effect was noted regarding time of year; the most noted effect was that larvae were 3.74 times more likely to be present in April than in January (95% C.L. = 2.07–6.83, $z = 4.438$, $P < 0.001$).

Salinity-mediated larval survivorship in Halocardina rubra

Survivorship of *H. rubra* larvae from early developmental stages (*i.e.*, Z_1 or early Z_2) to metamorphosis as juveniles differed significantly among salinities (Fig. 2A). Of the 25 larvae allocated per treatment, 22 (88%) and 18 (72%) survived and underwent metamorphosis into juveniles at 15‰ and 32‰, respectively, while only three (12%) did so at 2‰. This difference was apparent when survival probability was plotted against time (Fig. 2B). Cox proportional hazard models found significant differences among all three treatments, with larvae at 15‰ and 32‰ being 6.43 times (95% C.L. = 4.32–9.57, $z = 9.160$, $P < 0.001$) and 3.21 times (95% C.L. = 2.38–4.34, $z = 7.631$, $P < 0.001$) more likely to survive than those at 2‰. Larvae at 15‰ were also 2.0 times more likely to survive to settlement as juveniles than were those at 32‰ (95% C.L. = 1.27–3.15, $z = 2.995$, $P = 0.003$). Although there was a trend for salinity to influence larval survivorship as early as 5 d after the start of the experiment ($P = 0.079$, Poisson regression), this trend was not statistically significant until 10 d into the experimental period. At that time, larvae were 4.4 times as likely to survive in 15‰ and 4.0 times as likely to survive in 32‰ as those larvae receiving the 2‰ treatment ($P = 0.0132$, Poisson regression).

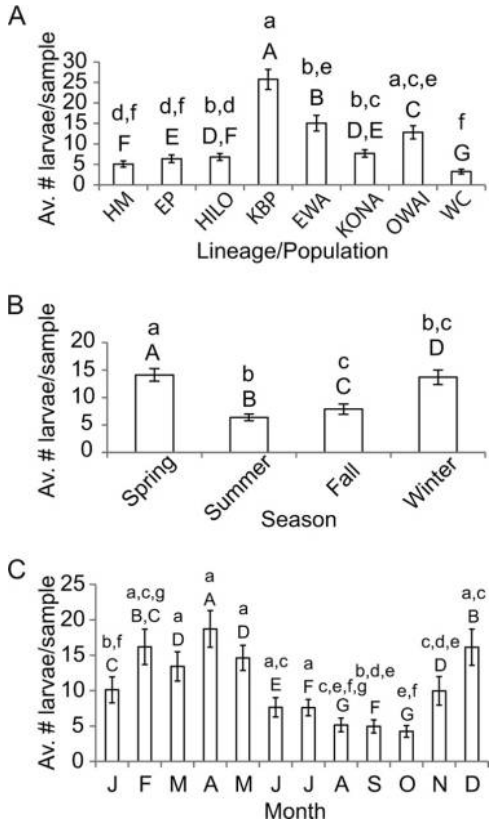

Figure 1. Reproductive output from eight colonies of *Halocaridina rubra*, monitored over a 5-y period under the same laboratory conditions. Variation in reproductive output was attributable to (A) genetic lineage/population of origin (locations follow Craft *et al.*, 2008), (B) season, and (C) month. Uppercase letters (A–G), denote significant groupings based on the number of larvae produced per sample period (approximately one per wk); lowercase letters (a–g) indicate significant groupings based on the likelihood of observing any larvae during the sample period (based on hurdle regression models). Error bars show ± SEM.

Development of ion-transporting tissues in Halocaridina rubra

Vital staining and confocal microscopy revealed "protogills" with the high MRC populations characteristic of adult gills (Fig. 3A) only in juveniles of *H. rubra* that had recently undergone metamorphosis (Fig. 3B, C). These

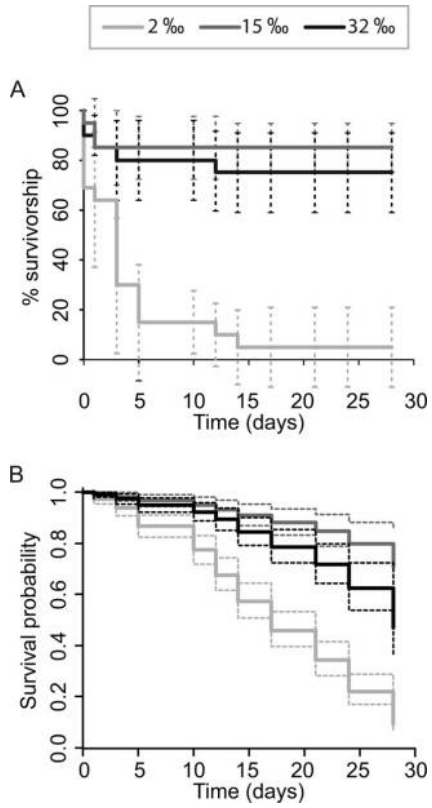

Figure 2. (A) Survivorship and (B) survival probability of larvae from *Halocaridina rubra* under three different salinities. For the survival analysis in (B), the results of a Nelson-Aalen non-parametric analysis (Nelson, 1969; Aalen, 1978) are presented; those from a Kaplan-Meier analysis (Kaplan and Meier, 1958) were qualitatively similar. Error bars in (A) and dashed lines in (B) represent 95% CI. Light gray line, 2‰ salinity; dark gray line, 15‰ salinity; black line, 32‰ salinity.

proto-gills were superficially similar to adult gills, but had only a few bud-like lamellae (*vs.* 10–16 plate-like or leaf-like lamellae in adults) and appeared underdeveloped with a low total surface area. Of the five larvae investigated per developmental stage, none of the Z_1–Z_4 individuals possessed recognizable gills (similar to those in Fig. 3B, C), whereas recognizable gills occurred in three of five juveniles. In the earlier developmental stages, a discernible, thin epithelial sheet, likely corresponding to the inner epithelium of the branchiostegite, had a large MRC population (arrow in Fig. 3D–G). Finally, structures in the lipid-rich yolk sac also stained heavily for dense populations of mitochondria.

Discussion

Development of larval salinity tolerance in Halocaridina rubra *illuminates ecology in the anchialine ecosystem*

This study demonstrates that the larvae of *H. rubra*, a member of the "anchialine clade" of the Atyidae, survive and develop most successfully in salt or brackish waters. Low salinity is a known barrier to larval development across a wide range of aquatic taxa (Zimmerman and Pechenik, 1991; Daniels *et al.*, 1996; Mann and Harding, 2003; Scott *et al.*, 2013; Ko *et al.*, 2014; Conley and Uye, 2015). Even though the adults of *H. rubra* are strongly euryhaline (Havird *et al.*, 2014a), our results imply that optimal larval development for this species requires waters of appreciable salinity, consistent with many other members of the family. While larval settlement and metamorphosis were higher at the 15‰ (88%) and 32‰ (72%) salinity treatments than was reported in previous studies (50% in Iwai, 2005; 33% in Couret and Wong, 1978; at 15‰ and 20‰, respectively), survivorship decreased drastically at 2‰ (to 12%). Vital staining for populations of MRCs offers a potential explanation as to why larvae, unlike adults, of *H. rubra* do not survive well at low salinity. Specifically, the Z_1–Z_4 developmental stages of *H. rubra* lack gills capable of ion transport. Instead, proto-gills were only apparent following settlement of juveniles, and, even then, only three of five individuals at this stage possessed them. A previous study using silver nitrate staining, a less precise way to identify MRCs, reported a similar lack of gills in early zoeal stages, with only one of five Z_4 larvae examined having proto-gills (fig. 3H of Havird *et al.*, 2014a). These results suggest that gills with ion-transporting capabilities usually only develop in the post-settlement, juvenile stage of *H. rubra*, with considerable variation in their development time among individuals. Although this variation may explain how 3 of 25 larvae survived at 2‰ and why survivorship was higher at 15‰ than 32‰, additional studies are needed to further correlate larval gill development and its timing with low-salinity survival in this species.

What does the lack of low-salinity tolerance in the early developmental stages of *H. rubra* mean for larval ecology in the anchialine ecosystem of Hawaii or elsewhere? Despite the fact that the larvae of *H. rubra* are positively phototaxic in the laboratory (Couret and Wong, 1978; Bailey-Brock and Brock, 1993), implying that they should be attracted to the epigeal portion of the ecosystem, they have never been found in anchialine habitats of the islands. Moreover, similar reports on the absence of larvae from the epigeal extend to other endemic anchialine caridean shrimp from around the Pacific Basin (Maciolek, 1983), and are supported by personal observations for species from anchialine habitats across the Ryukyu Islands of Japan (JCH and DAW). Furthermore, an amphidromous life cycle, consistent with a lack of larvae in anchialine habitats, has been suggested for the Japanese anchialine atyid *Caridina rubella* (Shokita, 1979). In this context, the results presented here offer an explanation, namely, that the deeper and saltier waters of the hypogeal portions of anchialine habitats (Holthuis, 1973; Sket, 1996; Iliffe, 2000; Pohlman, 2011; Havird *et al.*, 2014a) may result in greater larval survivorship. Notably, larvae of the glass shrimp *Palaemon debilis* occur in the

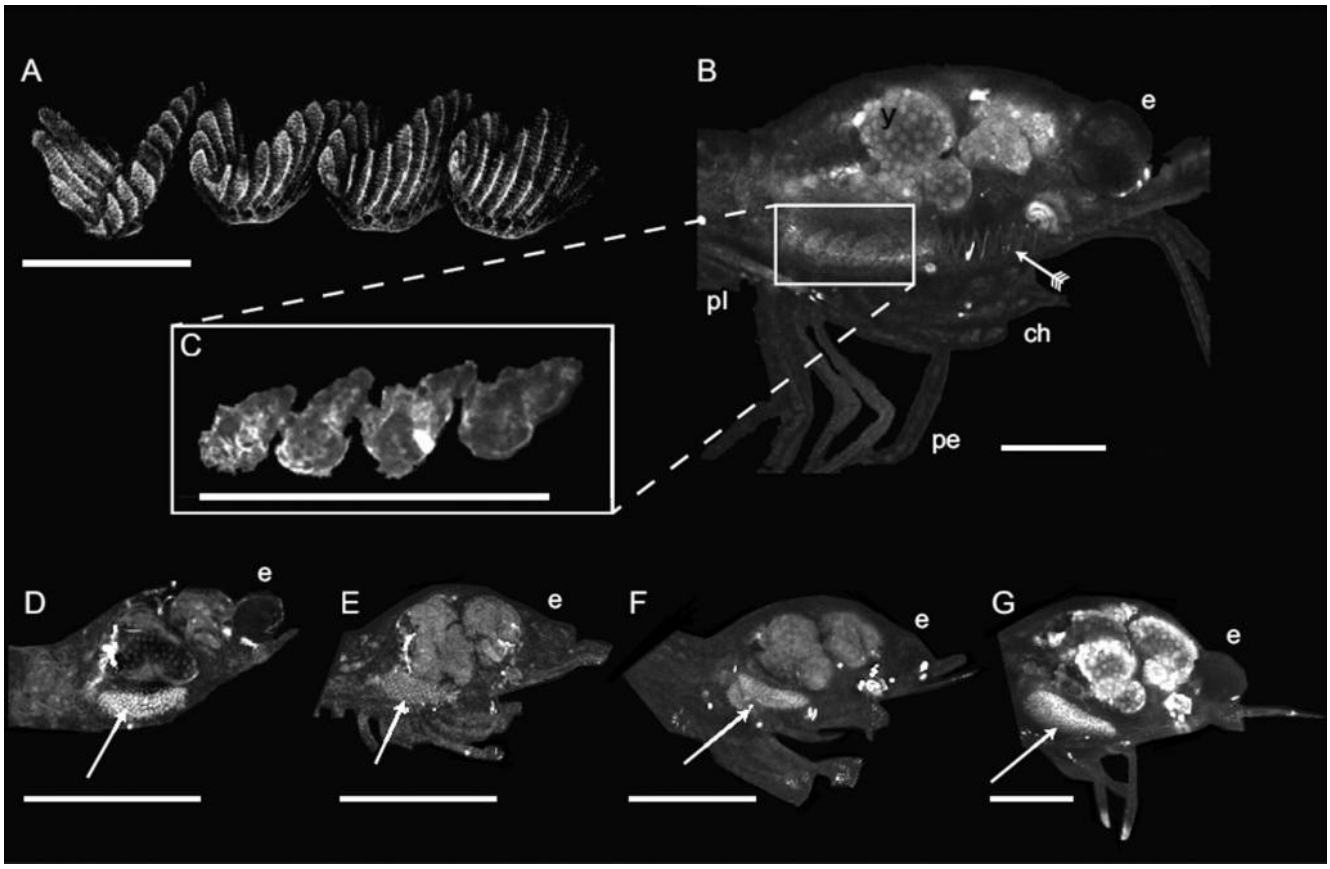

Figure 3. Vital mitochondrion-rich cell (MRC) staining in *Halocaridina rubra* using confocal microscopy of (A) adult gills (for reference), (B) a recently settled juvenile, (C) close-up of proto-gills in (B), and (D–G) zoeal stages Z_1–Z_4. Scale bars indicate approximately 1 mm. Feathered arrow in (B) shows the water stream from the pumping scaphognathite, the organ responsible for ventilating the gills (each peak likely corresponds to a single beat). Arrows in (D–G) indicate a possible ion-transporting epithelial sheet. *Abbreviations:* ch, chelipeds; e, eye; pe, periopods; pl, pleopods; y, developing yolk.

epigeal waters of Hawaii's anchialine habitats (Maciolek and Brock, 1974). A previous study of the related species *Palaemonetes argentinus* suggests that early developmental stages of glass shrimps, in spite of their lack of gills, have osmoregulatory capabilities similar to those of adults, allowing them to develop at lower salinities (Charmantier and Anger, 1999). Taken together, the larvae of *H. rubra* and other endemic anchialine caridean shrimp species are apparently and effectively confined to hypogeal waters. Unlike adults, larvae are apparently intolerant of the lower, fluctuating salinities of the epigeal waters, possibly due to a lack of osmoregulatory capabilities. How this facet of larval ecology might change, given expectations of rising, long-term epigeal salinities in anchialine habitats of Hawaii due to climate change (Marrack, 2014), warrants future consideration.

Osmoregulation may also explain in part why gravid females of *H. rubra* are absent from the epigeal portion of the anchialine ecosystem in Hawaii. For example, maintaining an egg mass and actively osmoregulating are both physiologically expensive processes. Therefore, gravid females in the wild may prefer hypogeal waters to avoid the energetic demands associated with lower salinities in epigeal waters. Support for this scenario comes from delayed reproduction in the brown shrimp *Crangon crangon* under low salinities in both the laboratory (Gelin *et al.*, 2001a) and in the wild (Gelin *et al.*, 2001b). While a study of survivorship and reproductive success of gravid females of *H. rubra* at low salinities could test this hypothesis, gravid females of other anchialine caridean shrimp species can be found in epigeal waters. This includes species that are within the "anchialine clade" of the Atyidae (JCH and DAW, pers. obs.) as well as the anchialine alpheids *Metabetaeus lohena* in Hawaii (Russ *et al.*, 2010) and *M. minutus* (Whitelegge, 1897) of Okinawa, Japan (JCH and DAW, pers. obs.). Another possibility is that the developing eggs or embryos carried by females may be intolerant of low salinity. Comparative studies of osmoregulation in embryos and the permeability of egg membranes among anchialine caridean species would further evaluate this hypothesis. Besides osmoregulation, the exact abiotic or biotic (*e.g.*, behavioral or

hormonal cues leading to a negative phototaxic response) factors that may be contributing to the absence of gravid females in the epigeal remains to be elucidated for *H. rubra*.

Although some larvae can osmoregulate while lacking gills (Charmantier and Anger, 1999), those of euryhaline and osmoregulating crustaceans generally tend to be osmoconformers until late developmental or juvenile stages, when gills capable of ion transport develop (Charmantier *et al.*, 2002; Cieluch *et al.*, 2004, 2005). For *H. rubra*, a thin epithelial sheet with a dense population of MRCs was identified by vital staining with both DASPMI (Fig. 3D–G) and silver nitrate (fig. 3D–G of Havird *et al.*, 2014a) in Z_1–Z_4 larvae near where the proto-gills develop in the post-settlement juvenile stage. This sheet is most likely the inner epithelium of the branchiostegite, which plays a role in osmoregulation in the early developmental stages of other crustaceans (Cieluch *et al.*, 2005, 2007; Boudour-Boucheker *et al.*, 2013). For example, the inner epithelium of the branchiostegite is characterized by high, salinity-mediated levels of Na^+/K^+-ATPase activity and MRC populations in the palaemonid shrimp *Macrobrachium amazonicum* (Boudour-Boucheker *et al.,* 2013). To the best of our knowledge, this study represents the first report of the branchiostegite in an atyid shrimp. However, while it likely represents an ion-transporting organ for the early larvae of *H. rubra*, the salinity challenge experiment suggests that the branchiostegite does not convey appreciable osmoregulatory ability to early zoeal stages of the species. Future experiments should examine salinity-mediated, hemolymph osmolality in the larvae of *H. rubra* to test such hypotheses regarding osmoregulatory capability.

Genetic and environmental influences on reproductive trends in Halocaridina rubra

Following about 5 y of maintenance under standardized conditions, colonies from seven of the eight recognized genetic lineages encompassed in *H. rubra* showed variability both in the number of larvae produced and whether larvae were likely to be present at a given sampling date. Surprisingly, the KBP colony—the only "mixed" population included in the study—encompassing individuals from both the West and South Oahu genetic lineages (*i.e.,* OWAI and EWA, respectively), was the most prolific. Notably, these represent the two most singularly prolific lineages examined in this study (Fig. 1), suggesting that the high level of reproduction could be an additive effect. Although it is possible that hybrid vigor (Shull, 1948) may be driving the increased reproductive output of the KBP colony in the laboratory, other crustaceans with a similar level of mitochondrial genetic divergence (*e.g.*, about 5% uncorrected (*p*) distances for *COI* and 16S-rDNA; Craft *et al.*, 2008) show dramatic fitness decreases by the F_2 generation due to incompatibilities between the mitochondrial and nuclear genomes of such individuals (Rawson and Burton, 2002; Ellison and Burton, 2006, 2008). Given this fact, it remains unknown if (or to what extent) offspring from this laboratory colony represent hybrid individuals between the lineages, and whether reproductive compatibility exists across the genetic lineages of *H. rubra*.

Reproduction in *H. rubra* appears to be influenced by genetic and environmental factors. For example, our finding that reproduction was high in the Spring is consistent with previous reports (Couret and Wong, 1978; Bailey-Brock and Brock, 1993). However, other studies did not document the peak during winter months that we found, instead suggesting that reproduction remains relatively low throughout Fall and Winter. One possibility for this difference is that only three of the *H. rubra* genetic lineages (*i.e.*, EP, EWA, and KONA) were previously examined. Notably, the reproductive peak identified in Winter persists when only these three lineages are analyzed (data not shown), suggesting that other factors are responsible for the discrepancy. One factor might be light regimes, which influence reproduction in many crustacean species (Chamberlain and Lawrence, 1981; Wang *et al.*, 2003). While artificial light was used here and in some earlier studies (Couret and Wong, 1978; Iwai, 2005), and high levels of reproduction have also been observed in *H. rubra* colonies exposed to natural light (JCH, DAW, and SRS, pers. obs.), future work that specifically investigates the influence of different light regimes on *H. rubra* reproduction might prove useful. Moreover, identifying additional environmental correlates of reproduction could aid in developing commercially viable aquaculture techniques for these shrimp in the ornamental trade (Weese and Santos, 2009).

The reproduction and development of organisms from the anchialine ecosystem have been studied only marginally, with *H. rubra* (Couret and Wong, 1978; Iwai, 2005) and the phylogenetically important remipede crustaceans (Koenemann *et al.*, 2007, 2009) being among the most detailed to date. A reason is that many anchialine larvae have a planktotrophic-feeding mode (Russ *et al.*, 2010; Weese *et al.*, 2013), rendering them difficult to rear under laboratory conditions. Furthermore, because larvae are rarely found in nature for most anchialine species in the first place, it should not be surprising that field-based studies of larval ecology in anchialine habitats are also lacking. For instance, the role of larvae in the otherwise depauperate food webs of the anchialine ecosystem remains unexplored. One recently proposed and non-traditional method for detecting larvae in the anchialine ecosystem involves measuring autofluorescence at near-ultraviolet wavelengths, since larvae from some species exhibit this property (Glenn *et al.*, 2013). Approaches like this one may increase knowledge in this understudied area. In any case, the differences in reproductive trends identified here serve as another indicator that the genetic lineages within *Halocaridina* are distinct biological entities, representing cryptic, but discrete, species (Santos,

2006; Craft *et al.*, 2008; Vaught *et al.*, 2014). We feel that the ability to maintain this diversity in the laboratory, together with the growing physiological information and genomic resources for the genus (Craft *et al.*, 2008; Havird *et al.*, 2014a, b) and the reproductive and developmental data presented here, further strengthen *Halocaridina* as an emerging model system in crustacean biology.

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