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GENETIC LINEAGE AND ENVIRONMENTAL CONDITIONS AS DRIVERS OF CHROMATOSOME VARIATION IN THE ANCHIALINE SHRIMP *HALOCARIDINA RUBRA* HOLTHUIS, 1963 (CARIDEA: ATYIDAE)

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ABSTRACT

Color variation among and within crustacean species is primarily due to morphological and physiological differences in specialized cells called chromatophores. Within Atyidae, individuals of *Halocaridina rubra* Holthuis, 1963, which is endemic to anchialine habitats (coastal ponds of mixohaline water under marine and terrestrial influences) of the Hawaiian Islands, range from bright red to nearly translucent in color. While *H. rubra* encompasses (at least) eight genetically diverged lineages based on analyses of mitochondrial genes, whether this phenotypic variation in coloration correlates to genetic background or if it statistically differs between lineages is unknown. Furthermore, if such properties in *H. rubra* exhibit plasticity under the environmental extremes that can be encountered in anchialine habitats has not been determined. Using previously published protocols for another atyid species, chromatosomal morphology and chromatic properties were quantified and statistically analyzed from 240 individuals of *H. rubra*, representing seven of the eight genetic lineages as well as one naturally mixed population ($n = 30$ per group), maintained under standardized laboratory conditions for >6 years. Analogous analyses were also conducted on animals before and after exposure to environmental extremes, including anoxia, desiccation, decreased salinity and high and low temperatures. Overall, the genetic lineages of *H. rubra* statistically differed from one another in varying numbers of chromatosomal properties while exposure to environmental extremes also elicited significant changes. Furthermore, these properties statistically differed among body parts of *H. rubra* in a manner inconsistent with previous studies. Notably, data on chromosomal properties generated via a streamlined protocol were well correlated with those from the previously published approach utilized here, suggesting such analyses for *H. rubra* (or other crustacean/atyid species) might be successfully conducted under field settings.

KEY WORDS: atyid, color variation, coloration, crustacean, methodology

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INTRODUCTION

Variation in external color and appearance, as well as the ability to change such properties, has evolved repeatedly among organisms (Thayer, 1909; Sumner, 1935; Cott, 1940; Rothschild, 1975) as mechanisms for eluding predators (Turner, 1961; Endler and Herrenkind, 1978), attracting mates (Zahavi, 1975; Farr, 1976), and trapping prey (Craig and Ebert, 1994; Hauber, 2002; Hoese et al., 2006). For example, marine benthic invertebrates such as cephalopods are especially adept at changing their color and patterning (Hanlon and Messenger, 1998; Sotka, 2005; Bandaranayake, 2006). Another group having similar abilities are the crustaceans, where these dynamic changes are primarily utilized for camouflage (Detto et al., 2008). In this context, both physiological and morphological processes are involved, with the former producing rapid (in minutes) transformations in coloration (Detto et al., 2008) by the movements of pigments within stellate cells called chromatosomes and the latter from longer-term (in days to years) and selective increases in particular types of chromatosome pigments (Robinson and Charleton, 1973). Typically, crustacean chromatosomes span approx. 200 μm and may exhibit one, or a variety of, pigments and color combinations (Boyle and McNamara, 2006).

For the majority of crustaceans, chromatosomes are comprised of tightly bound groups of 2-12 uninucleate chromatophores located under the exoskeleton (Bauer, 1981) and chromatosomal color changes are mediated via aggregation and dispersion of these cells (Fingerman and Tinkle, 1956). Within a given chromatophore, color variation is due to centripetal or centrifugal diffusion of pigment granules in the cytoplasm (McNamara, 1981) and rapid changes occur through an active motor response (Sutherland et al., 2008). Although chromatosomes have been described across a wide breadth of crustaceans (McNamara, 1981; Thurman, 1988; Bauer, 2004), the specifics of their properties for a single crustacean species in the context of encompassing multiple genetic lineages and varying environmental conditions have not been investigated.

Atyidae are important members of nearly all temperate and tropical fresh, as well as some brackish, water ecosystems (Bailey-Brock and Brock, 1993; Pringle et al., 1993; Pringle and Blake, 1994). One such example is the anchialine ecosystem, defined as coastal land-locked ponds, pools,

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and caves with subterranean connections to both freshwater aquifers and the ocean (Holthius, 1973; Sket, 1996). Thus, anchialine habitats can experience frequent and large fluctuations in environmental parameters such as salinity and temperature due to tidal cycles (Maciolek, 1986), with some considering them "extreme environments" (Jaume et al., 2009; Bishop and Iliffe, 2012). In the Hawaiian anchialine ecosystem, the small (approx. 10.0 mm as adults) endemic atyid *Halocaridina rubra* Holthuis, 1963 is the most common, widespread macro-organism and is considered a keystone grazer (Bailey-Brock and Brock, 1993). As the species name implies, the color of *H. rubra* is typically a bright red, so vibrant in fact, that shrimp can transform the appearance of water and benthic surfaces of an anchialine habitat when large numbers of individuals aggregate together (Bailey-Brock and Brock, 1993). Population analyses of *H. rubra* using mitochondrial genes such as cytochrome oxidase subunit I (COI) have identified (at least) eight genetically diverged (approx. 0.05 uncorrected *p* distance) lineages across the Hawaiian Islands, with each having a highly restricted and non-overlapping geographic distribution (Santos, 2006; Craft et al., 2008; Santos and Weese, 2011). Notably, while individuals from laboratory colonies of these lineages can exhibit the bright red color typical of the species, phenotypes within a lineage may also range from intermediates to nearly translucent (Fig. 1A). Furthermore, observations made between the lineages identified some as potentially possessing more coloration (Fig. 1B) relative to others (Fig. 1C), implying a relationship between chromatosomal properties and evolutionary genetic background. In this case, it remains unclear if this apparent variation is due to quantifiable differences in chromatosomal properties and whether such properties statistically differ between the genetic lineages of *H. rubra*. Along with this, whether, and the level to which, individual shrimp belonging to the same lineage exhibit plasticity in chromatosomal properties under the environmental extremes that can be encountered in anchialine habitats has yet to be determined.

In this study, the morphologic and chromatic properties of chromatosomes were quantified across seven genetic lineages, as well as one mixed population of two lineages, of *H. rubra* from colonies maintained under standardized laboratory conditions for >6 years. Along with this, chromatosome properties were quantified under anoxia, desiccation, decreased salinity, and high and low temperature, which represent situations that can be encountered in anchialine habitats in Hawaii and elsewhere (Maciolek, 1986; Sket, 1996; Seymour et al., 2007; Pohlman, 2011) and are conditions that can alter coloration in crustaceans (Detto et al., 2008). Here, chromatosomal properties were measured using established protocols from another atyid species (Flores and Chein, 2011) to test the hypotheses that: 1) morphologic and chromatic properties of chromatosomes differ statistically between the genetic lineages, and 2) extremes in the investigated environmental conditions elicit plasticity among properties in individual shrimp belonging to a single genetic lineage.

Materials and Methods

Laboratory Colonies of *H. rubra*

Halocaridina rubra were collected in Summer 2006 from eight different localities in the Hawaiian Islands (Fig. 2; Craft et al., 2008). These collections represent seven of the eight described genetic lineages within the species, with one (KBP) comprising a natural mix of two different lineages: the South (EWA) and West Oahu (OWAI) lineages (Craft et al., 2008). Animals were shipped to Auburn University, AL within ~2 days of collection. In the laboratory, shrimp were housed in separate 38 l aquaria under common conditions, including salinities maintained at 15‰, no circulating water or feeding, and minimal light exposure, i.e., darkness except for 12 hour light/dark cycles for one week every month. Shrimp were provided with baskets of porous volcanic rock to provide shelter as well as surface area for grazing on algae and microbes. This husbandry technique has resulted in consistent reproduction over the past about 8 years, with population sizes maintained at about 200 individuals per aquaria by periodical removal of larvae to additional aquaria.

Animal Acquisition and Acclimation

Data collection for this study began in June 2012, approx. 6 years following establishment of the *H. rubra* laboratory colonies. To minimize handling stress when collecting animals, plastic traps baited with *Spirulina*-tablets were placed in the established aquaria. After 2-3 hours, traps were emptied into individual 40 ml beakers and re-set until >30 shrimp per aquaria were acquired. Prior to being photographed, the acclimation procedures outlined in Flores and Chein (2011) were followed to allow for comparable pigment dispersion due to illumination (Ribeiro et al., 2004). Specifically, these acclimation procedures consisted of illumination by a fluorescent bulb (Model CL-151 65 W/46K, General Electric) monitored with a digital light meter (Model LI-189, Li-Cor) for 10 minutes before individual shrimp were photographed.

Image Capture and Analysis

Each shrimp was viewed with a light microscope (Model MX4300, Meiji Techno) at 4× magnification and images captured with an attached digital camera (Canon Rebel T2i DS126271), with individual *H. rubra* being placed laterally on glass slides, pereiopods facing out from the microscope and the rostrum pointing right. Shrimp were positioned using a disposable plastic transfer pipette and immobilized by wicking excess water away with a Kimwipe. No cover slips were used, as preliminary trials utilizing them resulted in mortality. Three images, on average, were acquired at various foci, allowing selection for a single high quality (in focus) image for analysis. Image acquisition duration was limited to 40 seconds to avoid handling stress (after Flores and Chein, 2011) and illumination settings on the microscope were kept at the same intensity for all photographs.

Photographs were analyzed with the NIS Elements package (AR 3.0, SP 7, Hotflix 8, Build 548, Laboratory Imaging-Nikon) using both Red, Green and Blue (RGB), and Hue, Saturation and Intensity (HSI) color spaces. For images of either the branchial region of the carapace or pleuron of the second pleomere, a circular domain of 1.0 mm^2 was centrally defined within the body part while a similarly positioned domain of 0.5 mm^2 was used for the exon of the uropod, which is smaller in area. The manual selection tool was first utilized to select all individual chromatosomes within the circular domain to measure their density and average area (see below). This was followed by detection of all chromatosomes within a circular domain via automatic thresholding, i.e., where an intensity value T for each pixel in an image is generated and either classifies a given pixel to an object or background point (Ridler and Calvard, 1978) based on color properties. Automatic thresholding was also utilized to measure the specific chromatosomal properties detailed below.

Chromatosomal Properties

The nine chromatosomal properties measured in this study mirror those of Flores and Chein (2011), including cell area and density, cell circularity, area fraction, hue, chroma (three (3) values) and brightness. These are defined as:

Cell density – number of chromatosomes in a 1.0 mm^2 area. Data from the 0.5 mm^2 domain for the exon of the uropod were multiplied by two for standardization to that of the other two measured regions.

Cell area – average area (in μm^2) of each chromatosome.

Cell circularity – average shape circle factor of each chromatosome, which characterizes the proximity of the chromatosome to an ideal circle.

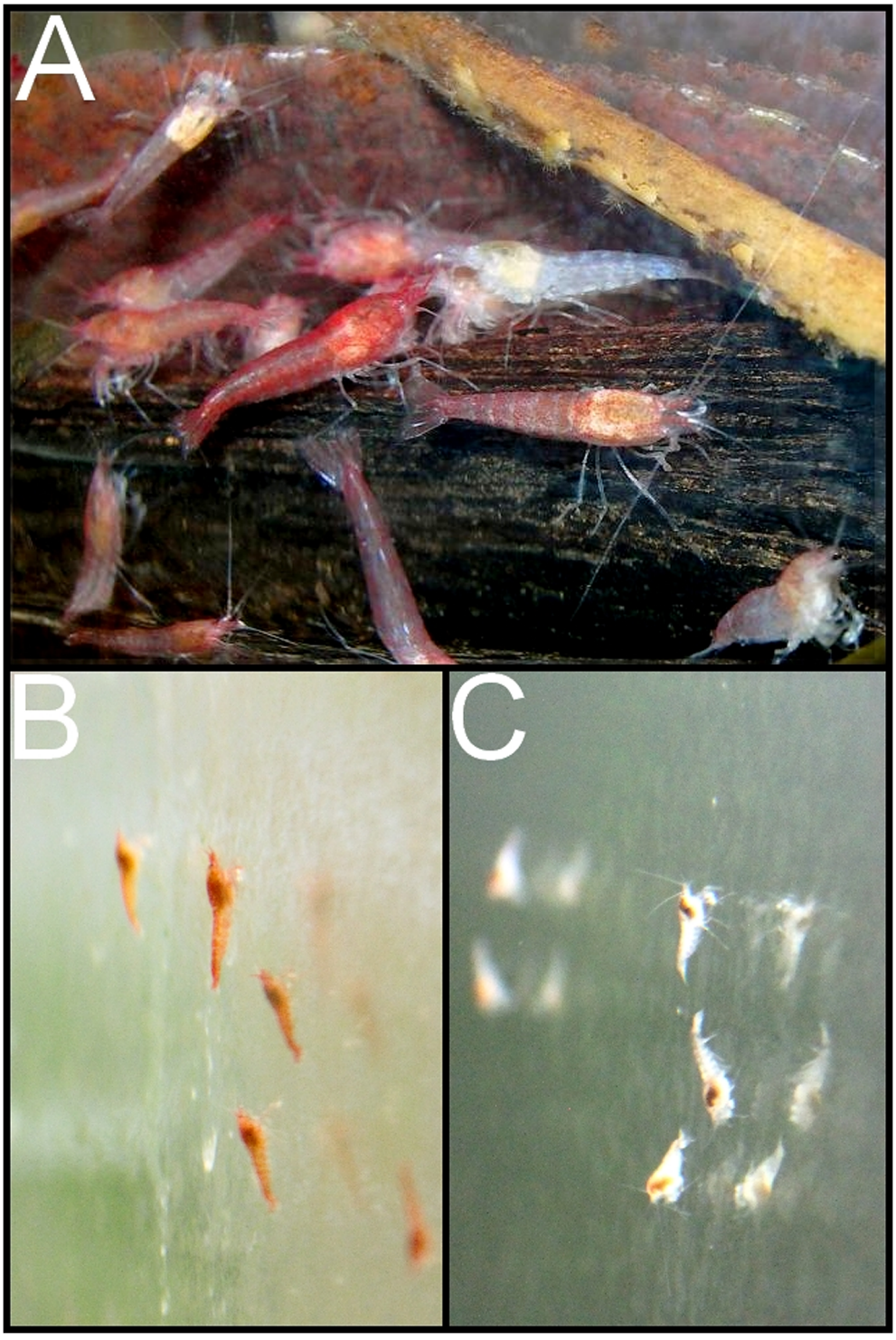

Fig. 1. Phenotypic variation in coloration among individuals of the anchialine endemic atyid *Halocaridina rubra* Holthuis, 1963, from the Hawaiian Islands. A, variation among individuals from the same (WC) genetic lineage; B, C, variation between individuals from different genetic lineages under laboratory conditions: Panel B individuals are from the KONA lineage while Panel C individuals are from the EWA lineage. Individual shrimp in all pictures are approx. 10 mm in total length. Photographs for Panels B and C contributed by Katie Webster.

An ideal circle has a value of 1.0; thus, the lower the circularity value, the more irregular the borders of the chromatosome.

Area fraction – fraction of the total measured area occupied by chromatosomes in a 1.0 mm^2 area.

Hue – "Hue typical" as measured in NIS Elements (NIS-Elements, Advanced Research User's Guide Ver. 3.00); the hue or color value with maximum frequency that represents the most frequent color in a chromatosome. A component of the Hue, Saturation and Intensity (HSI) color space.

Chroma – Representation of the "Mean Red," "Mean Blue" and "Mean Green" values. These three values are the statistical mean of intensity values for the color components present in a chromatosome as measured in

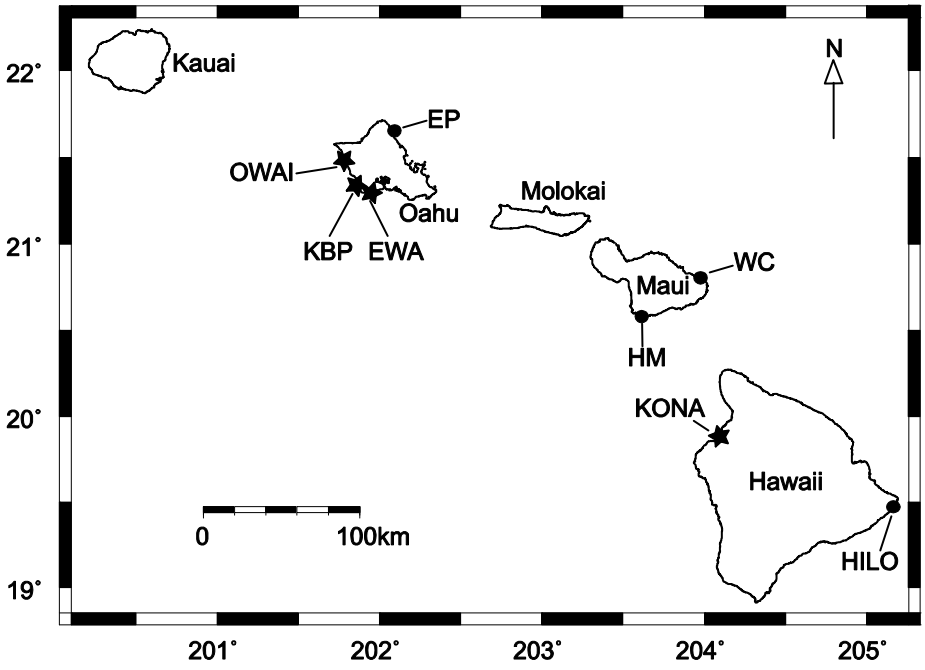

Fig. 2. Geographic origins from the Hawaiian Islands (after Craft et al., 2008) for the laboratory colonies of *H. rubra* utilized in this study. Chromatosomal properties were measured for 30 individuals at least once (indicated by filled circle) or twice (indicated by filled stars) from each genetic lineage/population. Acronyms for genetic lineages are: Windward Oahu (EP), South Oahu (EWA), East Hawaii (HILO), South Maui (HM), West Hawaii (KONA), West Oahu (OWAI) and East Maui (WC). Note that the population from KBP represents a natural mix of two different (EWA and OWAI) genetic lineages (Craft et al., 2008).

NIS Elements (NIS-Elements, Advanced Research User's Guide Ver. 3.00). Components of the Red, Green and Blue (RGB) color spaces.

Brightness – attribute representing the ability of chromatosomes to reflect a greater or lesser fraction of incident light.

Chromatosomal Properties Among *H. rubra* Genetic Lineages and Body Parts

For 30 shrimp from each of the seven *H. rubra* genetic lineages and the mixed population (referred to as KBP throughout), a single body part was photographed per individual: either the branchial region of the carapace ($n = 10$), the pleuron of the second pleomere ($n = 10$), or the exon of the uropod ($n = 10$) (after Flores and Chein, 2011). Data from these three body parts were used ($n = 30$ images per lineage) for comparisons among lineages. On the other hand, data from all eight groups were utilized in comparing chromatosomal properties between the three body parts ($n = 80$ images per body part). To determine the reproducibility of general trends preliminarily identified in the data, 30 additional individuals each from the South Oahu (EWA), West Hawaii (KONA) and West Oahu (OWAI) lineages as well as KBP were photographed and analyzed as described above. These were chosen since they spanned the diversity in chromatosomal properties measured among the eight laboratory colonies (see Results).

Chromatosomal Properties of *H. rubra* and Environmental Extremes

To examine the impact of environmental extremes on the chromatosomal properties of *H. rubra*, shrimp were exposed to anoxia and desiccation as well as decreased salinity and high and low temperatures. In this case, only the pleuron of the second pleomere was analyzed as previously described, as it produced the most reliable images. To eliminate potential variation in measurements due to differing evolutionary genetic background, only individuals from the South Maui genetic lineage of *H. rubra* (HM) were utilized for these experiments, with 10 shrimp per treatment, except in the cases of anoxia and salinity transfer treatments ($n = 9$). For anoxia, shrimp were photographed under normoxic (control) conditions prior to being transferred to an anoxic environment (created by N_2 bubbling) for 24 hours before being photographed again. Desiccation was simulated by placing shrimp into individual weigh boats, wicking away all water with a Kimwipe and leaving the animal exposed to air for 30 minutes. After this time interval, shrimp were placed back into water to ensure survival following the treatment and immediately photographed. To examine the effect of decreased salinity, animals were initially photographed after being chronically housed in 32‰ water (i.e., control), transferred to 2‰ for 24 hours and then photographed. Lastly, shrimp were photographed at room temperature (22°C) as a control then transferred to either 14°C or 32°C incubators in individual 40 ml beakers for 24 hours before being photographed again.

To determine whether animals exposed to elevated temperature underwent physiological stress, lipid peroxide (LPO) levels were assayed for control (22°C, $n = 8$) and treated (32°C for 24 hours, $n = 7$) shrimp. High LPO levels are a common indicator of cellular stress in shrimp (Downs et al., 2001; Boudet et al., 2013) as well as other marine invertebrates (Joyner-Matos et al., 2006). Levels of LPO were assessed as μM malondialdehyde using a Bioxytech® LPO-586 kit (Percipio Biosciences, Cat. No. 21012) and normalized to total mg protein, which was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. No. 23225), both following the manufacturers' protocols.

In addition to the image capture and analysis protocol described above, a streamlined protocol was also implemented for the anoxia experiment to test whether similar results could be generated under "simple" (field) conditions. Briefly, dorsal views of whole shrimp ($n = 10$) were photographed using a Canon PowerShot SD1200 IS digital camera against a white background both before and after 24-hour exposure to anoxia. ImageJ software (National Institutes of Health, Bethesda, MD, USA; Schneider et al., 2012) was then utilized in quantifying the number of, as well as the area fraction occupied by, chromatosomes on the dorsal portions of the posterior three pleura from the photographs before and after anoxia exposure.

Statistical Analysis

Statistical comparisons were performed using R v2.15.0 (R Core Team, 2013; code available on request). Specifically, ANOVAs with Tukey post-hoc analyses were performed to compare eight of nine chromatosomal properties among the *H. rubra* genetic lineages as well as among their body parts. The exception was chromatosome cell density; since these data consisted of counts, a Poisson regression was performed to identify significant groupings. Principal components analyses (PCAs) utilizing the Pearson product-moment correlation coefficient were also conducted to determine which chromatosomal properties best explained variance between the

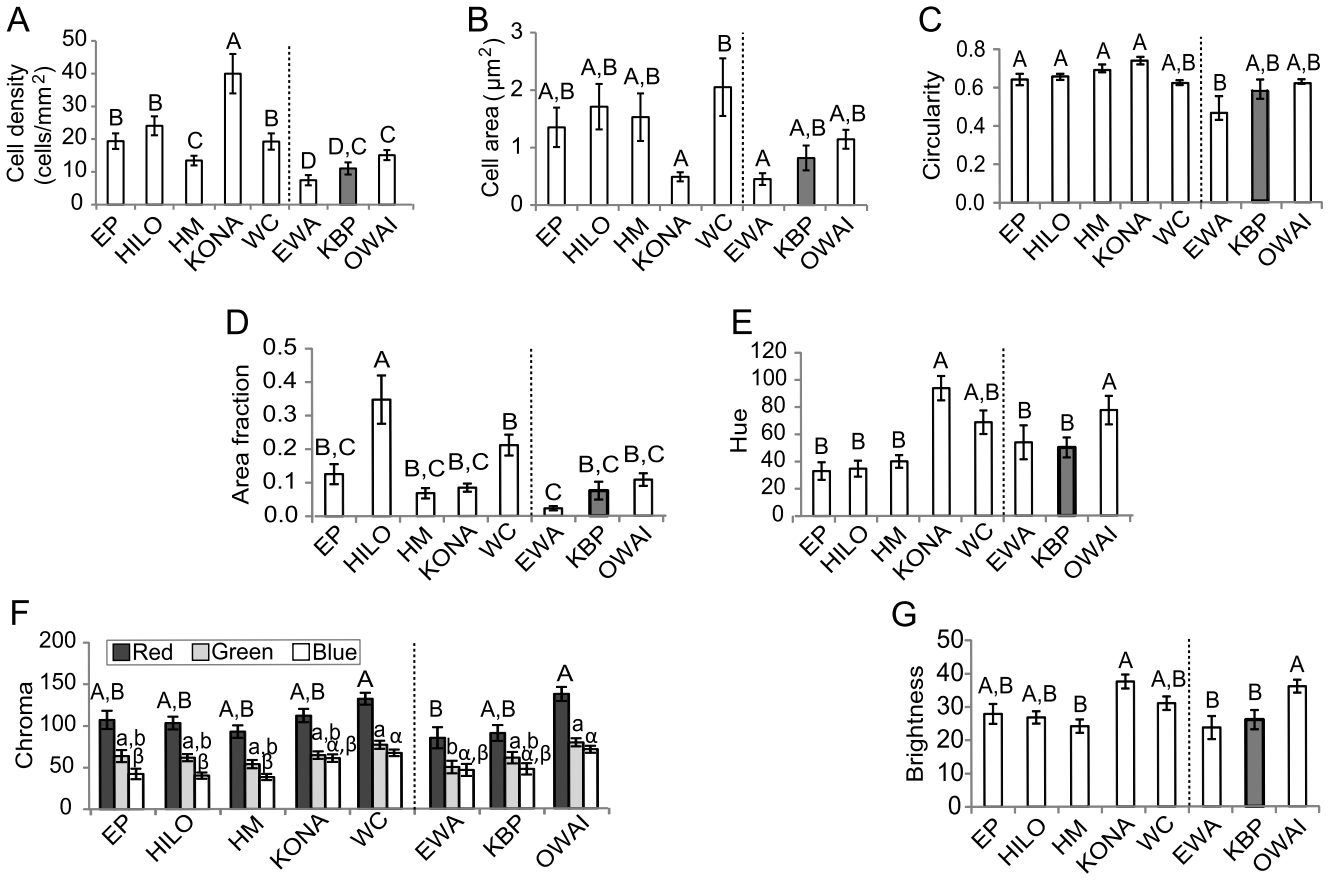

Fig. 3. Chromatosomal properties from each genetic lineage/population of *H. rubra* examined in this study from laboratory colonies (see Materials and Methods for additional details). A, cell density; B, cell area; C, circularity; D, area fraction; E, hue; F, chroma (comprised of three separate values, "Mean Red," "Mean Green," and "Mean Blue"); G, brightness using both Red, Green, and Blue (RGB) and Hue, Saturation and Intensity (HSI) color spaces. Acronyms for genetic lineages are: Windward Oahu (EP), South Oahu (EWA), East Hawaii (HILO), South Maui (HM), West Hawaii (KONA), West Oahu (OWAI) and East Maui (WC). Notably, the KBP population represents a natural mixture of individuals from both the EWA and OWAI genetic lineages (Craft et al., 2008). Given this, the KBP population as well as the EWA and OWAI genetic lineages are separated from the others by a dotted line and the KBP bar is filled with grey. Error bars depict the ±SEM. Uppercase letter(s) above individual bars designate statistically significant group(s) based on Poisson regression (chromatosome cell density only) or ANOVA tests with Tukey post-hoc analyses (other eight chromatosomal properties) ($n = 30$ individuals per lineage). For chroma, uppercase letters designate statistically significant groups for "Mean Red," lowercase letters designate statistically significant groups for "Mean Green," and Greek symbols designate statistically significant groups for "Mean Blue."

H. rubra genetic lineages as well as visualize relationships among the lineages. All 240 individuals examined for the between lineage comparisons (see above) were included, with the nine measured chromatosomal properties (where chroma is composed of three values: "Mean Red," "Mean Blue," and "Mean Green") being utilized and *H. rubra* genetic lineage treated as a qualitative variable. Based on the overall statistical results as well as the first PCA, a second PCA was performed which analyzed only three chromatosomal properties: cell density, area fraction, and brightness (see Results). Finally, a third PCA was performed to elucidate differences between body parts for all nine chromatosomal properties. These PCAs, along with the 95% confidence levels of categorical placements, were conducted with the package FactoMineR v1.24 (Lê et al., 2008) in R. Lastly, Student's paired *t*-tests were performed to statistically compare controls and environmental treatments as well as the quantification of chromatosomes and their area fraction under the streamlined protocol.

RESULTS

Chromatosomal Properties among *H. rubra* Genetic Lineages

Statistically significant differences in chromatosome cell density were identified among the *H. rubra* genetic lineages, with the EWA/HM/OWAI and KONA lineages having the lowest and highest cell densities, respectively, and the EP/HILO/WC lineages and KBP possessing intermediate densities ($P < 0.001$, Poisson regression, Fig. 3A). Properties related to chromatosome morphology were also found to be significantly different. For example, the WC lineage had the largest chromatosomal cell area and the KONA/EWA lineages had the smallest, with the other lineages or KBP being intermediate ($P < 0.001$, ANOVA, Fig. 3B). In regards to circularity, the EP/HILO/HM/KONA lineages possessed the most circular chromatosomes, the EWA lineage the least, and the OWAI/WC lineages and KBP were intermediate ($P < 0.001$, ANOVA, Fig. 3C). For area fraction, the EP/HM/KONA/OWAI/WC lineages and the KBP population were intermediate, with the EWA and HILO lineages having the lowest and highest areas encompassing chromatosomes, respectively ($P < 0.001$, ANOVA, Fig. 3D). Furthermore, the chromatic spectra of chromatosomes differed significantly among genetic lineages of *H. rubra*: with little variance, hue, chroma, and brightness were significantly higher in the KONA and OWAI

lineages relative to the EWA and other lineages/population ($P < 0.001$, ANOVA, Fig. 3E-G). Overall and in general, individuals of the EWA lineage had lower numbers of, smaller and less bright chromatosomes while those of the KONA lineage were brighter, larger and present in higher numbers, with lineages like OWAI or KBP lying in between (Fig. 3A-G). To determine the reproducibility of these trends, 30 additional individuals were photographed and analyzed from each of these four groups. Notably, this resampling of the EWA, KONA, and OWAI lineages and KBP resulted in the same general patterns in chromatosomal morphology and chromatic properties as originally measured (see supplementary data in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1937240x).

Principal components analysis (PCA) of the nine chromatosomal properties (Fig. 4A) segregated the *H. rubra* genetic lineages in a manner largely corresponding to results based on the individual properties (Fig. 3A-G). Eight of the nine chromatosomal properties (the exception being cell density) were significantly correlated ($P < 0.001$) with PCA Dimension 1, which explained 45.58% of the variation in chromatosomal properties while PCA Dimension 2 explained 18.81% of the variation and was primarily correlated with cell density, cell area, cell circularity, area fraction, and hue. On PCA Dimension 1, the OWAI and WC lineages were significantly separated from the EP/EWA/HM lineages and KBP ($P < 0.041$ for all comparisons) and on PCA Dimension 2, KONA was significantly separated from all other lineages and KBP ($P < 0.001$ for all comparisons, Fig. 4A). Overall, these results demonstrate that some *H. rubra* genetic lineages, such as KONA, OWAI and WC, can be clearly distinguished from others based on the nine chromatosomal properties examined here.

Both the individual analyses (Fig. 3A-G) and the first PCA (Fig. 4A) suggested examination of a reduced set of chromatosomal properties, i.e., cell density, area fraction, and brightness, might resolve potential differences between the *H. rubra* genetic lineages of EP/EWA/HM as well as KBP. Towards this end, a second PCA was performed which utilized only these three chromatosomal properties (Fig. 4B). In this case, PCA Dimension 1 explained 40.77% of the variation, with all three properties significantly correlated to it ($P < 0.001$), and KONA was significantly separated from WC and OWAI ($P < 0.03$). PCA Dimension 2 explained 32.34% of the variation and cell density and area fraction were significantly correlated to it ($P < 0.001$). On PCA Dimension 2, KONA and HILO, as well as EP, EWA and HM, were significantly separated from each other ($P < 0.005$ for all comparisons), with KPB overlapping both EWA and HM. Thus, separation between the lineages of EP, EWA and HM was much greater using the reduced set than in the initial analyses based on all nine (9) properties (Fig. 4A, B).

Chromatosomal Properties among Body Parts of *H. rubra*

On the basis of body parts, the cell density of chromatosomes was significantly different along the body of *H. rubra*, being highest for the branchial region of the carapace, intermediate in the pleuron of the second pleomere, and lowest in the exon of the uropod ($P < 0.001$, Poisson regression, Fig. 5A), although chromatosomes in the uropod had a larger area than the other two body parts ($P > 0.001$, ANOVA, Fig. 5B). Chromatosome circularity was also significantly different by body part, with the highest and low-

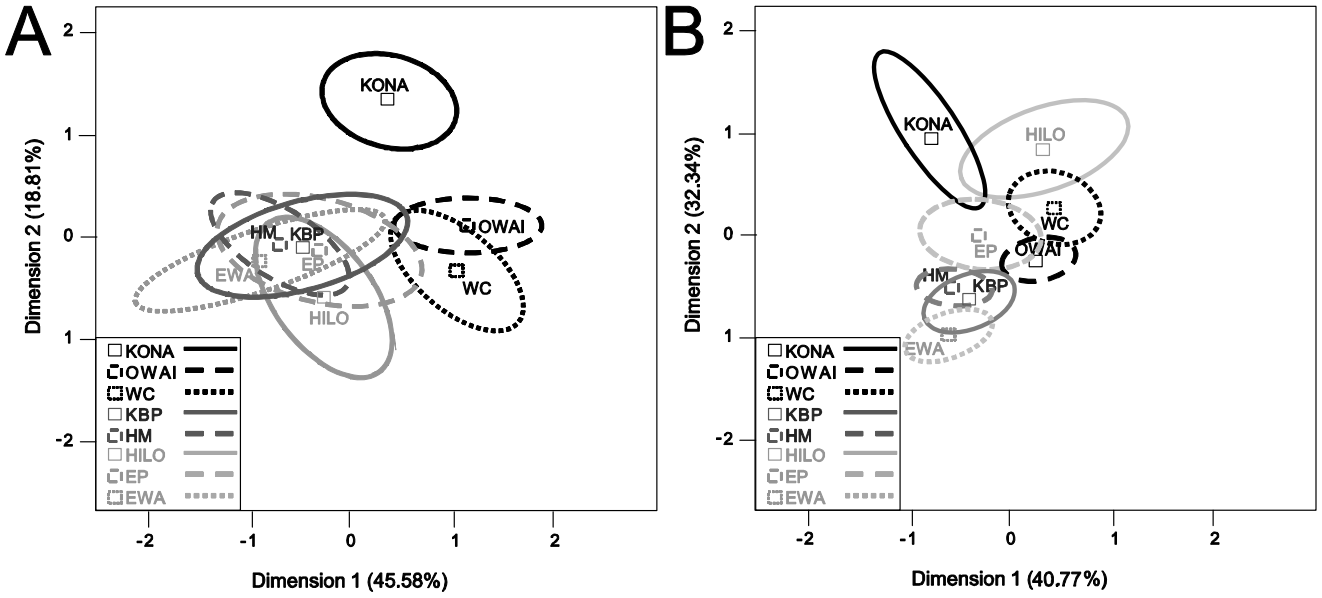

Fig. 4. Principal component analyses (PCAs) of the chromatosomal properties from each genetic lineage/population of *H. rubra* examined in this study from laboratory colonies (see Materials and Methods for additional details). A, PCA based on all nine (9) properties measured and analyzed in this study; B, PCA based on just three of the properties (cell density, area fraction, and brightness) measured and analyzed in this study. Square symbols represent barycenters (means) for a genetic lineage/population, with 95% confidence levels given by the ellipses. Acronyms for genetic lineages are: Windward Oahu (EP), South Oahu (EWA), East Hawaii (HILO), South Maui (HM), West Hawaii (KONA), West Oahu (OWAI) and East Maui (WC). Notably, the KBP population represents a natural mixture of individuals from both the EWA and OWAI genetic lineages (Craft et al., 2008).

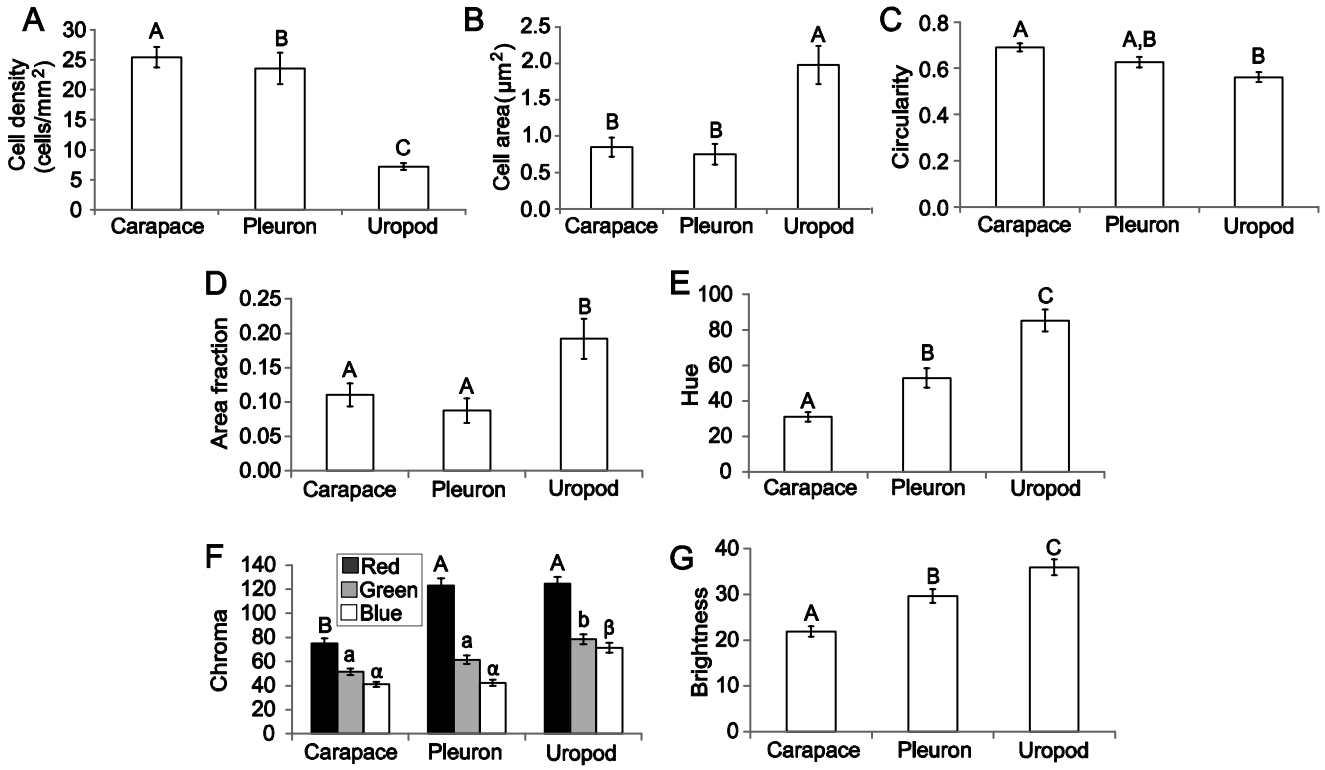

Fig. 5. Chromatosomal properties from each of the three body regions/parts (either the carapace, pleuron, or uropod) of *H. rubra* examined in this study from laboratory colonies (see Materials and Methods for additional details). A, cell density; B, cell area; C, circularity; D, area fraction; E, hue; F, chroma (comprised of three separate values); G, brightness using both Red, Green, and Blue (RGB) and Hue, Saturation and Intensity (HSI) color spaces. Error bars depict the ±SEM. Uppercase letter(s) above individual bars designate statistically significant group(s) based on Poisson regression (chromatosome cell density only) or ANOVA tests with Tukey post-hoc analyses (other eight chromatosomal properties) ($n = 80$ individuals per body region/part). For chroma, uppercase letters designate statistically significant groups for "Mean Red," lowercase letters designate statistically significant groups for "Mean Green," and Greek symbols designate statistically significant groups for "Mean Blue."

est circularity occurring in the carapace and uropod, respectively ($P < 0.001$, ANOVA, Fig. 5C). Along with this, the uropod had a greater area fraction of chromatosomes than the other two body parts ($P < 0.001$, ANOVA, Fig. 5D). Lastly, chromatic properties of hue and brightness were statistically higher in the uropod than the carapace and the pleuron while chroma was higher in the uropod and pleuron than the carapace ($P < 0.001$, ANOVA, Fig. 5E-G). When analyzed via PCA (Fig. 6), the three body parts were clearly separated along PCA Dimension 1, which explained 45.12% of the observed variation and included all properties except cell density. In contrast, the three body parts occupied the same relative position in PCA Dimension 2, which encompassed 20.53% of the variation and included cell density, cell area, cell circularity, area fraction, and hue.

Chromatosomal Properties of *H. rubra* under Environmental Extremes

Statistically significant differences were identified in chromatosomal properties under many of the tested environmental extremes, such as brightness and chroma for *H. rubra* under anoxic conditions (Student's paired *t*-test, Table 1). Notably, similar results were obtained when using the streamlined protocol: although the number of chromatosomes on the dorsal portion of the posterior three pleurons did not change, the overall "red" area fraction did statistically decrease after anoxia exposure ($P = 0.013$, Student's paired *t*-test, Fig. 7). Following desiccation, cell area ($P = 0.036$, Student's paired *t*-test, Table 1) and hue ($P = 0.007$, Student's paired *t*-test, Table 1) both significantly decreased and chromatosome density significantly increased ($P = 0.029$, Student's paired *t*-test, Table 1). In contrast, chromatosomes had statistically identical properties at 2‰ to those at the control salinity of 32‰ ($P > 0.08$ for all parameters, Student's paired *t*-test, Table 1). The area fraction also significantly increased at high temperature (32°C, $P = 0.038$, Student's paired *t*-test, Table 1) and lipid peroxide (LPO) levels confirmed that shrimp exposed to 32°C for 24 hours did undergo cellular stress, as LPO concentration in treated animals was approx. 20 fold higher than in those maintained at 22°C ($P < 0.001$, Student's paired *t*-test, Fig. 8). Furthermore, under low temperature (i.e., 14°C), area fraction significantly increased ($P = 0.038$, Student's paired *t*-test, Table 1) while chromatosome circularity significantly decreased ($P = 0.004$, Student's paired *t*-test, Table 1).

DISCUSSION

Chromatosomal Properties among *H. rubra* Genetic Lineages

Consistent with one of our initially proposed hypotheses, statistically significant differences were identified in chro-

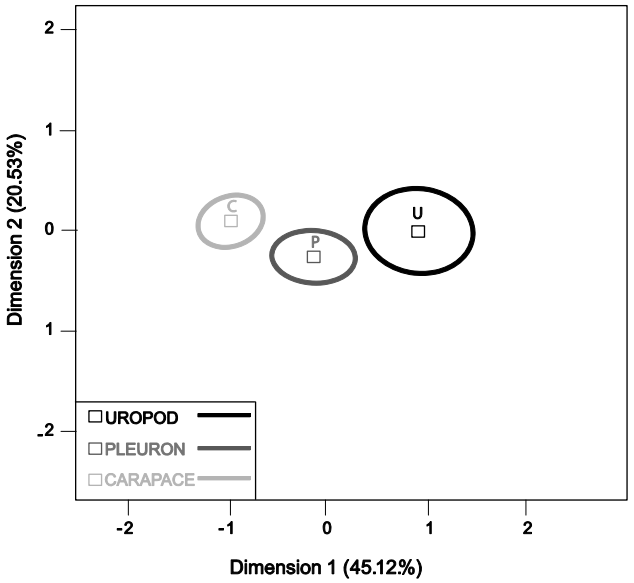

Fig. 6. Principal component analysis (PCA) of the chromatosomal properties from three body regions/parts (either the carapace, pleuron or uropod) of *H. rubra* examined in this study from laboratory colonies (see Materials and Methods for additional details). Square symbols represent barycenters (means) for a body region/part, with 95% confidence levels given by the ellipses.

matosomal properties between genetically diverged lineages of the Hawaiian anchialine endemic atyid *H. rubra* maintained under standardized laboratory conditions. Furthermore, this work demonstrates that the methodological approaches outlined in Flores and Chein (2011) can be extended to other species of atyid shrimp besides *Neocaridina denticulata* (De Haan, 1844). Of the nine chromatosomal properties examined here, those having the ability to best differentiate between the genetic lineages of *H. rubra* were the combination of cell density, area fraction, and brightness (Figs. 3A, D, G; Fig. 4B), and these three properties have been previously identified as being influential in coloration of either other crustaceans or aquatic species (Sugimoto, 2002; Tume et al., 2009). Overall, the most significant difference between the *H. rubra* lineages was chromatosomal cell density, where brighter or paler lineages possessed greater or lesser cell densities, respectively (Fig. 3A). For example, KONA had the highest cell densities (Fig. 3A) and was the most vibrant (Fig. 1B) while EWA had the lowest cell densities (Fig. 3A) and was nearly translucent (Fig. 1C). Thus, while chromatic and other chromatosomal properties do vary between them (Fig. 3B-G), cell density (Fig. 3A) appears to be the most significant driver underlying phenotypic variation among the *H. rubra* genetic lineages examined here.

Notably, utilizing all nine chromatosomal properties proved incapable of statistically distinguishing many of the *H. rubra* genetic lineages from each other via PCA; for example, the EP, EWA and HM lineages were statistically similar (Fig. 3A-G) and indistinguishable in this context (Fig. 4A). However, when taken together, a PCA conducted solely on cell density, area fraction, and brightness provided separation between all of the lineages, with the only overlap being between HM and KBP, with the latter represent-

Table 1. Chromatosomal properties ± SEM following exposure to environmental extremes for individual shrimp from the HM genetic lineage of *H. rubra*. Treatments of animals (n = 9-10) included anoxia, desiccation, decreased salinity, and high and low temperature. Chromatosomal properties before and after exposure are presented. * P = 0.05-0.10; ** P = 0.01-0.05.

Treatment	Cell density (cells/mm^2)	Cell area (1000 μm^2)	Circularity	Area fraction	Hue	Chroma (red)	Chroma (green)	Chroma (blue)	Brightness
Control (normoxia)	24.0 ± 3.28	0.13 ± 0.02	0.67 ± 0.08	0.08 ± 0.02	81.2 ± 18.9	183.2 ± 22.3	90.2 ± 16.2	74.3 ± 10.5	45.4 ± 6.2
One day anoxia	26.1 ± 3.30	0.14 ± 0.02	0.78 ± 0.09*	0.06 ± 0.01	103.4 ± 17.1	148.4 ± 18.2*	148.4 ± 18.2*	56.5 ± 7.1*	34.9 ± 4.3*
Control (Immersion)	22.0 ± 2.86	0.46 ± 0.14	0.73 ± 0.13	0.6 ± 0.05	43.91 ± 7.14	102.1 ± 31.33	39.0 ± 9.13	27.98 ± 7.16	22.10 ± 5.58
Desiccation (30 minute emmersion)	18.8 ± 8.75*	0.78 ± 0.58*	0.70 ± 0.09	0.10 ± 0.06	37.88 ± 7.62*	97.99 ± 31.16	34.45 ± 11.32	20.20 ± 5.56*	19.95 ± 5.68
Control (32‰)	28.6 ± 2.39	0.41 ± 0.06	0.77 ± 0.04	0.04 ± 0.01	48.64 ± 13.26	90.98 ± 13.67	35.67 ± 5.28	23.83 ± 3.75	19.66 ± 2.81
Low salinity (2‰)	27.9 ± 2.52	0.43 ± 0.73	0.81 ± 0.03*	0.04 ± 0.01	79.67 ± 11.15	90.9 ± 8.39	32.37 ± 2.46	27.68 ± 1.63	19.73 ± 1.54
Control (22°C)	29.1 ± 2.61	0.49 ± 0.11	0.74 ± 0.05	0.06 ± 0.02	41.82 ± 13.02	101.4 ± 10.19	35.93 ± 3.40	25.11 ± 5.115	21.23 ± 2.20
High temp. (32°C)	28.9 ± 1.93	0.45 ± 0.07	0.77 ± 0.04	0.08 ± 0.01*	48.5 ± 9.43	105.6 ± 8.94	41.9 ± 4.49	30.0 ± 4.73	23.20 ± 1.92
Low temp. (14°C)	23.7 ± 2.85	0.52 ± 0.07	0.65 ± 0.03**	0.045 ± 0.001*	47.81 ± 10.73	99.19 ± 7.74	40.5 ± 4.14	29.0 ± 3.70	22.04 ± 1.92

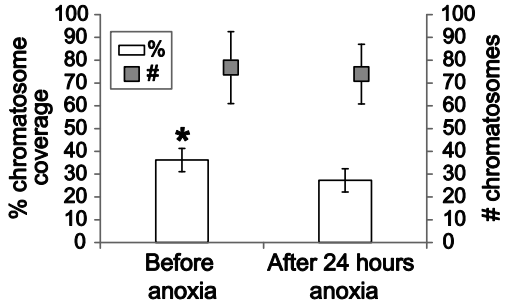

Fig. 7. Chromatosome coverage vs. number of chromatosomes for *H. rubra* in an anoxia exposure experiment where a streamlined protocol was followed, with pre- and post-images captured using a Canon PowerShot SD1200 IS digital camera and analyzed using Image J software. Chromatosome coverage was significantly higher prior to exposure to anoxic conditions and is represented by the bar graph ($P = 0.013$, Student's paired t-test). Squares indicate mean chromatosome numbers for the sampling ($n = 10$), with error bars representing 95% confidence intervals. No significant changes were observed in chromatosome number before or after exposure to anoxia ($P = 0.747$, Student's paired t-test).

ing a natural mix of shrimp from the EWA and OWAI lineages (of note, KBP occupies an intermediate position between these two lineages in PCA Dimensions 1 and 2 of Fig. 4B). Given this, particular chromatosomal properties appear to either provide more resolving power then others or the simultaneous analysis of multiple properties can mask significant differences, as is apparently the case for *H. rubra*. Thus, care should be taken when selecting which, and how many, chromatosomal properties are analyzed across closely related groups of crustaceans or other taxa.

Why might the different genetic lineages of *H. rubra* exhibit significant variation in the chromatosomal properties examined here? For other caridean species, such as the hippolytids *Heptacarpus sitchensis* (Brandt, 1851), *H. paludicola* Holmes, 1900 and *Hippolyte varians* Leach, 1814, highly distinctive color morphs are hypothesized to arise from differential responses of intraspecific genetic variation to environmental factors (Keeble and Gamble, 1900; Bauer, 1981, 1982). While this could be the case for *H. rubra*, such a scenario is less likely as all laboratory colonies examined here experienced similar, standardized environments for >6 years. Instead, the differing evolutionary histories between the lineages might best explain these differences in chromatosomal properties. If this is the situation, one might expect those *H. rubra* lineages having higher levels of similarity in their chromosomal properties to be more closely related to each other in a phylogenetic context. Unfortunately, attempts to infer a well-resolved phylogeny between the eight currently recognized *H. rubra* lineages have proven unsuccessful due to nearly all relationships being unresolved when utilizing only mitochondrial loci (Craft et al., 2008) as well as a lack of nuclear loci possessing appropriate phylogenetic signal (Santos and Weese, 2011). However, while it will be interesting to determine at a future time whether such correlations exist as well as their strengths, the appreciable amounts of genetic differentiation, high levels of ecological isolation, varying demographic histories (Santos, 2006; Craft et al., 2008; Santos and Weese, 2011) and now the significant differences in chromatosomal properties reported here between the lineages supports the contention that the genus *Halocaridina* encompasses a number of distinct and "cryptic species" across the Hawaiian Islands (Craft et al., 2008).

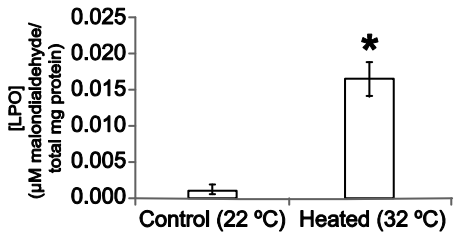

Fig. 8. Lipid peroxide (LPO) levels, quantified as μM malondialdehyde per total mg protein, for *H. rubra* ($n = 7$) following exposure to 32°C as a potential elicitor of stress. LPO levels were assessed with a Bioxytech® LPO-586 assay kit and measured using a BCA protein assay kit by following manufacturer protocols. Statistically higher LPO levels post-treatment imply shrimp are under stress at 32°C ($P < 0.05$, Student's paired t-test).

Chromatosomal Properties among Body Parts of *H. rubra*

Along with varying by genetic lineage, significant differences in the cell density, circularity and area fraction of chromatosomes as well as chromatic properties of hue and brightness were identified between the branchial region of the carapace, pleuron of the second pleomere, and exon of the uropod from *H. rubra* (Fig. 5A, C-E, G). This is consistent with previous reports that coloration along the body of individual shrimp can vary (Perkins, 1928) due to chromatosome number and size as well as different pigments being encompassed within them (Bauer, 1981; Flores and Chien, 2011). Based on the PCA of all nine chromatosomal properties (Fig. 6), while the three body parts occupied the same relative position in Dimension 2, Dimension 1 showed distinct separation between them (see Results). When taken together and in combination with previous precedent, the fact that chromatosomal properties can vary significantly between body parts in crustaceans like *H. rubra* implies such distinctions should be kept in mind when making comparisons either between different species or studies, particularly when methodological approaches may not be identical (see below).

Of the three body regions/parts examined here for *H. rubra*, one in particular was found to be the most easily photographed and analyzed in regards to chromatosomal properties, the pleuron of the second abdominal segment. This lies in contrast to other studies identifying uropods as the preferred body part for such analyses (Abrill and Ceccaldi, 1984; Flores and Chien, 2011) and highlights that methodological approaches might need to be optimized on taxa-specific bases. In the case of *H. rubra*, while uropods could be, and were, photographed and analyzed (see above), their small size compared to the carapace and abdomen, along with the need to physically manipulate them since they often aggregated together when *H. rubra* individuals were laid on their side (see Materials and Methods), made consistent photography more difficult than the other two body parts. Another advantage of utilizing the pleuron for *H. rubra* was that the analyzed area of 1.0 mm^2 encompassed this entire segment without overlap into adjacent body regions. Thus, for shrimp species in the size range of *H. rubra* (approx. 10.0 mm as adults), the pleuron, rather than

uropods, might likely be the best body part to target for effective analyses of chromatosomal properties.

Chromatosomal Properties of *H. rubra* under Environmental Extremes

Environmental extremes have historically been known to impact the chromatosome properties of crustaceans (Smith, 1930; Brown and Sandeen, 1948). Here, many of the extremes animals can potentially encounter in anchialine habitats were also found to significantly influence the chromatosomal properties of *H. rubra*, particularly those related to morphology of the chromosome. These changes are likely part of an overall response to stress (Detto et al., 2008), and LPO analyses confirmed that high temperature is in fact a stressor for *H. rubra* (Fig. 8). On the other hand, alterations in salinity did not elicit changes in any of the measured chromatosomal properties, suggesting such fluctuations do not induce a stress response in this species. This is consistent with reports of *H. rubra* occurring in, and tolerating, a range of salinities in nature (2-36‰) as well as under laboratory experiments (approx. 0-50‰) (Holthuis, 1973; Maciolek, 1983; Havird et al., 2014). Thus, while a significant component of variation in chromatosomal properties of *H. rubra* results from their specific evolutionary genetic background, changes within these properties can be driven by an animal's response to environmental conditions as well.

Though the data generated via the streamlined protocol for image capture were more rudimentary in nature, they correlated well with those obtained using the more sophisticated methodology for measuring chromosomal properties. In the future, this streamlined protocol could provide a means to perform such analyses for *H. rubra* (or other crustacean/atyid species) in the field. A first step in such a scenario would be to compare chromosomal properties among individuals from the same lineage found in "stressed" vs. pristine habitats. Stressors that could be examined include high levels of phosphates or other aquatic pollutants, the presence of invasive fishes, or other disturbances, all of which have the potential to elicit changes in the chromosomal properties of *H. rubra*. Ultimately, a large number of populations could be assessed for possible stress by utilizing a similar protocol to the streamlined one presented here and implemented by citizen scientists, enhancing conservation efforts of the species in the Hawaiian Islands.

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References

Abrill, M. O., and H. J. Ceccaldi. 1984. Influences of purified carotenoids on the composition of food in the pigmentation of *Penaeus japonicus* (Crustacea, Decapoda). Aquaculture 36: 217-228.

Bailey-Brock, J. H., and R. E. Brock. 1993. Feeding, reproduction, and sense organs of the Hawaiian anchialine shrimp *Halocaridina rubra* (Atyidae). Pacific Science 47: 338-355.

———, and ———. 1993. A unique anchialine pond in the Hawaiian Islands. International Review of Hydrobiology 83: 65-75.

Bandaranayake, W. M. 2006. The nature and role of pigments of marine invertebrates. Natural Product Reports 23: 223-255.

Bauer, R. T. 1981. Color patterns of the shrimps *Heptacarpus pictus* and *H. paludicola* (Caridea: Hippolytidae). Marine Biology 64: 141-152.

———. 1982. Polymorphism of colour pattern in the caridean shrimps *Heptacarpus pictus* and *H. paludicola*. Marine Behaviour and Physiology 8: 249-265.

———. 2004. Remarkable Shrimps: Adaptations and Natural History of the Carideans. University of Oklahoma Press, Norman, OK.

Bishop, R. E., and T. M. Iliffe. 2012. Ecological physiology of the anchialine shrimp *Barbouria cubensis*: a comparison of epigean and hypogean populations. Marine Biodiversity 42: 303-310.

Boudet, L. C., P. Polizzi, M. B. Romero, A. Robles, and M. Gerpe. 2013. Lethal and sublethal effects of cadmium in the white shrimp *Palaemonetes argintinus*: a comparison between populations from contaminated and reference sites. Ecotoxicology and Environmental Safety 89: 52-58.

Boyle, R. T., and J. C. McNamara. 2006. Association of kinesin and myosin with pigment granules in crustacean chromatophores. Pigment Cell Research 19: 68-75.

Brandt, J. F. 1851. Krebse, pp. 77-145, plates 5-6. In, A. T. von Middendorff (ed.), Reise in dem äussersten Norden und Osten Sibiriens während die Jaren 1843 und 1844 mit allerhöchster Genehmiggung auf Veranstaltung der Kaiserlichen Akademie der Wissenschaften zu St. Petersburg ausgeführt und in Verbinding mit vielen Gelehrten herausgegeben, 2(Theil 1). St. Petersburg.

Brown, F. A. Jr., and M. I. Sandeen. 1948. Responses of the chromatophores of the fiddler crab, Uca, to light and temperature. Physiological Zoology 21: 361-371.

Cott, H. B. 1940. Adaptive Coloration in Animals. Methuen, London.

Craft, J. D., A. D. Russ, M. N. Yamamoto, T. Y. Iwai Jr., S. Hau, J. Kahiapo, C. T. Chong, S. Ziegler-Chong, C. Muir, Y. Fujita, D. A. Polhemus, R. A. Kinzie III, and S. R. Santos. 2008. Islands under islands: the phylogeography and evolution of *Halocaridina rubra* Holthuis, 1963 (Crustacean: Decapoda: Atyidae) in the Hawaiian archipelago. Limnology and Oceanography 53: 675-689.

Craig, C. L., and K. Ebert. 1994. Colour and pattern in predator-prey interactions: the bright body colours and patterns of a tropical orbspinning spider attract flower-seeking prey. Functional Ecology 8: 616-620.

De Haan, W. 1833-1850. Crustacea. In, P. F. von Siebold (ed.), Fauna Japonica, sive descriptio animalium, quae in itinere per Japoniam, jussu et auspiciis superiorum, qui summum in India Batava Imperium tenent, suscepto, annis 1823-1830 collegit, notis, observationibus et adumbrationibus illustravit: ix-xvi, vii-xvii, i-xxxi, xii-xvii + 243 pp. Lugdunum Batavorum.

Detto, T., J. M. Hemmi, and P. R. Y. Backwell. 2008. Colouration and colour changes of the fiddler crab, *Uca capricornis*: a descriptive study. PLoS ONE 3: e1629.

Downs, C. A., J. E. Fauth, and C. M. Woodley. 2001. Assessing the health of grass shrimp (*Palaeomonetes pugio*) exposed to natural and Anthropogenic stressors: a molecular biomarker system. Marine Biotechnology 3: 380-397.

Endler, J. A., and W. F. Herrenkind. 1978. A predator's view of animal color patterns. Evolutionary Biology 11: 319-364.

Farr, J. A. 1976. Social facilitation of male sexual behavior, intrasexual competition, and sexual selection in the guppy, *Poecilia reticulate* (Pisces: Poeciliidae). Evolution 30: 707-717.

Fingerman, M., and W. D. Tinkle. 1956. Responses of the white chromatophores of two species of prawns (Palaemontes) to light and temperature. Biological Bulletin 110: 144-152.

Flores, E. E., and Y. Chien. 2011. Chromatosomes in three phenotypes of *Neocaridina denticulata* Kemp, 1918: morphological and chromatic differences measured non-invasively. Journal of Crustacean Biology 31: 590-597.

Hanlon, R. T., and M. B. Messenger. 1998. Cephalopod Behaviour. Cambridge University Press, Cambridge.

Hauber, M. 2002. Conspicuous colouration attracts prey to a stationary predator. Ecological Entomology 27: 686-691.

Havird, J. C., S. R. Santos, and R. P. Henry. 2014. Osmoregulation in the Hawaiian anchialine shrimp *Halocaridina rubra* (Crustacea: Atyidae): expression of ion transporters, mitochondria-rich cell proliferation, and hemolymph osmolality during salinity transfers. Journal of Experimental Biology, in press, DOI:10.1242/jeb.103051.

Hoese, F. J., E. A. J. Law, D. Rao, and M. E. Herberstein. 2006. Distinctive yellow bands on a sit-and-wait predator: prey attractant or camouflage? Behaviour 143: 763-781.

Holmes, S. J. 1900. Synopsis of the California stalk-eyed Crustacea. Occasional Papers of the California Academy of Sciences 7: 1-262, plates 1-4.

Holthuis, L. B. 1963. On red coloured shrimps (Decapoda, Caridea) from tropical land-locked saltwater pools. Zoologische Mededelingen 16: 261-279.

———. 1973. Caridean Shrimps found in landlocked saltwater pools at four Indo-West Pacific localities. Zoologische Verhandelingen 128: 1-7.

Jaume, D., B. Sket, and G. A. Boxshall. 2009. New subterranean Sebidae (Amphipoda: Gammaridea) from Vietnam and the SW Pacific. Zoosystema 31: 249-277.

Joyner-Matos, J., C. A. Downs, and D. Julian. 2006. Increased expression of stress proteins in the surf clam *Donax variabilis* following hydrogen sulfide exposure. Comparative Biochemistry and Physiology 145: 245-257.

Keeble, F., and F. W. Gamble. 1900. The color physiology of *Hippolyte varians*. Proceedings of the Royal Society 65: 461-468.

Lê, S., J. Josse, and F. Husson. 2008. FactoMineR: an R package for multivariate analysis. Journal of Statistical Software 25: 1-18.

Leach, W. E. 1814. The Zoological Miscellany; Being Descriptions of New, or Interesting Animals. Vol. 1. E. Nodder and Son, London.

Maciolek, J. A. 1983. Distribution and biology of Indo-Pacific insular hypogeal shrimps. Bulletin Marine Science 33: 606-618.

———. 1986. Environmental features and biota of anchialine pools on Cape Kina'u Maui, Hawai'i. Stygologia 2: 119-129.

McNamara, J. C. 1981. Morphological organization of crustacean pigmentary effectors. The Biological Bulletin 161: 270-280.

Nikon-NIS Elements Advanced Research. User's Guide Version 3.00. AR 3.0, SP 7, Hotflix 8, Build 548. Laboratory Imaging-Nikon, Tokyo.

Perkins, E. B. 1928. Color changes in crustaceans, especially in *Palaemonetes*. Journal of Experimental Zoology 50: 71-105.

Pohlman, J. W. 2011. The biogeochemistry of anchialine caves: progress and possibilities. Hydrobiologia 1: 33-55.

Pringle, C. M., and G. A. Blake. 1994. Quantitative effects of atyid shrimp (Decapoda: Atyidae) on the depositional environment in a tropical stream: use of electricity for experimental exclusion. Canadian Journal of Fisheries and Aquatic Sciences 51: 1443-1450.

———, ———, A. P. Covich, K. M. Buzby, and A. Finley. 1993. Effects of omnivorous shrimp in a montane tropical stream: sediment removal, disturbance of sessile invertebrates and enhancement of understory algal biomass. Oecologia 93: 1-11.

R Core Team. 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna.

Ribeiro, G. G., T. M. Lopes, C. A. Neves, L. E. M. Nery, and G. S. Trindade. 2004. Ultraviolet radiation induces dose-dependent pigment dispersion in crustacean chromatophores. Pigmented Cell Research 17: 545-548.

Ridler, T. W., and S. Calvard. 1978. Picture thresholding using an iterative selection method. IEEE Transactions on Systems, Man and Cybernetics 8: 630-632.

Robinson, W. G., and J. S. Charlton. 1973. Microtubules, microfilaments, and pigment movement in the chromatophores of *Palaemonetes vulgaris* (Crustacea). Journal of Experimental Zoology 186: 279-304.

Rothschild, M. 1975. Remarks on carotenoids in the evolution of signals, pp. 20-47. In, Coevolution of Animals and Plants. University of Texas Press, Austin, TX.

Santos, S. R. 2006. Patterns of genetic connectivity among anchialine habitats: a case study of the endemic Hawaiian shrimp *Halocaridina rubra* on the Island of Hawaii. Molecular Ecology 15: 2699-2718.

———, and D. A. Weese. 2011. Rocks and clocks: linking geologic history and rates of genetic differentiation in anchialine organisms. Hydrobiologia 677: 54-63.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9: 671-675.

Seymour, J. R., W. F. Humphreys, and J. G. Mitchell. 2007. Stratification of the microbial community inhabiting as anchialine sinkhole. Aquatic Microbial Ecology 50: 11-24.

Sket, B. 1996. The freshwater crab fauna (Crustacea: Decapoda: Brachyura) of the Philippines. IV. On the collection of Parathelphusidae from Bohol. The Proceedings of the Biological Society of Washington 109: 695-706.

Smith, D. C. 1930. The effects of temperature changes upon the chromatophores of crustaceans. Biological Bulletin 58: 193-201.

Sotka, E. E. 2005. Local adaptation in host use among marine invertebrates. Ecology Letters 8: 448-459.

Sugimoto, M. 2002. Morphological color changes in fish: regulation of pigment cell density and morphology. Microscopy Research and Technique 58: 496-503.

Sumner, F. B. 1935. Studies on protective color change. III. Experiments with fishes, both as predators and prey. Proceeding of the National Academy of Sciences of the United States of America 21: 345-353.

Sutherland, R. L., L. M. Mäthger, R. T. Hanlon, A. M. Urbas, and M. O. Stone. 2008. Cephalopod coloration model. I. Squid chromatophores and iridophores. Journal of the Optical Society of America 25: 588-599.

Thayer, G. H. 1909. Concealing-Coloration in the Animal Kingdom: an Exposition of the Laws of Disguise Through Color and Pattern. McMillian, New York, NY.

Thurman, C. L. 1988. Rhythmic physiological color change in crustacea: a review. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 91: 171-185.

Tume, R. K., A. L. Sikes, S. Tabrett, and D. M. Smith. 2009. Effect of background color on the distribution of astaxanthin in black tiger prawn color. Aquaculture 296: 129-135.

Turner, E. R. A. 1961. Survival values of different methods of camoflauge as shown in a model population. Proceedings of the Zoological Society of London 136: 273-284.

Zahavi, A. 1975. Mate selection: a selection for a handicap. Journal of Theoretical Biology 53: 205-214.

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