Grazing by an endemic atyid shrimp controls microbial communities in the Hawaiian anchialine ecosystem

Running head: Animal-microbial ecology in anchialine habitats

Justin C. Havird[A*], Pamela M. Brannock[B], Reyn M. Yoshioka[N,C], Rebecca C. Vaught[D], Kaile'a Carlson[E,F], Collin Edwards[N,G,H], Allison Tracy[N,I], Cornelia W. Twining[N,J], Yun Zheng[K], David Chai[L], Alan E. Wilson[M], Nelson G. Hairston Jr.[N], Scott R. Santos[O]

[A]Dept. of Integrative Biology, The University of Texas at Austin, Austin, TX
[B]Dept. of Biology, Rollins College, Winter Park, FL, USA
[C]Oregon Institute of Marine Biology, University of Oregon, Charleston, OR, USA
[D]Van Heron Labs, Huntsville, AL, USA
[E]Tropical Conservation Biology and Environmental Science, University of Hawaii at Hilo, Hilo, HI, USA
[F]Research Corporation of the University of Hawaii, Hilo, HI, USA
[G]Dept. of Biology, Tufts University, Medford, MA, USA
[H]School of Biological Sciences, Washington State University – Vancouver, Vancouver, WA, USA
[I]Smithsonian Environmental Research Center, Edgewater, MD, USA
[J]Dept of Fish Ecology and Evolution, Eawag, Kastanienbaum, Switzerland
[K]Dept of Civil and Environmental Engineering, Cornell University, Ithaca, NY, USA
[L]Hualalai Four Seasons Resort, Kailua-Kona, HI, USA
[M]School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, Auburn, AL, USA
[N]Dept. of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, USA
[O]Dept. of Biological Sciences, State University of New York at Buffalo, Buffalo, NY, USA

*correspondence: jhavird@utexas.edu; pbrannock@rollins.edu; rmy27@cornell.edu; rvaught@vanheronlabs.com; kailea_annandale@nps.gov; collin.edwards@tufts.edu; amt279@cornell.edu; cwt52@cornell.edu; yz499@cornell.edu; david.chai@fourseasons.com; wilson@auburn.edu; ngh1@cornell.edu; srsantos@buffalo.edu

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Abstract

Animals often shape environmental microbial communities, which can in turn influence animal gut microbiomes. Invasive species in critical habitats may reduce grazing pressure from native species and shift microbial communities. The landlocked coastal ponds, pools, and caves that make up the Hawaiian anchialine ecosystem support an endemic shrimp (*Halocaridina rubra*) that grazes on diverse benthic microbial communities, including orange cyanobacterial-bacterial crusts and green algal mats. Here, we asked how shrimp: 1) shape the abundance and composition of microbial communities, 2) respond to invasive fishes, and 3) whether their gut microbiomes are affected by environmental microbial communities. We demonstrate that ecologically relevant levels of shrimp grazing significantly reduces epilithon biomass. Shrimp grazed readily and grew well on both orange crusts and green mat communities. However, individuals from orange crusts were larger, despite crusts having reduced concentrations of key fatty acids. DNA profiling revealed shrimp harbor a resident gut microbiome distinct from the environment, which is relatively simple and stable across space (including habitats with different microbial communities) and time (between wild-caught individuals and those maintained in the laboratory for >2 years). DNA profiling also suggests shrimp grazing alters environmental microbial community composition, possibly through selective consumption and/or physical interactions. While this work suggests grazing by endemic shrimp plays a key role in shaping microbial communities in the Hawaiian anchialine ecosystem, the hypothesized drastic ecological shifts resulting from invasive fishes may be an oversimplification as shrimp may largely avoid predation. Moreover, environmental microbial communities may have little influence on shrimp gut microbiomes.

Keywords: *Halocaridina rubra*, 16S, 18S, selective grazing, microbiome, microbial consortia

Introduction

Animals play key roles in shaping microbial communities. In aquatic habitats, grazers such as fishes and invertebrates can alter both the biomass and composition of the microbial community through selective grazing and physical interactions (Hillebrand and Kahlert 2001; Szabó et al. 2020; Veach et al. 2018). However, microbes can also shape the numbers and species of animals in an ecosystem (Brett and Muller-Navarra 1997; Sehnal et al. 2021). For example, higher microbial productivity can support increased animal populations, and environmental microbes can shape the gut microbiomes of animal grazers, thus influencing their health and physiology (Spor et al. 2011).

While animal-microbe interactions have been previously explored in aquatic systems, it is unclear how grazing shapes microbial communities that vary among sites. For example, do the same grazers exert similar pressures on microbial communities dominated by eukaryotic algae vs. prokaryotic cyanobacteria? Although overall community biomass is often quantified in aquatic grazing studies, the key microbial players, especially bacterial taxa, are usually not identified, limiting our ability to determine how grazing drives community composition. The introduction of invasive species, especially those predating upon grazers, may also alter microbial communities *via* changing grazing patterns of native species, nutrient inputs, or complex trophic interactions (Finlay and Vredenburg 2007; Kurle et al. 2008). However, such studies are often correlative, comparing habitats with or without grazers or invasive consumers, because laboratory-based experiments are often not feasible (although see e.g., Corno and Jürgens 2006). Here, we address how shrimp grazing influences microbial communities, and, in turn, how microbial communities may affect shrimp populations in anchialine habitats of Hawaii.

Habitats belonging to the anchialine ecosystem are defined as landlocked coastal ponds, pools, and caves lacking surface connections to the ocean, but influenced by both marine and freshwaters through underground connections to the sea and underlying aquifer (Sket 1996). Although relatively rare worldwide (~1,000 known examples), anchialine habitats are most common (~600 described habitats) in the Hawaiian Islands (Bailey-Brock and Brock 1993). Many Hawaiian anchialine organisms are endemic, threatened, and/or have cultural significance.

The Hawaiian anchialine ecosystem is home to diverse microbial communities. Across the islands, habitats differ in their basin types (e.g., limestone, basalt, or mud), surroundings (e.g., very young lava fields to forested coastlands), and sizes/topography (e.g., from cracks in lava rock to small lakes). Benthic microbial communities from anchialine habitats of Hawaii have a high degree of endemism, with individual habitats possessing unique, highly diverse communities (Hoffman et al. 2018b), which may be the norm for this ecosystem worldwide (Kajan et al. 2021). In Hawaii, differences between communities are apparently driven by environmental factors (especially salinity), and community composition is relatively stable across seasons (Hoffman et al. 2018a; Hoffman et al. 2018b). Of particular interest are unique and striking laminated, orange cyanobacterial–bacterial crusts found in some habitats on the southern coast of Maui and west coast of Hawaii. These communities are composed of filamentous cyanobacteria, multicellular algae, diatoms, and other microorganisms and are structurally similar to laminated mats, stromatolites, or microbialites, as they are composed of ~4 layers with distinct constituents (Hoffman et al. 2020). Additional types of microbial communities in Hawaiian anchialine habitats include green mats dominated by algae, thin microbial films on bare rock, and communities supported by and living on leaf litter from overhanging trees and vegetation.

In the Hawaiian anchialine ecosystem, the endemic atyid *Halocaridina rubra* (Holthuis 1963) is the most abundant and widespread macro-organism (Bailey-Brock and Brock 1993). These small (ca 1 cm long) shrimps are the primary grazers on microbial communities and may maintain the diversity of the orange crusts, as habitats with invasive fishes have been reported to become overgrown with filamentous green algae due to shrimp suppression or extirpation (Bailey-Brock and Brock 1993; Capps et al. 2009; Carey et al. 2011). This is supported in tropical freshwater streams where other atyid species act as primary grazers and the abundance and composition of algal communities is strongly influenced by shrimp grazing (March and Pringle 2003; Pringle 1996). Some even suggest atyid grazing may act to maintain a “garden” of algae (De Souza and Moulton 2005), similar to the intertidal limpet *Lottia gigantea* (Stimson 1970). One correlative analysis of epilithon communities in Hawaiian anchialine habitats did find that ponds with lower *H. rubra* abundances had higher epilithon biomass, suggesting shrimp grazing

plays an important role in maintaining benthic communities (Dalton et al. 2013). However, experimental studies of *H. rubra* grazing on different substrates are lacking.

Furthermore, it is unclear how microbial communities affect *H. rubra* populations and shape macro-ecological patterns. While shrimp can be abundant in anchialine habitats with orange crusts, it is unknown if these communities are adequately nutritious to shrimp, given that cyanobacteria generally lack metabolically critical fatty acids (e.g., (Twining et al. 2021). It is also unknown if grazing might dictate the gut microbiomes in these shrimp, which could translate into different patterns of growth, health, and disease (Spor et al. 2011). Habitats with increased nitrogen and phosphorous concentrations do have increased epilithon biomass as well as more and larger shrimp (Dalton et al. 2013). However, detailed microbial surveys from both environmental communities and shrimp guts are lacking. Thus, while microbial ecology in Hawaii's anchialine ecosystem is subject to both "top-down" and "bottom-up" processes (Dalton et al. 2013; Dudley et al. 2017; Sakihara et al. 2015; Seidel et al. 2016), many questions remain.

Here, we explore animal-microbial ecology in the Hawaiian anchialine ecosystem using a combination of DNA profiling of microbial communities, diel shrimp abundance surveys of diverse habitats, and field and laboratory experiments. Specifically, we hypothesized that: 1) grazing by *H. rubra* alters the relative abundance and composition of environmental microbial communities and 2) *H. rubra* grows slowest when grazing on orange crusts, which may be nutritionally poor. We also examined gut contents of *H. rubra* with DNA profiling to determine: 1) if gut microbiomes represent a non-random portion of the environmental microbiome, as predicted if *H. rubra* grazing is selective and 2) the presence of an endemic gut microbiome, including how stable it is through time, and the extent to which such gut communities are consistent across habitat types. Finally, we provide further evidence on how invasive fishes may result in significant microbial community shifts through altered shrimp grazing.

Materials and Methods

Environmental microbial community sampling

We characterized microbial communities from the benthic substrate of anchialine habitats across the Hawaiian Islands (Fig. 1, Table S1) using sampling and processing strategies described previously (Hoffman et al. 2018a; Hoffman et al. 2018b; Hoffman et al. 2020). Briefly, sterile spoons or spatulas were used to collect ~100 g of benthic material (i.e., orange cyanobacterial-bacterial crusts, green algal mats, mud, and leaf litter) that was preserved in RNAlater (ThermoFisher, CA, USA) and frozen until DNA extraction (usually weeks later). DNA extraction was performed with MoBio PowerSoil DNA Isolation Kits (MOBIO, CA, USA) following the manufacturer's protocol except that bead-beating, rather than vortexing, was used to homogenize samples.

Many of the environmental samples analyzed here have been described previously based on sampling efforts in 2010 and 2011 (Hoffman et al. 2018a; Hoffman et al. 2018b; Hoffman et al. 2020), including three fishless habitats with orange crust communities: Hanamanioa (HM) and Skippy's Pond (SKIP) on Maui as well as Pohue Bay (PB) on Hawaii (Fig. 1). Each site was sampled from several locations per habitat across multiple seasons. Orange crusts have ~4 distinct layers that were sometimes sampled separately as an orange top layer, a second orange layer, and pink and green bottom layers. Samples were also collected from habitats without orange crusts: Waianae (OWAI), a small fishless pond with mud/limestone substrate on Oahu; Waianapanapa Cave (WC), a fish-invaded cave with mud/leaf litter on Maui; Puhi' Ula Cave (PU), a fishless cave with basalt pebble substrate on Hawaii; and a large fish-invaded pond at Pu'uhonua O Hōnaunau National Historical Park (PUHO3) with mud substrate (Fig. 1).

Also included here are new environmental samples from additional Hawaiian anchialine habitats. These include a fishless restoration habitat at Pu'uhonua O Hōnaunau National Historical Park (Res1) which was created to mitigate the destruction of other anchialine habitats in the area, with mud and algae substrate at the time of sampling (two different locations) in summer 2010. Furthermore, six habitats on Hawaii were sampled in January 2013. Three were at or near the Waikoloa Resort and all had orange crusts: two habitats at Kapalaoa Bay (AB and AC; formerly called `Anaeho`omalu Bay by Dalton et al., 2013) and one at the resort itself (WAI),

which were each sampled in two locations. Three habitats were also sampled at Hualalai Resort (HA, HB, and HC), either at one, two, or three locations depending on size, each with green mats. Along with preserving samples in RNAlater, single "live" samples from WAI and HC were stored in environmental water and shipped to Auburn University where DNA was immediately extracted (~3 days post-collection) to determine how sampling and shipping benthic samples might influence characterization of microbial communities.

We also broadly assessed taxonomic composition and biomass of orange crust and green mat communities at Kapalaoa/Waikoloa and Hualalai habitats, respectively. Briefly, broad taxonomic categories and their relative abundances were examined via microscopy and chlorophyll *a* concentration was used as a proxy for epilithon biomass. See Supplementary Methods for details.

H. rubra gut sampling

To examine gut contents of *H. rubra*, we sampled wild-caught individuals from SKIP, HM, and PB in summer 2010 as well as Kapalaoa/Waikoloa and Hualalai habitats in January 2013. Complete digestive tracts were dissected from shrimp (n = 2-4 per habitat) following anaesthetization on ice, making sure not to include any muscle or nervous tissues. For Kapalaoa/Waikoloa and Hualalai habitats, shrimp were held in filtered pond water with no substrate in a 250 mL chamber with a 300 µm mesh screen 1 cm above the bottom for 24 hours. We assumed this resulted in shrimp being "cleared" of gut contents as fecal pellets fell through the screen and were unavailable for re-consumption. This was performed to screen for a resident gut microbiome and reduce potential microbes from transient food sources, although gut clearing was not feasible at the other sites. Guts were washed with sterile water and then preserved in RNAlater before being shipped to Auburn University for DNA extraction using a Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol.

Laboratory grazing experiment and diel surveys

To examine how *H. rubra* grazing alters environmental microbial communities, a laboratory experiment was performed with microcosms containing proxy microbial communities and variable shrimp densities (Fig. S1). Each microcosm consisted of a 2.8 L

199 rectangular tank filled with 2 ppt salinity water (Instant Ocean, VA, USA; salinities of sampled
200 habitats ranged from 2-35 ppt) and small ceramic tiles (3 x 3 x 1 cm) cultured with an algal
201 community from a small pond on the Auburn University campus. Tiles were placed in a mesh
202 bag and submerged in the pond for 2 weeks before being incubated in the lab in 2 ppt water for
203 an additional 2 weeks with algal substrate from the same pond and *Gambusia affinis* fishes to
204 further seed the microbial communities. Following this culturing regime, tiles had a noticeable
205 green/brown algal film on them (Fig. S1A). Preliminary data showed that shrimp readily grazed
206 on this film (Supplemental movie 1) and that algae and cyanobacteria were major components
207 of this community. Thus, while *bona fide* anchialine microbial communities were not employed,
208 these cultured tiles were deemed *a priori* to be a suitable proxy for our purposes in this
209 experiment. Tiles were randomly placed in the 24 microcosms, with 9 tiles per microcosm stood
210 "on edge" so that ~27 cm^2 of grazing area was available per tile, or ~243 cm^2 per microcosm
211 (Fig. S1B). Tiles were allowed to incubate for an additional 2 weeks in the microcosms before
212 shrimp were added.
213 To simulate ecologically relevant levels of grazing pressure in the microcosms, shrimp
214 densities were quantified during diel surveys of anchialine habitats in 2010 and 2011, as
215 described previously (Havird et al. 2013) (Supplementary Methods). Habitats surveyed included
216 fish-invaded and fishless habitats with and without orange crust communities across Maui and
217 Hawaii (Fig. 1). Based on these surveys, each microcosm received one of four shrimp density
218 treatments (*n* = 6 microcosms per treatment): 1) "no" (zero shrimp) to simulate daytime
219 densities in fish-invaded habitats and acting as a negative control, 2) "low" (5 shrimp, 206
220 shrimp m^{-2}) simulating nighttime densities in fish-invaded habitats, 3) "medium" (15 shrimp,
221 617 shrimp m^{-2}) simulating densities of fishless habitats, and 4) "high" (25 shrimp, 1029 shrimp
222 m^{-2}) representing the highest densities observed in diel surveys. Shrimp used in the experiment
223 were from a laboratory colony initially collected from Hanamanioa, Maui, and kept in the
224 laboratory for >2 years prior to the experiment. *Halocaridina* represents at least eight divergent
225 genetic lineages based on mitochondrial DNA analyses (Craft et al. 2008; Santos 2006), and
226 those from HM belong to the South Maui lineage. Shrimp were acclimated to 2 ppt water for
227 one month prior to being added to the microcosms, which were arranged randomly in a 6 x 4
228 grid in the laboratory near a window for the duration of the experiment.

To determine how grazing by *H. rubra* alters environmental microbial abundance, a single tile was randomly removed from each microcosm before the addition of any shrimp (i.e., time = 0 days) as well as at 1, 2, 3, 7, 14, 28, and 52 days after shrimp addition and cleaned of algal film. Chlorophyll *a* concentration was then quantified using fluorometry as described in Sartory and Grobbelaar (1984) (see Supplemental Methods).

Tile and shrimp gut microbial communities were also opportunistically sampled for DNA profiling during this experiment to determine: 1) how these proxy communities compared to *bona fide* anchialine microbial communities of the Hawaiian Islands; 2) if shrimp grazing altered microbial community composition of tiles; and 3) whether shrimp grazing on tiles had a distinct gut microbiome compared to wild-caught individuals. Such samples, though not collected systematically, offer a complementary snapshot to the experiment. Briefly, a small portion (i.e., 1 cm^2) of the tile community was sampled from the "high shrimp" microcosms 0, 3, and 24 days after shrimp addition (*n* = 4 samples per time point were analyzed). Additionally, a single shrimp was removed for gut content profiling from microcosms at 3, 24, and 33 days after shrimp addition. These microcosms included one control for shrimp grazing where shrimp were kept without substrate to graze (*n* = 4-6 samples per time point were analyzed). Guts were processed from laboratory shrimp using the same method as for wild-caught individuals, except that DNA was immediately extracted from guts without being preserved in RNAlater. Lastly, fecal pellets that accumulated in one "high shrimp" microcosm were pooled for DNA extraction and profiling 24 days after shrimp addition. DNA extraction from tile samples and fecal pellets utilized the same protocol described previously for environmental samples.

Fatty acids in orange crusts vs. green mats

Cyanobacteria lack nutritionally important fatty acids (FA) present in green algae and diatoms, but orange crusts, while dominated by cyanobacteria, also contain green algae and diatoms. Green mats, while dominated by green algae, also contain cyanobacteria. To assess the nutritional quality of these mixed resource substrates, we compared the percent of total FAs in each. Of particular focus were the polyunsaturated FAs linoleic acid (LIN, 18:2ω-6), α-linolenic acid (ALA, 18:3ω-3), arachidonic acid (ARA, 20:4ω-6), and eicosapentaenoic acid (EPA, 20:5ω-3). Benthic substrate samples were collected in June 2021 from the same three habitats

with orange crusts at Waikoloa (WAI) and Kapalaoa Bay (AB, AC) described above and from two green mat habitats at Hualalai Resort (HA and Ho`onanea because HB and HC were exposed at low tide at time of sampling). Single samples from each habitat (except two from opposite sides of Ho`onanea, which is a large pond) were processed following methods of Taipale et al. (2016) and Yoshioka et al. (2019) (see Supplemental Methods).

Growth rate experiment

To assess the quality of orange cyanobacterial crust vs. green algal mat communities as food sources for *H. rubra*, we measured specific somatic growth rates of individual shrimp in a 2 × 2 design, with shrimp from each substrate type fed each substrate type. The experiments were performed using shrimp and substrate collected from the three Waikoloa/Kapalaoa-Bay (orange crust) and three Hualalai (green mat) habitats described above and carried out in 20 clear 5.7 L microcosms (35L × 21W × 12.5H cm) situated in an outdoor covered area and shielded from direct sunlight. Each microcosm contained 75 µm filtered water and one or more resource rocks collected from the appropriate pond types (ca. 5 cm diameter and naturally coated with either orange crust or green algal mat). Shrimp were collected by hand-netting from each habitat, with 18-19 individuals placed in each microcosm. Overall, five microcosms contained shrimp, water and resource rocks from green mat habitats; five contained shrimp, water and rocks from orange crust habitats; five contained shrimp from green mat habitats and water and rocks from orange crust habitats; and five contained shrimp from orange crust habitats and water and rocks from green mat habitats.

To obtain initial *H. rubra* mass at the start of the experiment, four or five individuals from each microcosm were separately oven dried at 70 °C for 24 h and weighed on a Mettler Toledo MX5 microbalance. Shrimp carapace lengths (anterior tip of rostrum to dorsal posterior carapace edge) were measured using electronic Vernier calipers, and shrimp condition was calculated as mass/length. The remaining 14-15 shrimp in each microcosm were allowed to feed on the provided resource rocks for 61 days. Water and resource rocks were replaced weekly with used resource rocks returned each week to the ponds from which they were collected. Shrimp fed readily on both substrates, producing large quantities of fecal pellets which were collected at each water change. These fecal samples were preserved in RNAlater

and shipped to Auburn University for DNA extraction as described previously. Notably, fecal pellets from the orange crust treatments were consistently a clear-yellow color while those from the green algal mat treatments were consistently dark green. There was always substantial orange crust or green mat left on rocks when they were exchanged, and there was little to no mortality in any treatment combination. At the end of the experiment, shrimp were dried, weighed, and measured in the same manner as at the start of the experiment.

Because measuring *H. rubra* mass involved terminal sampling and mass was necessary to estimate condition and growth rate, the same individuals could not be measured at the start and end of the growth experiment. Thus, the mean initial weights and mean final weights of shrimp from each treatment and replicate were utilized to calculate the specific somatic growth rate (SSGR with units day^{-1}) as:

$$SSGR = [(\ln W_2 - \ln W_1) / t]$$

Where W_1 and W_2 are the initial and final mean weights, respectively, and t is the duration of the experiment (61 days).

DNA profiling and microbial community analyses

The DNA sequencing scheme utilized here for microbial community profiling has been described previously (Hoffman et al. 2018b) (see Supplemental Methods). Importantly, two different markers were analyzed for each sample, the *Bacteria*-specific hypervariable V6 region of *16S-rRNA* and the *Eukarya*-biased hypervariable V9 region of *18S-rRNA*, to capture bacterial and eukaryotic communities more fully in each sample. Quantitative Insights Into Microbial Ecology (QIIME v. 1.9.1; Caporaso et al. 2010) was used to quality filter sequencing reads, cluster operational taxonomic units (OTUs), assign their taxonomy, and create diversity matrices. For V9, any OTUs that were classified as Malacostraca, which represented a high proportion of reads from the shrimp gut samples, were removed as they were likely from *H. rubra*. Plymouth Routines in Multivariate Ecological Research (PRIMER-e v. 7) was used to produce non-metric multidimensional scaling (nMDS) ordinations and perform permutational

multivariate analyses of variance (PERMANOVA) and PERMDISP tests (Anderson 2006), largely following (Brannock et al. 2014) (see Supplemental Methods).

Statistical analyses

We compared *H. rubra* abundances from fish-invaded and fishless habitats during day and night surveys, using a mixed effect model with a random effect of quadrant to account for repeated sampling. Predictors included were fish status (invaded vs. fishless), time of survey (day vs. night), and their interaction. As observations were extremely right-skewed, we used a log-normal error distribution (similar results were obtained using a gamma distribution and a zero-inflated log-normal distribution). We evaluated predictors with marginal hypothesis tests using the **Anova()** function in the *car* package (Fox and Weisberg 2019).

To determine if shrimp grazing altered chlorophyll *a* concentration in the laboratory grazing experiment, we assumed chlorophyll *a* concentration changed at a constant per-density rate (e.g. exponential growth or decline) based on shrimp density. We fit a linear mixed model of log chlorophyll *a* concentration, including as predictors time in days, an interaction between time and shrimp density (i.e., no, low, medium or high), and a random effect of microcosm to control for microcosm-level variation in sunlight exposure. Because initial chlorophyll *a* concentration should not have differed between treatments, we fit a single intercept rather than one per treatment. We evaluated predictors with marginal hypothesis tests as above. We compared rates of growth/decline in Chlorophyll *a* concentration between treatments with Tukey posthoc analysis using the **emtrends()** function in the *emmeans* package (Lenth 2022).

Differences in particular fatty acids (FA) between orange crusts and green mats were analyzed using a Wilcoxon rank sum test adjusted for clustering (Rosner et al. 2003) in the R package *clusrank* (Jiang et al. 2020). For the growth rate experiment, initial size differences between *H. rubra* from orange crust vs. green mat habitats were examined using a Welch's two sample *t*-test to compare weight, length, and condition between habitat types for shrimp prior to starting the growth rate experiment. To test for drivers of *H.* rubra growth rate, we fit general linear models (GLM) with growth rates of one of shrimp mass, length or body condition as response variables, and habitat of origin, experimental food source (orange crust or green

mat) and their interactions as predictors. All statistical analyses were conducted in R 4.0.3 (R Core Team 2021), and all mixed models were fit using the *lme4* package (Bates et al. 2015).

Data availability

All new sequence data generated here are publicly available in NCBI's SRA database under BioProject PRJNA767902. Sequences that have been previously described are under BioProject PRJNA325159. Raw data from the diel surveys, laboratory grazing experiment, and growth rate experiment are available via FigShare at DOI 10.6084/m9.figshare.16709632 (https://tinyurl.com/36eu2b77) along with R code used in statistical analyses and outputs from the PERMANOVA and PERMDISP tests.

Results

Microbial community composition varies across anchialine habitats

As reported previously (Hoffman et al. 2018b), benthic samples from anchialine habitats with orange crusts have distinct microbial communities compared with samples from habitats with green mat or mud substrates. The inclusion here of new sites did not alter this finding as distinct clusters from these habitat types were formed in analyses of the *Bacteria*-specific V6 ($P < 0.001$, $t = 5.26$, PERMANOVA, orange vs. brown diamonds in Fig. 2A, Fig. S2) and *Eukarya*-biased V9 regions ($P < 0.001$, $t = 4.92$, Fig. 2B, Fig. S2). Microscopic analyses of the samples from habitats at Kapalaoa/Waikoloa and Hualalai revealed the orange crusts consisted of various Cyanobacteria, including very tightly packed filaments of *Lyngbya* and a few of *Planktothrix* (formerly *Oscillatoria*), consistent with Bailey-Brock and Brock (1993), and much less common fine filaments of *Anabaena*; also uncommon were Chlorophyta like colonial green *Gomontia* and highly branched filamentous *Oedocladium*. In green algal mats, Chlorophyta were dominated by the filamentous green alga *Cladophora* with some cells of *Scenedesmus*, and the diatoms *Diatoma* and *Cymbella*. Also present in the green algal mats were *Lyngbya* filaments. Based on this microscopic analysis, cyanobacteria were at relatively high proportions in orange crusts, while eukaryotic algae dominated in the green algal mats (Fig. S3). These results were generally confirmed by DNA profiling: Kapalaoa/Waikoloa ponds with orange crusts had especially high proportions of Cyanobacteria, while Hualalai ponds with green algae tended to have higher proportions of Chlorophyta (Figs. S4 and S5).

Diel surveys show H. rubra is absent during the day in fish-invaded habitats

In nearly all fish-invaded anchialine habitats, *H. rubra* was completely absent during the daytime survey (KAHO54 being the sole exception) but was found in appreciable numbers at night (Fig. 3). Presumably, *H. rubra* had retreated underground during the day in fish-invaded habitats. In contrast, *H. rubra* was present during both daytime and nighttime surveys in fishless habitats, although in two of five comparisons abundances were greater at night in fishless habitats as well (Fig. 3). We found significant effects of all predictors in the mixed-effects model: day vs night, fish present vs absent, and their interaction (chi-square = 643.4, 61.92, and 350.2, respectively; $P < 0.001$ in all cases). There was an estimated 237 (95% CI: 116-

484) and 271 (95% CI: 133-554) individuals/m^2 during day and night, respectively, in habitats without fish, while 1 (95% CI: 0.6-2.1) and 42 (95% CI: 24-73) individuals/m^2 were present during day and night, respectively, in fish-invaded habitats.

Grazing by H. rubra alters microbial abundance in proxy communities

Across grazing treatments of the proxy tile communities seeded with pond algae, chlorophyll *a* concentration decreased over time in the medium and high density treatments, but remained stable or increased in the low and no shrimp treatments (Fig. 4). Chlorophyll *a* concentrations decreased by 0.040 μg cm^{-2} day^{-1} and 0.041 μg cm^{-2} day^{-1} in the medium and high shrimp treatments, respectively, but increased (0.006 μg cm^{-2} day^{-1}) or remained nearly stable (decreasing by 0.005 μg cm^{-2} day^{-1}) in the no and low shrimp treatments, respectively (Fig. 4). The treatment-by-time effect was significant (chi-square = 71.24, $P < 0.001$), with no and low densities having statistically indistinguishable growth rates ($P = 0.41$), medium and high densities having indistinguishable growth rates ($P = 0.997$), but no and low densities differing significantly from medium and high ($P < 0.001$ in each case). Across the 52 day experiment, these differences in among treatments led to dramatically reduced chlorophyll *a* concentrations in the medium and high shrimp treatments (Fig. S6).

In PERMANOVA comparisons, tile communities were distinct from anchialine communities sampled from the field when summing all tiles and field collections together ($P < 0.001$). But like field samples, tile communities had appreciable numbers of cyanobacterial and algal OTUs (Fig. S4 and S5), including many specific OTUs being shared and abundant in both communities. Perhaps relatedly, shipping a "live" crust sample in environmental water over several days caused the microbial community to shift markedly compared with those collected in the field (see Xs in Fig. 2, Fig. S7). Most importantly, *H. rubra* readily grazed on tile communities (Fig. S6, Supplementary movie 1).

We found evidence that grazing by *H. rubra* also altered the composition of tile microbial communities. For both the *Bacteria*-specific V6 and *Eukarya*-biased V9, tiles at the beginning of the experiment were similar to field communities, but they became more dissimilar as the experiment progressed (green circles in Fig. 2, Fig. S8). However, all

PERMANOVA comparisons suggested tile communities were not statistically different at time points up to 24 days into the experiment ($P > 0.170$ for all comparisons).

H. rubra fecal pellets reflect benthic microbial communities

Fecal pellets from *H. rubra* were collected during both the growth rate and laboratory grazing experiments (on Day 7 and Day 33, respectively) and in all cases the microbial communities of fecal pellets were most like the environmental samples on which shrimp were grazing (asterisks in Fig. 2, Fig. S9). In PERMANOVA comparisons, microbial communities of *H. rubra* fecal pellets were always significantly different from gut communities of experimental shrimp ($P < 0.05$) but were often not statistically different from environmental substrate samples. Specifically, *Bacteria*-specific V6 and *Eukarya*-biased V9 data from fecal pellets of the growth rate experiment were more similar to the materials on which *H. rubra* were grazing than to the environment from which the shrimp originated. For example, shrimp grazing on orange crusts had fecal pellets similar to orange crusts regardless of their habitat of origin (see insets in Fig. 2, Fig. S9).

Orange crusts have a lower content of long-chain polyunsaturated fatty acids

Green algae-dominated mats had a much greater content of metabolically important highly unsaturated fatty acids (HUFAs) compared with orange cyanobacteria-dominated crusts, a finding consistent with previous research (reviewed by Twining et al. 2021). Green mats had an order of magnitude greater percentage of the HUFAs arachidonic acid (ARA) and eicosapentaenoic acid (EPA) than orange crusts (Table S2), and 1.6 times the percentage of linoleic acid (LIN). The HUFA alpha-linolenic acid (ALA) content did not differ significantly between the two benthic substrate types (Table S2) and docosahexaenoic acid (DHA) content was negligible in both. Although the differences among substrates for ARA, EPA and LIN are quantitatively large, the low statistical power of sampling from only two or three ponds of each type resulted in differences that were marginally non-significant, while ALA was distinctly non-significant (Table S2). Notably, each of 30 different fatty acids comprised at least 0.5% of total identified from both substrate types (data available via FigShare at DOI 10.6084/m9.figshare.16709632).

446

447 *Orange crusts have larger H. rubra*

448 *Halocaridina rubra* from Kapalaoa/Waikoloa habitats with the orange crusts were
449 significantly heavier ($P = 0.002$), longer ($P = 0.032$), and had higher body condition ($P < 0.001$)
450 when freshly collected from their ponds (and prior to beginning the growth rate experiment)
451 compared with those from Hualalai habitats with green algal mats (Fig. 5A). Substrate biomass,
452 measured as chlorophyll *a* concentration, did not differ significantly between ponds at
453 Kapalaoa/Waikoloa and Hualalai ($P = 0.178$, *t*-test, Fig. S10). Growth rates for *H. rubra*
454 originating from green algal mat habitats were greater than those from orange crust habitats (P
455 $= 0.028$, Fig. 5E), but there was not a significant effect of substrate or an interaction of origin
456 and substrate ($P > 0.684$). Shrimp from the orange crust habitats did not have a growth rate
457 significantly different from zero ($P = 0.360$).

458

459 *H. rubra has a resident gut microbiome*

460 The guts of *H. rubra* had a microbial community distinct from the benthic substrates on
461 which they graze in analyses of both *Bacteria*-specific V6 and *Eukarya*-biased V9 (triangles vs.
462 diamonds or circles in Fig. 2, Figs. S4, S5). Specifically, gut samples formed a unique cluster in
463 nMDS analyses compared with any field or laboratory collected samples ($P < 0.001$ for both, $t =$
464 3.37 and 5.13 for V6 and V9 respectively, PERMANOVA, Fig. 2, S11). As reported previously
465 (Hoffman et al. 2018b), benthic substrates from Hawaiian anchialine habitats are diverse: on
466 average ~1500 Bacteria-specific V6 and 975 *Eukarya*-biased V9 OTUs were identified per
467 sample, respectively. Gut microbiomes from *H. rubra* not only had distinct taxa compared to
468 environmental microbial communities, but were also considerably less diverse than benthic
469 communities, averaging just 361 and 94 OTUs per sample for *Bacteria*-specific V6 and *Eukarya*-
470 biased V9, respectively. From the V6 data, gut bacteria were dominated by the phylum
471 Fusobacteria, except for one wild-caught individual from Kapalaoa Bay dominated by
472 Gammaproteobacteria and one individual from the laboratory grazing experiment that had a
473 high proportion of cyanobacteria (Fig. S4). Guts from *H. rubra* had a diverse micro-eukaryotic
474 community based on the V9 data, including alveolates, Stramenopiles, and Fungi along with
475 various bacterial groups given that our V9 primers do amplify *Bacteria* as well (Fig. S5). Not

surprisingly, most sequencing reads from the *Eukarya*-biased V9 primers were annotated as being from crustaceans and were therefore removed as they were likely from the *H. rubra* gut tissue itself. Dissected guts generally yielded low DNA quantities and a total of 24 samples were excluded based on downstream analyses, particularly those collected from wild-caught individuals and amplified with the *Bacteria*-specific V6 primers. As a general caveat, we point out that different DNA extraction methods were used on shrimp guts and environmental samples, which may have caused some differences between the sample types but likely does not fully explain these results.

H. rubra gut microbiomes are stable across environments, but can be variable among populations

The gut microbiome communities of *H. rubra* were stable over differing environments and across time. For example, wild-caught individuals from HM had presumably grazed only on the native orange crusts in natural habitats, while shrimp from the laboratory grazing experiment originated from HM, but had been grazing on laboratory-grown algae for over two years. Despite this long-term dietary change, no significant difference between wild-caught (n = 2) and laboratory-housed (n = 6) individuals was detected in *Bacteria*-specific V6 using PERMANOVA tests ($P > 0.3$, $t < 1.16$ in all comparisons, "Gut_tile" vs. "Gut_HM" samples in Fig. 2A). Likewise, gut microbial communities were indistinguishable between wild-caught (n = 4) and laboratory-housed (n = 4-8) individuals based on *Eukarya*-biased V9 ($P > 0.1$, $t < 1.08$ in all comparisons, Fig. 2B). Lastly, there was no statistical difference in *H. rubra* gut contents from the microcosm experiment from shrimp that were housed with vs. without grazing substrate ($P > 0.4$, $t < 1.12$ for all).

In general, there were also limited differences in gut microbial communities of *H. rubra* from wild-caught individuals of different populations. While comparisons were not possible using *Bacteria*-specific V6 (see above), the *Eukarya*-biased V9 identified only a single comparison as statistically significant: those from the AC pond at Kapalaoa on Hawaii vs. those from SKIP on Maui (both with orange crusts, $P = 0.032$, $t = 1.48$, Fig. 2B, Fig. S12). All other comparisons were not statistically significant (including those involving gut microbial communities from sites with orange crusts vs. green mats). However, a general divide in gut

506 microbial communities from *H. rubra* populations at Hualalai and Kapalaoa/Waikoloa (i.e., west
507 coast of Hawaii) vs. those from Maui and the south coast of Hawaii is apparent in the NMDS
508 plot (Fig. 2B, Fig. S13).

Discussion

Animal-microbe interactions in the anchialine ecosystem

Across the world, the anchialine ecosystem is home to both endemic macro-organisms and microbial communities. In the Hawaiian Islands, both the atyid shrimp *Halocaridina rubra* and orange cyanobacterial-bacterial crusts are endemic to anchialine habitats. While previous studies have investigated interactions between *H. rubra*, the microbial communities on which they graze, and the invasive fishes that can be predators of the shrimp (Bailey-Brock and Brock 1993; Dalton et al. 2013; Sakihara et al. 2015; Seidel et al. 2016), these have been largely correlative surveys. Furthermore, the specific microbes under investigation have not been identified. It also remains unclear if "top-down" effects from invasive fishes produce a drastic shift from orange cyanobacterial crusts to green algae-dominated communities as previously hypothesized (Bailey-Brock and Brock 1993). Here, we used a combination of DNA profiling of microbial communities, diel surveys, and field and laboratory experiments to gain novel insights into animal-microbe interactions in Hawaii's anchialine ecosystem. Our main findings are that grazing by *H. rubra* shapes the abundance and composition of microbial communities, but while invasive fishes cause shrimp to graze only at night, this may not lead to drastic shifts in microbial community structure. Microbial communities may also affect shrimp populations: shrimp from orange crust habitats were in better condition, but grew more slowly than those from green mat habitats. Finally, we provide an initial description of the *H. rubra* gut microbiome, which is stable across space and time and may be largely independent of environmental microbial communities.

Results from our microcosm experiment (Figs. 2 and 4) demonstrate that grazing pressure from ecologically relevant densities of *H. rubra* alters the biomass of microbial communities. Even in the low shrimp treatment, there was a trend for lower chlorophyll *a* concentration at the end of the experiment compared with the no-shrimp control, where chlorophyll *a* concentration increased due to algal growth (Fig. 4). These trends are consistent with those of a field-based experiment in Hawaii (Sakihara et al. 2015) where artificial agar plates were deployed in multiple anchialine habitats and colonized by natural microbiota. Similar to our results, there was a clear pattern of lower chlorophyll *a* concentration in the presence of grazing shrimp. Our results and those of Sakihara et al. (2015) complement each

other in showing that grazing reduces epilithon biomass both in nature and in the laboratory when shrimp density is controlled.

Shrimp grazing also likely alters the composition of microbial communities. Sakihara et al. (2015) showed that grazed communities had higher autotrophic index (AI) and C:N ratios, appeared more diverse (based on microscopic analyses), and were green compared to the ungrazed communities, which were brown and composed largely of diatoms. This aligns with the idea that atyids may act as "gardeners" by promoting the growth of algae as a "crop" (De Souza and Moulton 2005; Stimson 1970). Stable isotope analyses of *H. rubra* are also consistent with this, as shrimp appear to incorporate carbon derived from algae (Capps et al. 2009). Similarly, Dalton et al. (2013) documented greater AI and a markedly higher C:N in ponds lacking fish and with higher densities of shrimp. Our data also support this, although not statistically confirmed, as both the *Bacteria*-specific V6 and *Eukarya*-biased V9 communities in the grazing experiment consistently changed over 24 days (Fig. 2, Fig. S8). Taken together, these results suggest *H. rubra* exerts selection on microbial community composition via grazing, either by selective consumption, physical disturbance by shrimp feeding, discouraging other grazers, or other mechanisms. One intriguing possibility is that shrimp grazing promotes algal growth while preventing conditions favoring diatom growth, as demonstrated for some detritivore fishes (Flecker 1996).

It is possible, given that *H. rubra* grazing can alter the abundance and composition of benthic microbial communities, that altered grazing by shrimp in fish-invaded habitats may lead to a shifts from orange crusts to algae-dominated communities (Bailey-Brock and Brock 1993). In fish-invaded ponds shrimp grazing is greatly reduced, because the shrimp largely avoid predation risk by retreating underground during the day and emerging at night to graze when fish are not actively feeding (Capps et al. 2009; Carey et al. 2011), although predation can still occur in at least some habitats (Havird et al. 2013). The diel surveys we report here further support this behavior pattern and extend it to additional anchialine habitats across the Hawaiian Islands (Fig. 3).

Because *H. rubra* continues to graze at night, altered shrimp grazing by invasive fishes may not shift orange crust communities to green mat communities, as previously hypothesized (Bailey-Brock and Brock 1993; Capps et al. 2009; Carey et al. 2011). On the other hand, even

though shrimp may largely avoid predation through diel migration, community shifts could still be possible due to altered shrimp behavior via the "ecology of fear" (Brown et al. 1999; McIntosh et al. 2004). Here, we only included environmental substrate samples from orange crust habitats that lacked fish at the time of sampling (one pond at Kapalaoa Bay did have a few fish, but shrimp were still present during the day). In a previous DNA profiling study using the same rRNA markers, fish-invaded orange crust communities from Hawaii (e.g., MAKA3 and KBI1) were included with the fishless PB habitat examined here (Hoffman et al. 2018b). In that study, PB had very similar microbial communities to fish-invaded habitats (it fell in between them in NMDS analyses), suggesting differences between the orange crusts may be due to geography or other site-specific environmental factors and not necessarily fish invasion. Moreover, many fish-invaded habitats in West Hawaii (i.e., Kapalaoa/Waikoloa, MAKA, KBI, KAHO) have retained their orange crusts despite being fish-invaded for ten or more years. Unfortunately, fishless anchialine habitats on Hawaii's Kona coast are becoming increasingly rare (e.g., most Kapalaoa/Waikoloa ponds are now invaded), owing to alien poecilids like *Gambusia* spp. being some of the most globally invasive species in aquatic ecosystems, especially in Hawaii (Holitzki et al. 2013). While this makes directly comparing fish-invaded vs. fishless habitats difficult, the hypothesis that fish invasion drives drastic changes in benthic epilithon communities is likely an oversimplification, given that fish-invaded habitats have similar microbial communities to those without them and can retain the orange crusts for many years post-invasion.

H. rubra was abundant in anchialine habitats with both orange cyanobacterial-bacterial crusts and green algal mats. Our experiments confirm that shrimp readily graze on both substrate types and either grow rapidly or maintain body weight when fed either in captivity (Fig. 5). These results are intriguing because it is well established that cyanobacteria generally lack metabolically critical ω-3 and ω-6 long-chain polyunsaturated fatty acids (LCPUFA) and for that reason are nutritionally poor food for consumers, including aquatic crustaceans (e.g., Brett and Muller-Navarra 1997; Twining et al. 2021). Consistent with these broad patterns, we found the concentrations of the ω-3 LCPUFA EPA and the ω-6 LCPUFA ARA were an order of magnitude greater in green mats than orange crusts (Table S2). However, both substrate types were found to have a high content of ALA and LIN, the precursors of EPA and ARA, respectively

(Table S2). The FA content of orange crusts, though much lower in EPA and ARA than green mats, is apparently sufficient to support healthy shrimp populations, possibly because algal productivity is high in orange crusts despite low stocks. Furthermore, green mats and orange crusts, while dominated by green algae and cyanobacteria, respectively, are also not monocultures (Figs. S3-5). For example, orange crusts also contained green algae and diatoms in appreciable abundances. Alternatively, *H. rubra* may be physiologically capable of converting precursors ALA to EPA, and/or LIN to ARA, as is the case for some animals (Twining et al. 2021), including atyids and other crustaceans (Lau et al., 2009; Twinning et al. 2017).

At the start of the growth experiment, *H. rubra* from green mat habitats were significantly smaller and in lower condition than those from orange crust habitats (Fig. 5A), and yet the shrimp from green mat ponds were able to grow well during the experiment in the presence of either substrate. In contrast, the larger and better condition shrimp from orange crust habitats did not grow, but maintained body condition on both substrates. One possibility may be that as crustaceans, *H. rubra* has either determinate growth or a greatly reduced increase in body mass as adulthood is approached (Maszczyk and Brzezinski 2018). It may be, then that at the time of our growth study younger *H. rubra* occurred in the specific habitats with green mats while older individuals occupied those with orange crust habitats examined here, explaining why the latter exhibited no growth in our experiments. In fact, the Tōhoku/Fukushima tsunami of 2011 reached Hawaii Island and filled near-shore anchialine pools at the Hualalai site with ocean water containing fish that subsequently excluded *H. rubra* from the affected habitats. Following fish removal, juvenile *H. rubra* dominated, and these juveniles very likely had not matured by the time of our study in 2013 (D. Chai, pers. obs.) Assigning individual *H. rubra* to particular anchialine habitats is difficult in any case because shrimp move freely belowground and may emerge in different ponds. *H. rubra* apparently reproduces exclusively in the higher salinity waters found underground (Havird et al. 2015) and populations at Kapalaoa/Waikoloa and Hualalai share mitochondrial cytochrome oxidase subunit I (COI) haplotypes (Craft et al. 2008; Santos 2006), suggesting movement between habitats may be common despite their separation by ~15 km.

Our work and previous studies suggest complex "top-down" and "bottom-up" processes influence overall community composition in the Hawaiian anchialine ecosystem

(Bailey-Brock and Brock 1993; Capps et al. 2009; Carey et al. 2011; Dalton et al. 2013; Dudley et al. 2017; Sakihara et al. 2015; Seidel et al. 2016). Our microcosm grazing experiment and that of Sakihara et al. (2015) provide clear evidence that grazing by *H. rubra* alters microbial communities, while Dalton et al. (2013) found ponds with higher nutrient inputs supported more microbial and shrimp biomass. However, extending these experiments in the future may prove difficult, given the challenges of bringing *bona fide* anchialine microbial communities into the laboratory. Sakihara et al. (2015) deployed artificial agar plates into anchialine habitats that underwent colonization during their experiment, but it is unclear how these communities reflect natural ones. Excluding shrimp from areas within natural, low-salinity ponds using electrical fields (Lourenço-Amorim et al. 2014) or combining field-deployed tiles/plates with DNA profiling may be a useful method to further investigate the effects of shrimp grazing on microbial communities.

H. rubra has a stable resident gut microbiome

While crustaceans are among the most ecologically diverse invertebrates, inhabiting marine, freshwater, and terrestrial habitats, the microbial communities associated with them are only just beginning to be examined, and primarily for commercially important species. While communities from hemolymph, skin, and hepatopancreas have been described, gut microbiomes have been the most extensively characterized (Gil-Turnes et al. 1989; Jung et al. 2021; Wang and Wang 2015). Crustacean gut communities can change during development, under different disease states, and with environmental fluctuations (Cicala et al. 2020; Cornejo-Granados et al. 2017; Gainza et al. 2018). It has also been hypothesized that these communities play a major role in the ability of crustaceans to colonize new habitats (Cannicci et al. 2020). For example, true marine isopods lack a substantial intestinal microbiome while terrestrial isopods digest leaf litter *via* gut microbes (Zimmer et al. 2001). However, some have suggested that most crustaceans lack persistent gut microbiomes because of their peritrophic matrix, a chitinous sheath continuously excreted from the midgut epithelium that surrounds microbes and ingested material, potentially preventing the establishment or stability of gut microbial communities (Martin et al. 2019).

Here, we show for the first time that *H. rubra* possesses a unique gut microbiome when compared with the environmental microbial communities on which it grazes (Fig. 2, Fig. S1). While most animals possess gut microbes that aid in digestion (Russell et al. 2014; Sommer and Bäckhed 2013), some arthropods do seem to lack a resident gut microbial community (Hammer et al. 2017). The *H. rubra* gut microbiome also appears to be highly stable (although sample sizes were small for some comparisons), as we detected no difference between gut communities of wild-caught shrimp and those from the same habitat but maintained in the laboratory for over two years (Fig. 2, Fig. S11). Microbiomes of freshly collected animals from orange crust and green mat ponds were also similar (Fig. 2). On a shorter timescale, this microbiome was not significantly altered when shrimp were provided, or denied, substrate in our grazing experiment (Fig. S4, S5). While gut microbes are commonly vertically or horizontally inherited, they are also usually contingent on the environment (Grieneisen et al. 2021). For example, gut microbiomes in freshwater copepods shifted based on their food source in laboratory experiments (Eckert et al. 2021) and cultured whiteleg shrimp have different microbiomes than wild-caught individuals (Cornejo-Granados et al. 2017). However, some crustaceans apparently have stable gut microbiomes, like *H. rubra*, including the intertidal isopod, *Pentidotea resecata,* whose gut microbiome was unaltered when fed three different macrophyte diets (Gustafson 2020). Similarly, farmed and wild-caught mud crabs also have comparable gut microbiomes (Apine et al. 2021). Future studies exploring the effect of other environmental factors on the *H. rubra* gut microbiome and whether they are transmitted across generations would aid in assessing their stability. Larvae of *H. rubra* are lecithotrophic (i.e., yolk-bearing), making the vertical transmission of a stable gut community from mother to offspring a possibility. The potential coevolution between these shrimp and symbiotic microbes that may aid in digestion is an intriguing direction for future work.

The gut microbiome of *H. rubra* is not particularly diverse and appears to contain a few "core" members. Of the few hundred OTUs per gut identified using *Bacteria*-specific V6, only 9 were present at an abundance of 1% or higher when averaged across all samples, and just 5 of these were found in all samples. These core OTUs included Gammaproteobacteria (i.e., Vibrionaceae, Pseudoalteromonadaceae, Oceanospirillales, and Alteromonadaceae) and one Cyanobacteria (*Synechococcus sp*.). However, a single bacterial species dominated the *H. rubra*

688 gut bacterial microbiome, accounting for 62% of gut V6 reads overall: the fusobacterium
689 *Cetobacterium somerae*. Originally isolated from human feces (Finegold et al. 2003), *C. somerae*
690 is now known to be a major component of gut microbiomes in freshwater fishes, and can also
691 produce vitamin B12 (Larsen et al. 2014; Tsuchiya et al. 2008). Although *C. somerae* was
692 relatively rare in the wild shrimp collected from Kapalaoa/Waikoloa and one individual from the
693 laboratory grazing experiment, it made up the vast majority (82%) of the V6 reads from the
694 other shrimp gut samples.
695 Similar to *Bacteria*-specific V6, *Eukarya*-biased V9 gut communities were not particularly
696 diverse, containing only a few OTUs found at appreciable abundances in multiple gut samples
697 from *H. rubra*. Only 21 OTUs averaged at least 1% abundance across all samples (ranging from
698 1%-5%), and no OTU was found in all gut samples. The most abundant OTUs were from animals
699 (nemerteans), fungi (Saccharomycetes), Alveolates (apicomplexans, ciliates, and
700 dinoflagellates), Stramenopiles, chlorophytes, charophytes, and uncultured marine eukaryotes.
701 However, no group was particularly common across samples, suggesting that unlike the
702 fusobacterium *C. somerae*, *H. rubra* lacks core eukaryotic members in its gut microbiome and
703 these may instead represent grazed items (at least in samples where guts were not cleared, see
704 below) or opportunistic commensal organisms. Some abundant OTUs also likely represent
705 environmental DNA, including ones identified as being from fishes, possibly from the ingestion
706 of mucus by *H. rubra*.
707 Our analyses of *H. rubra* fecal pellets from the growth rate experiment is consistent with
708 grazing having little effect on the shrimp gut microbiome. Fecal pellets were always a passive
709 reflection of the communities on which they grazed (Fig. 2, Fig. S9), suggesting ingested
710 microbes are either digested or passed without colonizing the gut. However, fecal pellets did
711 remain in the experimental chambers for up to a week before being removed, thus some of the
712 similarities to environmental samples may be due to colonization after being passed. Notably,
713 pellets from treatments with orange crusts were always pale yellow, while those from
714 chambers with green algal mats were always dark green, even when first passed. This suggests
715 that at least some microbes (or their DNA) pass somewhat intact through the digestive track of
716 *H. rubra*.

Our initial survey of the *H. rubra* gut microbiome raises several questions. For example, do different shrimp populations have different gut microbial communities? Because gut microbes can be vertically inherited, different genetic lineages of the same species could possess different microbial communities (Macke et al. 2017), possibly as a result of coevolution and co-speciation (Moran and Sloan 2015). Here, we examined shrimp gut microbiomes from 9 sites representing two genetic lineages of *H. rubra* (identified through mitochondrial COI; Craft et al., 2008) and while gut communities from Waikoloa/Kapalaoa-Bay and Hualalai were somewhat different than others (Fig. 2, Figs. S12-S13), these differences do not correspond to the described genetic lineages. For example, all sites from Hawaii examined here are inhabited by members of the same *H. rubra* COI lineage (West Hawaii, Craft et al., 2008) but the gut microbiome of those from PB grouped with those from Maui (South Maui lineage) to the exclusion of other Hawaii samples (Fig. 2, Fig. S13). One explanation for why guts from Waikoloa/Kapalaoa-Bay and Hualalai appeared different may be because shrimp from these habitats were cleared of gut contents before sampling and may therefore be more reflective of a true resident gut microbiome, while the same was not feasible for the other sites. Thus, a more thorough and consistent sampling of *H. rubra* from across the islands is required to further explore co-segregation and discordance between shrimp populations and their gut microbiomes.

We also found that amplifying microbial communities from *H. rubra* digestive tracks was not straightforward. Given their small size (~5-8 mm), dissecting an intact digestive track while preventing contamination from other tissues and obtaining enough material for sequencing is a difficult task. We initially tried to physically separate gut contents from the shrimp digestive track itself, but this proved impossible. We therefore elected to exclude crustacean OTUs bioinformatically, but this resulted in discarding large quantities of data (up to 99% in some cases). Future studies should consider pooling guts from the same habitat or treatment to more reliably obtain sufficient DNA quantities for sequencing while primarily focusing on prokaryotic organisms in the process.

Conclusions

Overall, our analyses demonstrate that grazing by *H. rubra* can drive the abundance and composition of environmental microbial communities in the Hawaiian anchialine ecosystem. Shrimp populations and these habitats, in general, are threatened by coastal development, invasive species, and climate change (Bailey-Brock and Brock 1993; Marrack 2016). While drastic community shifts from orange crusts to green mats may not be driven solely by the presence of invasive fishes (e.g., increased nutrients may play a large role in these shifts; Dalton et al. 2013), altered patterns of shrimp grazing will most certainly affect benthic microbial communities. On the other hand, the type of microbial community in a habitat may have relatively small effects on shrimp growth rates or gut microbiomes. While shrimp from orange crust habitats were larger, shrimp grew well on green mat communities. Shrimp gut microbiomes also appear to be structured largely independently of the environmental microbial community. This may help explain why *H. rubra* is a common and key feature of Hawaiian anchialine habitats regardless of the location, size, or microbial makeup of the particular habitat.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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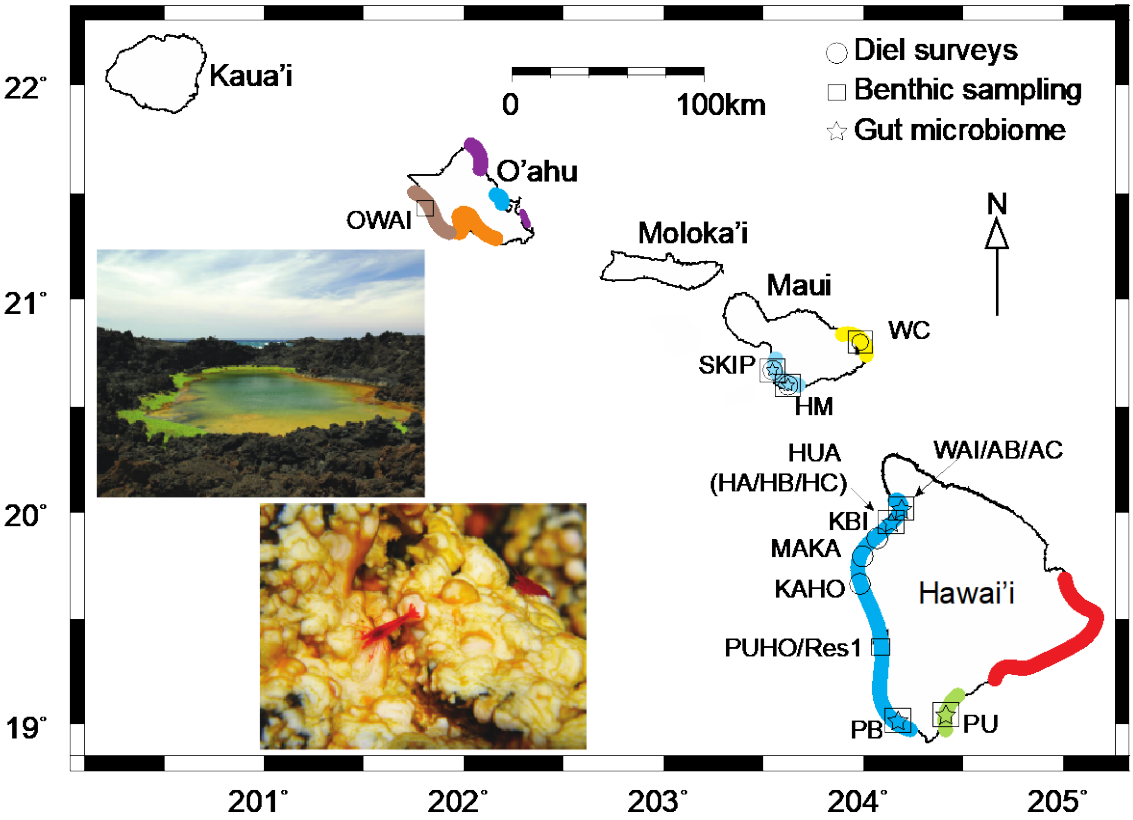
952 Figures and captions



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954 Fig 1. Map of anchialine habitats across the Hawaiian Islands examined here. Site symbols
955 indicate: circles – sites where diel surveys of shrimp abundances were conducted, squares –
956 sites sampled for benthic microbial sequencing, and stars – sites where shrimp gut microbiomes
957 were sequenced. Colors along the coastlines represent approximate distributions of different
958 *Halocaridina rubra* genetic lineages (Craft et al. 2008) as identified by mitochondrial DNA.
959 Photo inserts show a habitat (SKIP) with the orange crust and a close-up of *H. rubra* grazing on
960 the crust (courtesy of D.A. Weese).

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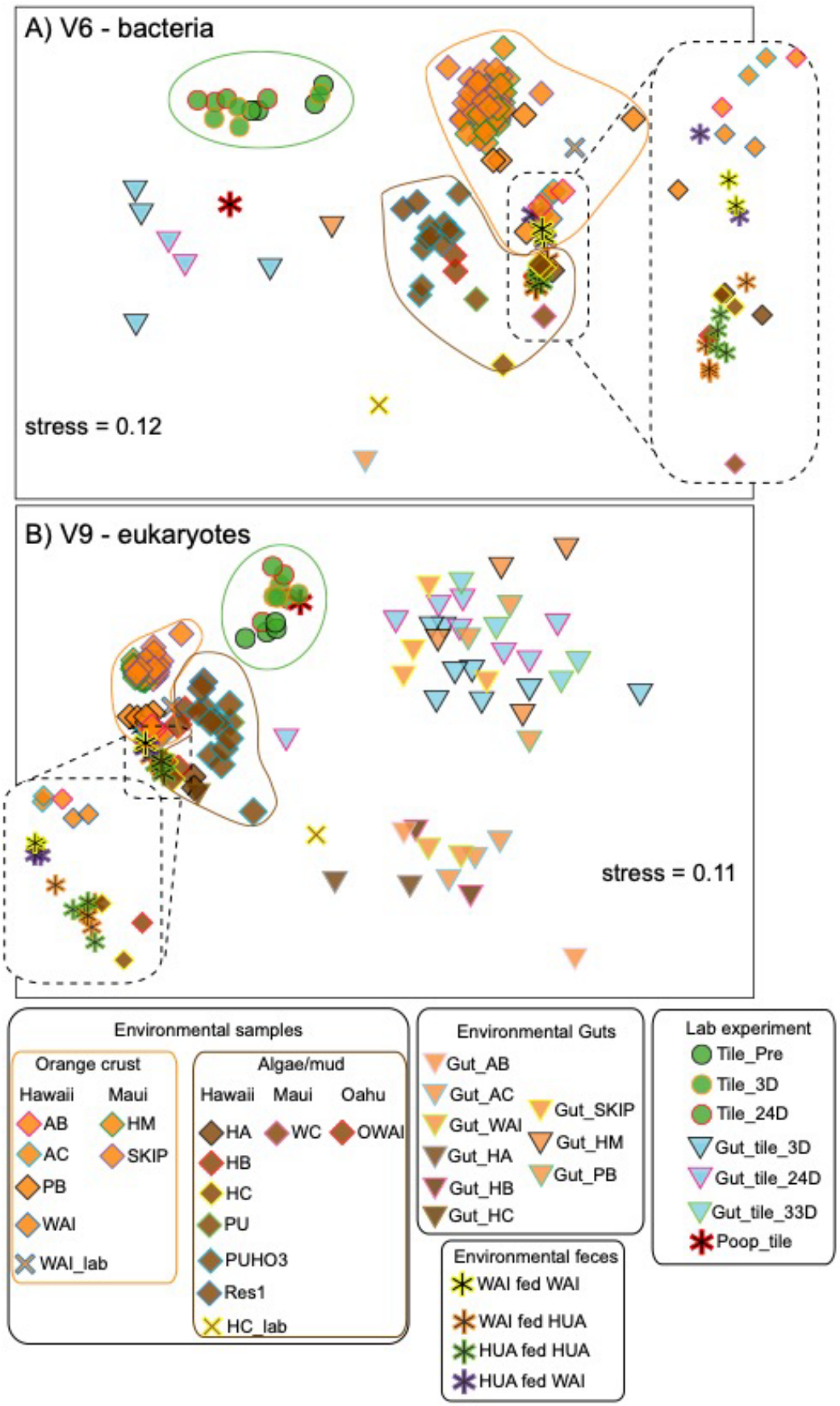
A) V6 - bacteria
stress = 0.12
B) V9 - eukaryotes
stress = 0.11
Environmental samples
Orange crust
Hawaii
Maui
AB
AC
PB
WAI
WAI_lab
HM
SKIP
Algae/mud
Hawaii
Maui
Oahu
HA
HB
HC
PU
PUHO3
Res1
HC_lab
WC
OWAI
Environmental Guts
Gut_AB
Gut_AC
Gut_WAI
Gut_HA
Gut_HB
Gut_HC
Gut_SKIP
Gut_HM
Gut_PB
Environmental feces
WAI fed WAI
WAI fed HUA
HUA fed HUA
HUA fed WAI
Lab experiment
Tile_Pre
Tile_3D
Tile_24D
Gut_tile_3D
Gut_tile_24D
Gut_tile_33D
Poop_tile

962

963 Fig. 2. NMDS ordination using the Binary Jaccard dissimilarity index of samples grouped by
964 origin and analyzed in this study. Samples were generated with A) *Bacteria*-specific V6
965 hypervariable region of the *16S-rRNA* and B) *Eukarya*-biased V9 hypervariable region of the
966 *18S-rRNA* genes. Samples from habitats with orange cyanobacterial-bacterial crusts are colored
967 orange, those from algal or mud communities are brown, and those from the laboratory
968 microcosm grazing experiment are green and blue. Environmental benthic microbial samples
969 represented as diamonds, *Halocaridina rubra* gut samples as triangles, *H. rubra* fecal pellets as
970 stars, proxy microbial communities from tiles as circles, and environmental benthic samples
971 shipped to the laboratory in water prior to DNA extraction indicated with Xs. Insets with dashed
972 lines are blowup of tightly clustered samples to increase clarity.
973

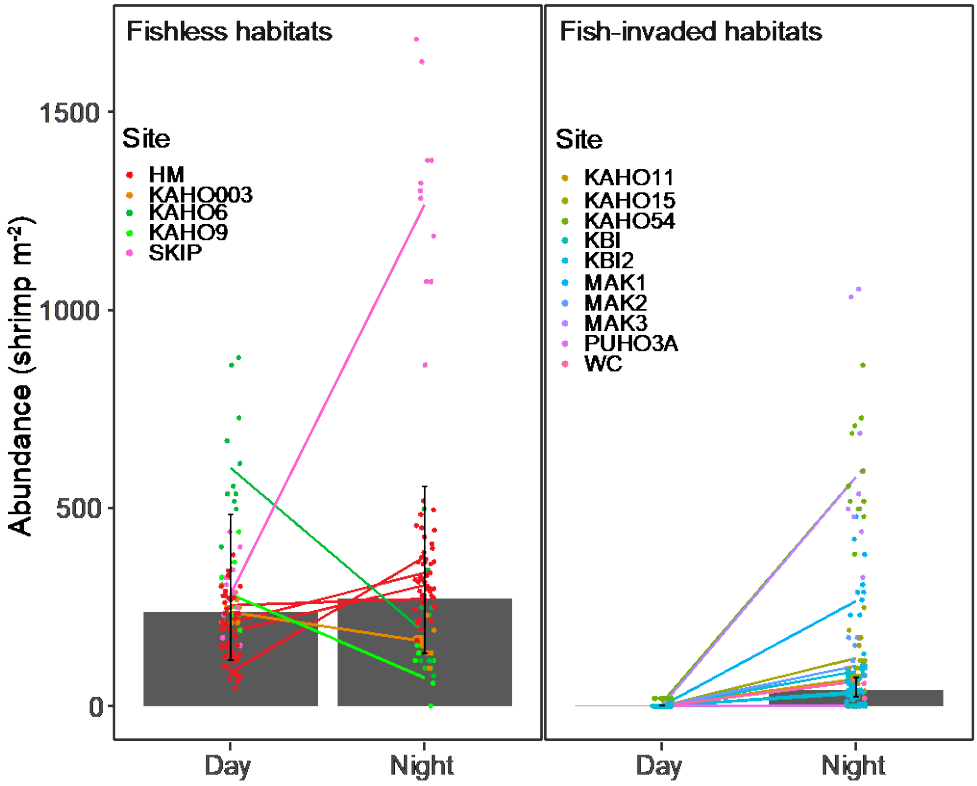

Fig. 3. Diel surveys of *Halocaridina rubra* from fishless and fish-invaded anchialine habitats. Bars show estimated abundance from the best-fitting generalized linear mixed model ± 95% confidence interval (log-normal distribution, random effect of quadrat to account for repeated measures). Points represent individual observations, colored by site. Lines connect the average for day and night at each site (calculated on a log scale to be consistent with model fitting). Time (day or night), fish (present or absent), and their interaction were all significant (chi-square test, $P < 0.001$ in all cases). For HM and KBI2, 4 locations were surveyed within a single habitat. WC refers to the "open" portion of the cave (Havird et al. 2013). N = 11 per sampling time and location.

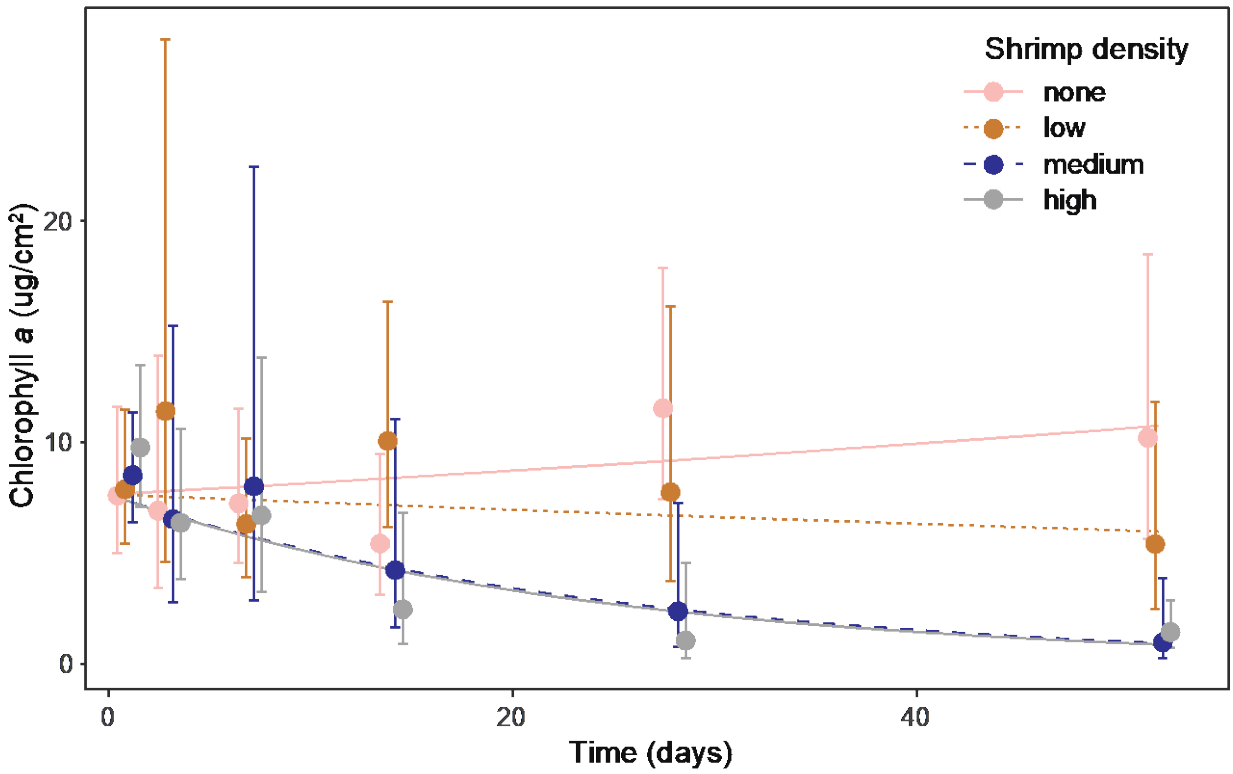

Fig. 4. Chlorophyll *a* concentration of proxy microbial communities from tiles in the laboratory microcosm grazing experiment after variable densities of *Halocaridina rubra* were allowed to graze for up to 52 days. Lines represent prediction of exponential growth/decline from the best fitting linear mixed model (log abundance as response, separate slope for each treatment, same intercept for all treatments). Points and error bars show the mean and +/- 1 standard deviation of the raw data (calculated on a log scale to be consistent with model fitting). Data from initial measurements and measurements on day 2 are excluded for visual clarity. Rate of change in "none" and "low" treatments differed significantly from "medium" and "high" treatments ($P < 0.001$ in all cases), but "none" and "low" did not differ from each other ($P = 0.412$), and neither did "medium" and "high" ($P = 0.996$) (Tukey's Honest Significant Difference test). $N = 6$ per time and treatment.

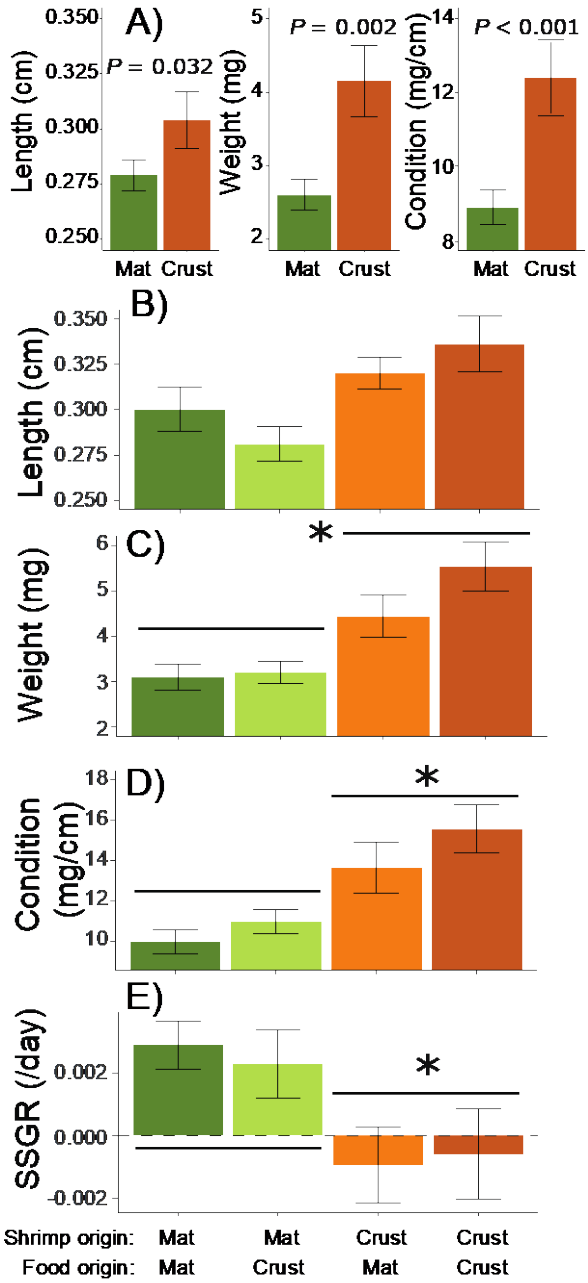

Fig. 5. Characterization of *Halocaridina rubra* during the growth experiment. A) Prior to the growth experiment, shrimp from the orange crust habitats were longer, heavier, and in better condition. At the end of the 61 day growth experiment, there was no statistical difference among treatments (i.e, shrimp from either habitat type fed either substrate) in A) length ($P > 0.156$) although shrimp from orange crust habitats were B) heavier ($P < 0.001$) and C) in better condition ($P = 0.001$) than those from green mat habitats at the end of the experiment. D) Shrimp from habitats with green mats grew more (as calculated by specific somatic growth rate

1006 [SSGR with units day^{-1}]) than those originating from orange crust habitats (P = 0.028) (significant
1007 effects of habitat origin are shown with asterisks). No effect of food type or the interaction
1008 between food type and habitat origin was detected in any of the three measured traits. N = 4-5
1009 for A) and N = 14-15 for B)-D). Means ± SEM are shown.
1010