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Original Article

Intrapopulation variability in coloration is associated with reproductive season in the crayfish *Faxonius virilis*

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Abstract

Animal coloration has a wide range of biological functions and may be subject to different, sometimes conflicting, selective pressures. In crustaceans, the evolution of coloration is relatively unstudied, despite the broad range of colors and color patterns, which includes variability at multiple levels. Freshwater crayfish are known to show color variability within species and populations, as well as intra-individual variability, but the function, if any, of crayfish coloration is largely unknown. Here, I report on an experiment to understand patterns of color variability in the crayfish *Faxonius virilis* and show that variation is strongly correlated to ontogenetic changes from a summer non-reproductive form to a fall reproductive form. Crayfish showed comparatively little inter- and intra-individual color variation in their non-reproductive form, but substantial variation at both levels in the reproductive form. Transition to the reproductive form was associated with the development of greener or bluer coloration localized to the chelae on a subset of individuals, but these changes showed no clear correlation with sex or body size. Future investigations should focus on determining whether differences in color between individuals in the mating season are associated with any physiological or behavioral differences, or with differential susceptibility to predation.

Key words: crayfish, color variation, reproductive form, seasonal changes.

Animal coloration may serve a number of biological functions, including species recognition (Alatalo et al. 1994; Couldridge and Alexander 2002; Drury et al. 2015; Dyson et al. 2020), crypsis and aposematism (Rojas and Endler 2013; Nokelainen et al. 2019; Pellitteri-Rosa et al. 2020; Nekaris et al. 2021), communication among conspecifics and heterospecifics (Ligon et al. 2014; Li et al. 2022), and physiological maintenance (e.g., of internal temperature; Geen and Johnston 2014; Smith et al. 2016; Rogalla et al. 2022), and consequently, it may evolve as a function of many, sometimes conflicting, selective pressures (reviewed in Cuthill et al. 2017; Franklin et al. 2020; Postema et al. 2023). For example, in some contexts, selection may favor decreased brightness or lower contrast against a background color or pattern, which can reduce detectability and assist in predator avoidance. On the other hand, selection may favor increased conspicuousness through bright coloration or distinctive patterning, which may function as signals to other individuals. For example, sexually selected coloration can carry with it increased predation risk, as has been shown for fish (Endler 1980; Johnson and Candolin 2017) and lizards (Amdekar and Thaker 2019). Thus, a suite of complex and interacting selective pressures has led to high natural variability in animal coloration at intra- and interspecific levels.

Coloration may vary intraspecifically from population to population (Rand 1990; Morrongiello et al. 2010; Moreno-Rueda et al. 2021) or by sex (Owens and and Hartley 1998; Svensson et al. 2008; Rojas and Endler 2013; Cooper et al. 2016; Storniolo et al. 2021; Suárez -Tovar et al. 2022). In other cases, coloration varies within a population over time. Variability in time can be a result of genetic differences among permanent color morphs in a population (Osawa and Nishida 1992; Tuttle 2003; Pryke 2007; Suárez-Tovar et al. 2022) or it may result from ontogenetic or seasonal changes (Ra'Anan and Sagi 1985; Svensson et al. 2008; Ciccotto et al. 2014; Hartzell 2017; Fresnillo et al. 2019; Nokelainen et al. 2019; Baling et al. 2020; Pellitteri-Rosa et al. 2020; Yoshikawa et al. 2020; Nekaris et al. 2021; Storniolo et al. 2021; Suárez-Tovar et al. 2022) and/or short-term (over minutes or hours) physiological changes (Vroonen et al. 2012; Ligon et al. 2014; Keren-Rotem et al. 2016; Takeshita 2019; Suárez-Tovar et al. 2022). In some cases, selective pressure has apparently resulted in within-individual variability in coloration, with pronounced differences in the color of different body regions, which have evolved in response to different selective pressures. For example, in fiddler crabs, *Austruca lactea*, carapace coloration is darker than claw coloration, and the carapace alone shows rapid darkening under stressful conditions; Takeshita (2019) theorized that these differences between claw and carapace coloration could reflect different signaling functions for these 2 body regions. In shore skinks, Baling et al. (2020) reported that dorsal surfaces show greater camouflage coloration than ventral surfaces, and that ventral coloration varied by sex and age, which may indicate a function for ventral color in sex recognition.

In malacostracan crustaceans, existing research has focused on how coloration and color patterns function in various contexts. In some taxa, coloration may function primarily as

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protection from predators. Color polymorphism or polyphenism in prawns of the genus *Hippolyte* has been shown experimentally to function as background matching crypsis in a variable environment (Duarte et al. 2018; Green et al. 2019). Other examples of crypsis through either background matching (similarity in coloration and pattern with the background) or disruption (patterns that interfere with the detection of an object's edges and shape; Stevens and Merilaita 2009) have been documented in a number of crab species (Caro 2018), and the shore crab *Carcinus maenus* has been shown to undergo rapid (within 2 h) color changes that improved background matching crypsis (Stevens et al. 2014). In the shrimp, *Hippolyte obliquimanus*, 2 different color morphs are associated with crypsis in a heterogeneous environment (Duarte et al 2016), and a congener *H. varians* uses both background matching behavior and color change to maintain crypsis (Green et al. 2019). Background matching crypsis in marine isopods was shown experimentally to reduce predation risk (Hultgren and Mittelstaedt 2015). Other investigations have focused on describing temporal and spatial variability in crustacean coloration. For example, Yoshikawa et al. (2020) reported ontogenetic changes in color patterns of the walking legs of hermit crabs (*Clibanarius virescens* Krauss, 1843), though it remains unclear if this color change serves any adaptive function. Ontogenetic color changes in rock crabs (*Cancer irroratus* Say, 1817) are apparently associated with reduced susceptibility to fish predators as crabs increase in size (Palma and Steneck 2001). Shore crabs (*Carcinus maenas* Linnaeus, 1758) also show ontogenetic changes in color patterns that may be associated with different crypsis strategies as individuals grow (Nokelainen et al. 2019).

In other cases, coloration has been shown to serve signaling functions, including intra- and interspecific communication. Coloration has been well-studied in fiddler crabs of the genus *Austruca*, in which males' enlarged claws are used in territorial and courtship displays. For example, Dyson et al. (2020) reported that females of *A. mjoebergi* (Rathbun 1924) likely use the coloration of male claws for species recognition but have no preferences for claw colors, which fall within a species-specific range, suggesting that male claw coloration is not subject to sexual selection. However, in another fiddler crab species, *A. lactea*, Takeshita (2019) reported that males showed seasonal changes in coloration, but these changes varied for different body regions. In this species, coloration of different body regions may serve different purposes; for example, carapace coloration may function in thermoregulation and/or crypsis as well as in sexual signaling, while coloration of the chela is likely to function mostly or entirely in signaling to potential mates or territorial competitors. More recently, Li et al. (2022) found that food availability affects the brightness of the ultraviolet signals on the male's major claw, which, in turn, affects success in courtship. Signaling coloration has also been explored in the mantis shrimp *Neogonodactylus oerstedii* (Hansen, 1895), a species in which colored patches on the raptorial appendages are visible in a meral spread display, and the degree of luminance of meral patches affects the duration and intensity of conflicts (Franklin et al. 2016). Variability in dorsal coloration, on the other hand, seems to function as camouflage from fish predators in a habitat with variable backgrounds (Franklin et al. 2020). In the freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879), variable coloration, in combination with other morphological traits, distinguishes males of different developmental morphotypes and correlates with different male mating strategies (Ra'Anan and Sagi 1985).

Freshwater crayfish show a wide range of coloration, both within and between species, yet the causes of color variability in crayfish have largely been understudied (Shuster 2020). Sacchi et al. (2021) reported differences in saturation and brightness between body regions and by sex for the astacid crayfish *Pacifastacus leniusculus* (Dana, 1852) and suggested that color patterns in this species may be a result of conflicting selection for camouflage and conspicuousness for communication. Sexual dimorphism is present in the parastacid *Cherax quadricarinatus* (von Martens, 1868), in which males have soft red patches on both claws that may function as a signal to conspecifics and/or as a sensory structure (Karplus et al. 2003). Alternatively, other lines of investigation suggest that at least some color variation in crayfish may have no adaptive significance. Using a comparative phylogenetic approach using 466 crayfish species, Graham and Padilla Perez (2024) found that conspicuous coloration has evolved more than 50 times in the included taxa and was over-represented among semi-terrestrial species, which spend daylight hours in burrows. The investigators suggested that, in these cases, bright coloration may be selectively neutral, and its relatively frequent occurrence may be driven by small population sizes and low gene flow.

The capacity for color vision in crayfish is not thoroughly understood, but prior work suggests that crayfish may have at least 2-receptor color vision (Goldsmith and Fernandez 1968; Wald 1968). Wald (1968) determined that the eyes of the crayfish, *Faxonius virilis*, have 2 types of visual receptors, corresponding to absorption at about 435 and 565 nm. The compound eyes of crayfish have rhabdoms comprising 8 retinular cells; of these, 7 have been characterized as green (longer wavelength) receptors and the eighth as a violet (shorter wavelength) receptor (Cummins and Goldsmith 1981). In a study focusing on the longer wavelength receptors, Crandall and Cronin (1997) reported a relatively narrow range (522–530 nm) in spectral absorption among crayfish species despite the wide phylogenetic and ecotype diversity (i.e., burrow- and cave-dwelling species as well as species that inhabit lotic or lentic surface waters) included in their investigation. In an investigation of seasonal variability in spectral sensitivity in *Procambarus clarkii*, Hariyama (2004) found that "summer-type" crayfish had narrower sensitivity that peaked at 600 nm and contained only retinal. On the other hand, "winter-type" crayfish (which had been housed at low temperature and light conditions for an extended period) had broader spectral sensitivity and contained 2 chromophores, retinal and 3-dehydroretinal. Hariyama (2004) proposed the hypothesis that increased spectral sensitivity during mating seasons may provide greater contrast between the body or body regions against a muddy or rocky substrate, allowing individuals to distinguish, for example, the chelae of conspecifics from the background. There have been few empirical investigations directly examining visual acuity and propensity for color vision in crayfish, and their ability to distinguish the different components of color under natural conditions remains unexplored. However, van der Velden et al. (2008) reported that individuals of *Cherax destructor* are able to recognize familiar conspecifics based on differences in the shape of their faces but did not show similar ability to distinguish conspecifics based on differences in facial color. In *P. clarkii* and *C. destructor*, Suryanto et al. (2023) found that

individuals showed preferences for or repulsion from backgrounds of different colors.

The cambarid crayfish, *F. virilis* (Hagen 1870), is distributed over much of northeastern North America, and it is common in streams, rivers, and lakes on rocky or muddy substrates (Hamr 2002). The species exhibits a wide range of coloration, including yellow, olive, brown, and blue, with dappled or spotted patterns on some body parts (Thacker et al. 1993; Hamr 2003). Momot and Gall (1971) reported on the occurrence of blue "color phase" *F. virilis* in Michigan lakes, and found that blue individuals were scarce, comprising no more than 1% of the total catch. They also reported no evidence for differential predation rates on blue and brown phase individuals, suggesting that neither form was better camouflaged than the other. Thacker et al. (1993) studied *F. virilis* from a number of populations that showed local variability in coloration. They reported that individuals of *F. virilis* from a "blue morph" population were generally more active than were individuals from "brown morph" population and that in ~4-week reciprocal transplants, some individual crayfish showed a shift in coloration to be more similar to the coloration characteristic of the local population. That study also reported no evidence for differences in susceptibility to predation between the 2 color forms (Thacker et al. 1993), and the ecological and evolutionary significance of the 2 forms remains unclear. In 2 other cambarid species, *Cambarus bartonii bartonii* (Fabricius 1798) and *Faxonius obscurus* (Hagen 1870), Hartzell (2017) reported no evidence for ontogenetic color change and suggested that the presence of green and brown forms in both species must be driven by other factors.

The current investigation provides new insight into color variability in the North American cambarid crayfish *F. virilis*. Like all cambarid crayfish, individuals of this species undergo cyclical alternation of forms, between "form II" or non-reproductive morphology and "form I" or reproductive morphology (Hagen 1870; Creaser 1933). Form alternation is more easily diagnosable in males, which experience changes to the shape of the gonopods in addition to changes in chela size relative to body size. However, females of at least some species also undergo form alternation reflected in changes to the annulus ventralis (the seminal receptacle), the relative width of the abdomen, and/or to relative chela size (Wetzel 2002; Buřič et al. 2010; Kawai and McLay 2023). In temperate regions of North America, the life history of *F. virilis* has a clear annual cycle (Hamr 2002; personal observation). By late spring, both males and females, which have overwintered, generally undergo a molt from form I morphology to form II morphology. Summer is a period of molting and growth for both adults and juveniles, with both males and females undergoing one or more molts. By late summer, mature males and females molt into form I morphology, and the fall mating season begins. Individuals are thought to live around 2 years, with some individuals molting to form I in their first fall and reproducing in year 1, and others overwintering as juveniles and molting to form I in their second fall (Caldwell and Bovbjerg 1969; Weagle and Ozburn 1972; Mitchell and Smock 1991). In males, form alternation from non-reproductive to reproductive is associated with a greater allocation of growth to chelae as opposed to body length (Cabrera and Griffen 2023). In a congener, *F. obscurus*, the claws of reproductive form crayfish are stronger than those of non-reproductive crayfish for both males (Graham et al. 2023a) and females (Graham et al. 2023b).

In addition to morphological differences, form I and form II crayfish differ in behavioral traits. Tierney et al. (2008) reported that form II males of *F. rusticus* (Girard 1852) spent more time inside shelters than did form I males, and that form II males showed less agonistic behavior in intraform groups than did form I males. Atkinson et al. (2023) reported that form I individuals of *F. virilis* of both sexes sheltered less and were quicker to locate a food item than were form II individuals. Guiaşu and Dunham (1997a, 1997b) found no difference in fight dynamics in intraform contests in *Cambarus robustus* (Girard, 1852), but a later study showed that form I males of *C. robustus* are dominant to form II males (Guiaşu and Dunham 1998). Martin and Moore (2010) considered intersexual agonistic interactions in *F. rusticus* and found that reproductive form had a significant effect on contest outcome. Although males won most contests across all treatments when contests involved form I females and form II males, contests were equally likely to be won by either sex. Finally, in *F. quinebaugensis*, Warren et al. (2009) found that form I females showed significantly reduced agonistic behavior relative to form II females, but that males showed no such difference between forms.

In central Massachusetts, individuals of *F. virilis* show color variation similar to that described by Thacker et al. (1993), with some individuals showing brown or yellowish body coloration with blue coloration largely restricted to the chelae and others showing brown coloration over body and chelae. Moreover, blue coloration seems to occur more frequently in the fall, when crayfish are in their reproductive form, and in larger individuals (personal observation), suggesting that the coloration could be associated with conspecific signaling. This investigation tests the hypothesis that individuals show an ontogenetic color transition between brown and blue morphs in the transitions between the form II and form I morphology that is associated with physiological and behavioral changes related to reproduction. Furthermore, this investigation compares color variability based on size and sex, since coloration could be a signal used in inter- and/or intrasexual communication. I report coloration data from a group of crayfish, which was collected and maintained in the laboratory in early summer through mid-fall, to document individual changes in coloration over time and across the form-change molt.

Materials and Methods

Crayfish collection and care

Crayfish were collected by hand from the Mill River in the Blackstone River watershed in June 2022 and those that were not missing any limbs were returned to the campus of Worcester Polytechnic Institute, where they were housed individually in plastic bins 33.0 × 19.7 × 11.4 cm filled with tap water. Each crayfish received a PVC tube of ~15 cm in length for use as a shelter, but no material was added as a substrate. Polystyrene egg crate was used to cover the containers to prevent crayfish from escaping. A timer was used with fluorescent lighting to mimic the natural day–night cycle, with on/off times updated every 2 weeks during the period of the experiment. Temperature was maintained at ~22 °C. Each container received a ~50% water change 2 times per week, and crayfish were fed 3 times per week on an alternating diet of commercial rabbit food and commercial shrimp pellets. At the end of the final photography day, crayfish were held for a

further 7 days to monitor for molting or death and were then released at their collection site.

Digital photography

Photographs were taken and analyzed after the general approach developed by Bergman and Beehner (2008), which was shown to be both accurate and precise even across different light conditions, including natural light. Generally similar methods have been applied to investigate coloration in a range of taxa, including fish (Moran et al. 2017; Hemingson et al. 2022), reptiles (Sacchi et al. 2013; Ligon 2014), birds (Stoddard et al. 2016; Romano et al. 2021), mammals (Higham et al. 2021), invertebrates (O'Hanlon et al. 2017; Sacchi et al. 2021), and plants (Brothers and Atwell 2014; del Valle et al. 2018). Initial photographs were taken 18–21 days after collection. First, all crayfish were measured for carapace length and length of the right chela and were inspected for reproductive status. Before the first round of photographs, all males were confirmed by inspection of gonopod shape to be in form II (non-reproductive) morphology, and all females had no visible signs of glair gland development. After measurements, each crayfish was placed into a ~475 mL disposable plastic cup and covered in a layer of crushed ice. Crayfish were allowed to chill for ~10 min to reduce movement. Photography took place in the WPI greenhouse under natural light, filtered by clear glass windows using a Nikon Coolpix P7000 digital camera. Crayfish were removed from the ice bath, briefly blotted to minimize dripping water, and placed on a black foam background next to a Calibrite ColorChecker Classic Mini color standard card. Crayfish recover quickly from chilling (personal observation), so both the dorsal and ventral surfaces were photographed in rapid succession. A unique random letter or letter combination was included in each of the photos taken on a given day to allow photos to be analyzed without knowledge of the individual identity of each animal.

After being photographed, each crayfish was returned to its original bin and monitored for recovery, which took place in all cases within a few minutes of removal from the ice. The same photography procedure was repeated 2 more times with 7 days between each photography day. After the third photograph in the summer series, crayfish were held in the laboratory with feeding and water changes as above until the second series of photographs began in September, a gap of ~7 weeks. During this period, crayfish were monitored for molting or death, and molted crayfish were examined for any changes in reproductive form. By September 1, all male crayfish had molted into form I reproductive morphology as confirmed by inspection of the gonopods, and all females showed at least initial development of the glair glands indicated by opaque white coloration visible at the bases of the pleopods and on the ventral surfaces of the uropods. At this time, crayfish were re-measured and the series of 3 fall photographs took place using the identical procedure as the summer photographs. All photographs in both the summer and fall series took place at approximately the same time of day between 10 and 11 am.

At the start of the summer series, there was a total of 30 crayfish included in the sample (16 males, 14 females). Before the completion of the summer series of 3 photos, 2 crayfish died (1 male, 1 female) and their photos were removed from all analyses, leaving a final sample of 15 males and 13 females in the dataset.

RAW image files were converted to DNG format, and the software package ColorChecker Camera Calibration (v.2.2.0; X-Rite, Inc.) was used to create a custom color profile for each individual photograph. Using the plugin Camera Raw in Adobe Photoshop 2023, individual color profiles were applied to each photograph to adjust colors in the photograph to known color values in the color chart. To control for different light conditions, the white balance was adjusted using the color-neutral gray squares on the ColorChecker chart, and exposure was adjusted manually by matching red–green–blue values in the white square to 243 ± 5.

In Adobe Photoshop, the lasso tool was used to select pixels from 5 body regions, 4 on the dorsal surface and, 1 on the ventral surface (Fig. 1). These include 2 locations on the dorsal surface of the chelae (the pollex and the manus) and one location on the ventral surface of the chelae (the manus). It also includes 2 locations on the dorsal surface of the body: the carapace just anterior to the cervical groove and the second abdominal somite. These regions were chosen because prior experience with these populations indicated that color differences between individuals, where present, were most obvious (to human eyes) on the chelae (personal observation). Therefore, the 5 regions were selected to compare measures from the chelae to those from regions that may not show such pronounced differences, like the dorsal carapace and abdominal segments. Pixels were selected to avoid areas where light was reflected off water on the crayfish; these areas consisted of the parts of the crayfish that are relatively flat, like the center of the carapace, and were similar across most photographs. However, because each photograph was unique, there was some variability in the exact parts of the exoskeleton chosen for analysis from photo to photo, including between photos taken on different dates of the same individual. RGB levels were recorded and were then converted to the hue, saturation, and value (HSV) system. In this system, hue corresponds to the color (a circular parameter ranging from 0 to 1, though sometimes reported as an angle between 0° and 360°).

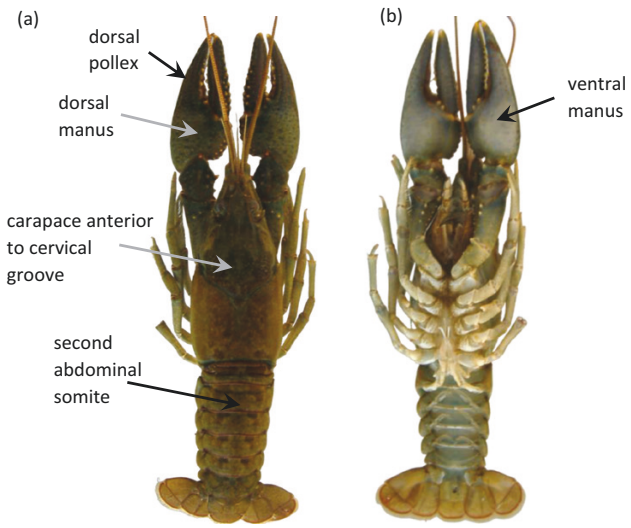

Figure 1 Five body regions included in color analysis of photographs. Four regions were sampled from photographs of the dorsal surface (a), and one region was sampled from photographs of the ventral surface (b). The same regions were targeted in each photograph, but specific pixels were chosen to avoid areas of reflected light, which were similar but not identical in all photographs.

A hue of "0" corresponds to red, ~0.33 or 120° corresponds to green, and ~0.66 or 240° corresponds to blue. Because of the circular nature of this parameter, a value of "1" or 360° also corresponds to red. Saturation refers to the amount of gray in the color (ranging from 0 to 1, where lower values correspond to a grayer or more faded color), and value refers to the brightness of the color (ranging from 0 to 1, with lower values corresponding to darker colors).

Data analysis

All analyses were carried out in R (version 4.1.1) (R Core Team 2021). Separate linear mixed models were used to explore the relationship between each of the dependent variables of hue, saturation, and value for each photographed body region and factors including body size, sex, and season, for a total of 3 models per body region. All models were constructed using the package "brms" (Bürkner 2017), which interfaces with Stan (Stan Development Team 2020). Models were run with uninformative priors and 4 chains and consisted of 5,000 iterations with a warm-up of 1,000. All models converged with low among-chain variability with Rhat = 1. Posterior predictive checks and trace plots were used to check for adequate mixing. All models used a Gaussian distribution with an identity-link function.

Individual crayfish identity and photo number of the series of 6 photos were included as random effects in all models, and full models included fixed effects of season, sex, carapace length, and all 2-way interactions. Carapace length was included as the measurement taken for each season; color measurements taken from a particular crayfish in the summer, for example, included that crayfish's carapace length before the summer series, while color measurements taken from that crayfish in the fall included its carapace length before the fall photo series. Because all crayfish were molted at least once between the summer and fall photo series, virtually all crayfish showed an increase in size between the 2 series; only one crayfish had the same measurement in the summer and fall series.

In addition to full models, I also ran reduced models with all combinations of main effects and 2-way interactions, as well as a model with only the random effects of individual identity and photo number. Sets of models for each dependent variable were compared using the "LOO" leave one out cross-validation function in brms (Vehtari et al. 2017). Significance was evaluated by examination of posterior means and 95% credible intervals (CI). Effects that had 95% CIs, which did not include zero, are reported as significant.

Results

A total of 15 male and 13 female crayfish were included in both the summer and fall photograph series. In this dataset, the most pronounced differences in coloration were between seasons, with fall reproductive form crayfish showing different color parameters than summer non-reproductive form crayfish (Fig 2). However, these differences were not consistent across all body regions. In particular, hue, the parameter that captures color value, showed significant seasonal variation for all 3 chela regions but not for the body regions on the carapace or the abdomen (Fig. 3, Table 1). On average, chelae showed more blue or green coloration in the fall, whereas in the summer, chela coloration tended to be primarily brown, similar in color to the carapace and abdomen. Furthermore, variability in chela coloration was elevated in the fall, higher than both chela coloration variability in the summer, and coloration of the carapace and abdomen in either season (Fig. 3, Supplementary Fig. S1). Some individuals showed little seasonal change in chela coloration, whereas others showed substantial increases in hue in their reproductive form relative to their non-reproductive form. Finally, there was no evidence of any difference in hue between males and females or based on differences in body size.

The data also reveal differences in saturation, or the amount of grayness in the color, based on multiple factors. The most consistent effect across body regions was again a seasonal difference: all measured body regions showed significantly lower saturation in the fall except for the dorsal carapace, which showed no difference between the seasons (Fig. 3, Table 1). Thus, reproductive crayfish showed generally grayer coloration compared with non-reproductive crayfish. There were also some significant effects of carapace length on saturation measures of the chela (Table 1), with larger crayfish showing lower saturation values, but these effects were generally small (Supplementary Fig. S2). Finally, for the dorsal surface of the abdomen, there were seasonal differences based on sex. Males showed lower saturation measures for the abdomen than females in their non-reproductive form, but this difference was small (Fig. 3, Table 1).

For value, which quantifies the brightness or lightness of a color, there were significant differences between the crayfish in their summer non-reproductive form and in their fall reproductive form for all 5 body regions. Crayfish were generally lighter in the fall than in the summer. There were also small but significant differences in value based on the interaction of season and sex for measures of the dorsal and ventral manus surfaces (Fig. 3, Table 1).

Discussion

This investigation demonstrates that color in *F. virilis* shows variability at several levels. First, the data showed clear within-individual variability in coloration. Some of this variability was consistent throughout the experiment; specifically, ventral surfaces are generally paler, reflected in consistently lower measures for saturation and higher measures for value brightness for the ventral surface of the chela. Darker and more saturated dorsal coloration may reflect selection for color-matching to the rocky substrate of the species's natural habitat. Future investigations could include tests of this hypothesis considering the range of background coloration that individuals of *F. virilis* experience in their natural habitat. Other within-individual variability was more complex and showed ontogenetic change. For example, in the summer non-reproductive period, there was little variability in coloration of different body regions on the dorsal surface, but in the fall reproductive period, there was a pronounced difference in the hue and saturation measures of the chelae as opposed to other body surfaces. Reproductive form crayfish had claws, which were on average greener or bluer than non-reproductive form crayfish, while body coloration hues of the carapace and abdomen remain largely unchanged between the reproductive and non-reproductive forms. The chelae of reproductive crayfish also had lower saturation measures than those of non-reproductive crayfish, indicating that the coloration of these body regions was generally less intense in the reproductive form.

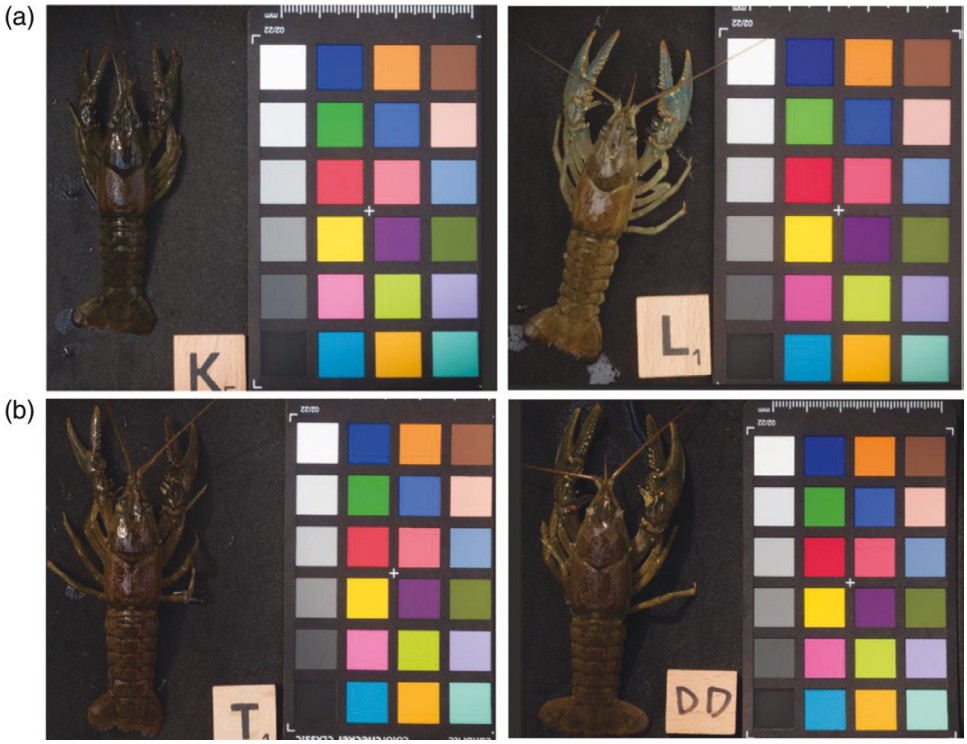

Figure 2 Examples of crayfish showing transition between form II (left panels) and form I (right panels). The images in panel (a) show a male that transitioned from a generally brown coloration in form II to a blue-clawed coloration in form I, whereas images in panel (b) show a male that had little color change associated with the form II to form I transition.

However, there was also a pronounced increase in inter-individual variability in both hue and saturation measures of the chelae as crayfish transitioned from non-reproductive to reproductive form. Some individuals showed a larger magnitude of change in hue and saturation of the chelae; these individuals had distinctly blue or green coloration of the chelae, especially on the dorsal surface. Other individuals showed minimal or no color change between their non-reproductive and reproductive forms. Thus, prior reports of "blue" and "brown" morphs of *F. virilis* (e.g., Momot and Gall 1971; Thacker et al. 1993) may refer to ontogenetic shifts associated with reproductive cycles.

Although the evolutionary and ecological significance of crustacean color variation is not well-studied, there is a better understanding of the physiological and genetic mechanisms that contribute to such variation. External coloration in malacostracan crustaceans is attributed to carotenoid pigments; blue coloration in lobsters, for example, is attributed to a high concentration of crustacyanin, while a yellower coloration results from a higher concentration of crustochrin (Buchwald and Jencks 1968; Cianci et al. 2002; Tlusty and Hyland 2005). The crustacyanin gene family is unique to crustaceans, and Wade et al. (2009) suggested that the gene family likely originated early in the evolution of malacostracans and may have contributed substantially to their evolutionary success. The study found that the spatially variable deposition of crustacyanin complexes in the exoskeleton is key in driving the diversity of colors and color patterns observed in this group.

The function of color patterns and color variation is much less understood in crustaceans, especially crayfish. Shuster (2020) provided an extensive review and analysis of color and color patterns in crayfish, but few investigations have provided insight into whether or how variable coloration is used for signaling to conspecifics and/or heterospecifics. Physiological investigations indicate that crayfish may be capable of distinguishing among at least some colors (Goldsmith and Fernandez 1968; Wald 1968; Cummins and Goldsmith 1981), suggesting that there is at least the potential for adaptive evolution of color for communication, though this has not been empirically demonstrated in any crayfish species. On the other hand, color variability in *F. virilis* could be selectively neutral, as seems to be the case in a number of other crustaceans. For example, Caro et al. (2019) found no support for the hypothesis that color dimorphism in coconut crabs, *Birgus latro*, results from selection for camouflage in different microhabitats, and suggested that the 2 color morphs may represent a neutral polymorphism in this species. Likewise, João et al. (2023) suggested that variable coloration in adults of the land crab *Johngarthia lagostoma* is probably selectively neutral rather than a form of camouflage in a variable environment because adults of this species are rarely subject to predator attack. In crayfish, Graham (2023) pointed out that color variation is relatively common in semi-terrestrial burrowing species, which are unlikely to be able to detect conspecific coloration underground and suggested that color variation in at least some crayfish species may be selectively neutral. However, it should be noted that Graham (2023) focused on color variation that is "verifiably different" from average coloration for a species or a population—in other words, variants that are striking outliers and immediately distinguishable from the rest of a population by human observers, a phenomenon that is well-documented in crayfish. It may be necessary to distinguish such variants from the "normal" range of color variation that may exist in a population for the purposes of understanding the adaptive significance of color evolution.

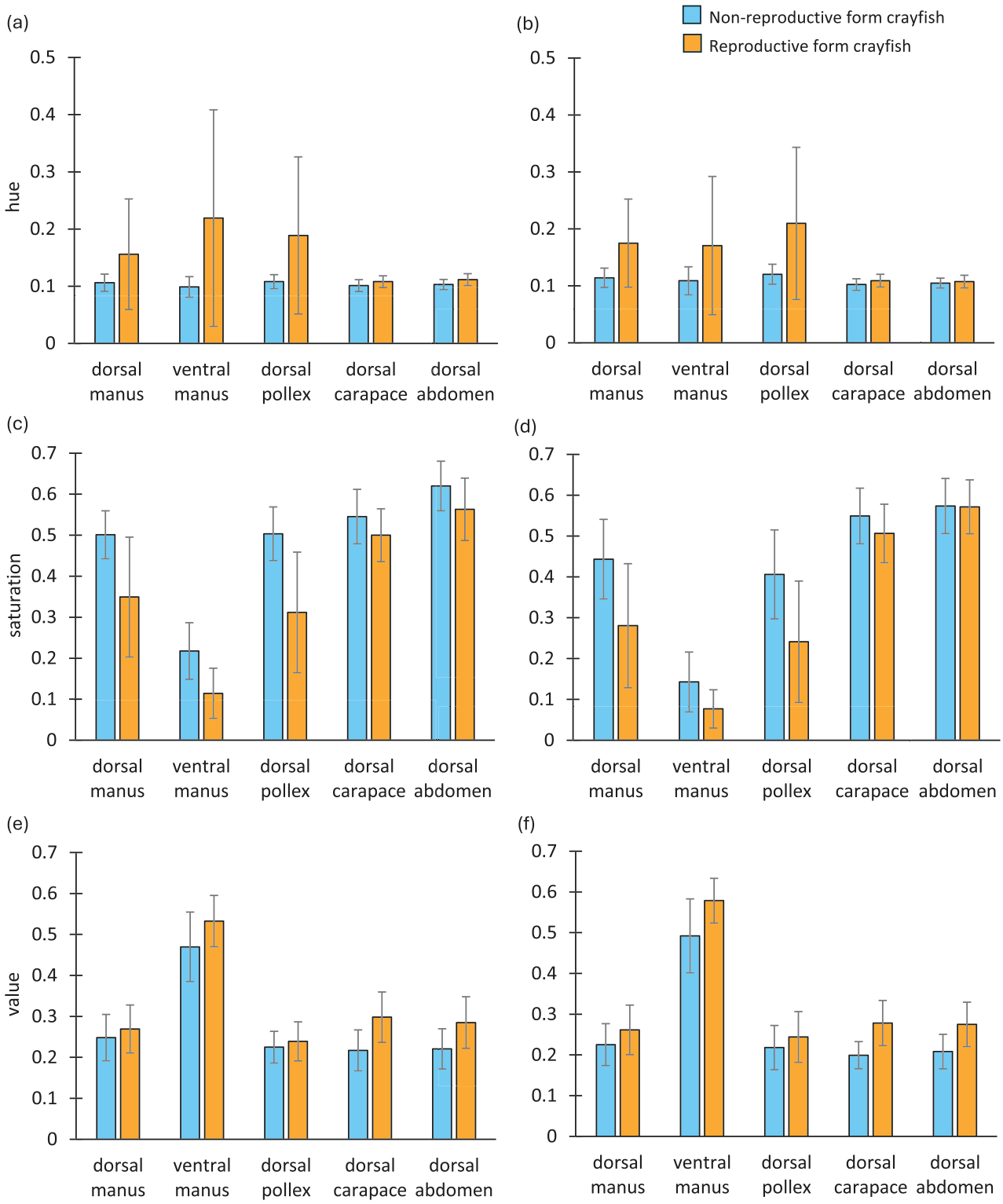

Figure 3 Mean values for hue, saturation, and value for each measured component of the carapace shown for summer, when crayfish were in their non-reproductive form (blue bars) and fall, when crayfish were in their reproductive form (orange bars). All 3 parameters can theoretically range from 0 to 1. For hue, values close to 0 indicate redder color, with higher numbers indicating progression through the visible spectrum. For saturation, lower values indicate more grayness. For value, lower numbers indicate more darkness. Panels (a), (c), and (e) show data for female crayfish, whereas panels (b), (d), and (f) show data from male crayfish. Error bars show standard deviation.

In the current investigation, there are a number of possible explanations for the observed changes in coloration. First, it is important to note that there were no consistent patterns related to either sex or body size. Both carapace length and sex were significant predictors in a few cases. For example, "sex" was a significant predictor of value and saturation for the ventral surface of the chela; there was no corresponding effect of sex on color measures of the dorsal surface of the

chela, and it is not clear whether this represents a meaningful difference between males and females in chela coloration. In addition, carapace length also was a significant predictor of some measures of saturation in the chela. However, the data from this study yield no convincing evidence that either sex or body size is correlated with color differences. This is different from the insights reported by Sacchi et al. (2021) for *P. leniusculus*, which did show differences in saturation and brightness related to sex and size; the authors suggested that these patterns of color variation may indicate that color functions in communication and exists as a compromise between selection for conspicuousness and camouflage. Conversely,

Table 1 Predictor effects from LMMs for dependent variables of hue, saturation, and value for 5 different body regions, indicated in Figure 1.

Body region		Predictor	Estimate ± SE	95% CI
Dorsal surface of manus	Hue	Season	**0.23 ± 0.1**	**0.04, 0.42**
		Carapace length	0.01 ± 0.01	0.00, 0.02
		Sex	0.01 ± 0.02	-0.00, 0.05
		Season × carapace length	0.01 ± 0.00	-0.01, 0.00
	Saturation	Season	**-0.56 ± 0.17**	**-0.88, -0.23**
		Carapace length	-0.01 ± 0.02	-0.06, 0.03
		Sex	0.32 ± 0.41	-0.47, 1.15
		Season × carapace length	0.01 ± 0.00	0.00, 0.02
		Sex × carapace length	-0.01 ± 0.01	-0.04, 0.01
	Value	Season	**0.17 ± 0.07**	**0.02, 0.31**
		Carapace length	0.03 ± 0.01	0.00, 0.06
		Sex	0.33 ± 0.23	-0.11, 0.78
		Season × carapace length	-0.01 ± 0.00	-0.01, 0.00
		Sex × carapace length	-0.01 ± 0.01	-0.03, 0.00
		Season × sex	**0.04 ± 0.02**	**0.01, 0.07**
Dorsal surface of pollex	Hue	Season	**0.09 ± 0.02**	**0.05, 0.12**
	Saturation	Season	**-0.71 ± 0.16**	**-1.02, -0.40**
		Carapace length	**-0.04 ± 0.01**	**-0.06, -0.02**
		Sex	-0.07 ± 0.04	-0.15, 0.01
		Season × carapace length	**0.02 ± 0.00**	**0.01, 0.03**
	Value	Season	**0.02 ± 0.01**	**0.01, 0.04**
		Sex	0.00 ± 0.02	-0.01, 0.04
Carapace anterior to cervical groove	Hue	Season	0.03 ± 0.02	0.00, 0.06
		Carapace length	0.00 ± 0.01	0.00, 0.00
		Sex	0.04 ± 0.02	-0.11, 0.03
		Sex × carapace length	0.00 ± 0.00	0.00, 0.00
	Saturation	Season	-0.04 ± 0.02	-0.07, -0.01
		Carapace length	0.00 ± 0.00	-0.01, 0.01
	Value	Season	**0.19 ± 0.06**	**0.07, 0.31**
		Carapace length	0.01 ± 0.00	0.00, 0.02
		Sex	-0.02 ± 0.02	-0.06, 0.02
		Season × carapace length	0.00 ± 0.00	-0.01, 0.00
Dorsal surface of second abdominal somite	Hue	Season	0.04 ± 0.02	0.00, 0.07
		Carapace length	0.00 ± 0.00	-0.01, 0.00
		Sex	-0.02 ± 0.04	-0.09, 0.05
		Season × carapace length	0.00 ± 0.00	0.00, 0.00
		Sex × carapace length	0.00 ± 0.00	0.00, 0.00
		Season × sex	-0.01 ± 0.00	-0.01, 0.00
	Saturation	Season	**-0.31 ± 0.01**	**-0.50, -0.11**
		Carapace length	0.01 ± 0.02	-0.02, 0.04
		Sex	0.10 ± 0.27	-0.43, 0.65
		Season × carapace length	0.01 ± 0.00	0.00, 0.01
		Sex × carapace length	-0.01 ± 0.01	-0.02, 0.01
		Season × sex	**0.06 ± 0.02**	**0.02, 0.10**
	Value	Season	**0.07 ± 0.01**	**0.05, 0.08**
		Sex	-0.01 ± 0.02	-0.05, 0.02

Table 1. Continued

Body region		Predictor	Estimate ± SE	95% CI
Ventral surface of manus	Hue	Season	**0.17 ± 0.06**	**0.07, 0.34**
		Carapace length	0.06 ± 0.05	-0.04, 0.17
		Sex	0.00 ± 0.01	-0.01, 0.02
		Season × carapace length	-0.06 ± 0.03	-0.11, 0.00
	Saturation	Season	**-0.42 ± 0.10**	**-0.62, -0.23**
		Carapace length	**-0.04 ± 0.01**	**-0.06, -0.01**
		Sex	-0.29 ± 0.21	-0.72, 0.11
		Season × carapace length	0.01 ± 0.00	0.00, 0.02
		Sex × carapace length	0.01 ± 0.001	0.00, 0.02
	Value	Season	**0.31 ± 0.08**	**0.16, 0.46**
		Carapace length	0.03 ± 0.02	0.00, 0.06
		Sex	0.02 ± 0.29	-0.56, 0.59
		Season × carapace length	-0.01 ± 0.00	-0.01, 0.00
		Sex × carapace length	0.00 ± 0.01	-0.02, 0.02
		Season × sex	**0.04 ± 0.02**	**0.01, 0.08**

See "Materials and Methods" section for details on model selection. Significant predictors (those whose 95% CI does not include 0) are indicated in bold.

the most convincing pattern of color variation in the current study of *F. virilis* occurs in the ontogenetic transition from non-mating to mating form.

These color changes in *F. virilis* may be selectively neutral, neither the result nor the target of adaptive evolution, if, for example, they are associated with other physiological changes. Alternatively, a seasonal shift in coloration may reflect seasonal differences in the visual perception of predators. For example, fall coloration could represent better camouflage given changing environmental conditions. However, the current study showed not only a color difference between seasons but also elevated variability in color within the fall mating season, with only a subset of the sample of crayfish undergoing a color shift associated with the transition to mating form. The elevated intrapopulation color variability only during the mating season suggests that color may function in some way in interspecific communication or may be associated with physiological or behavioral differences related to mating interactions, and future work should focus on elucidating any such functions. Thacker et al. (1993) reported behavioral differences, for example, in activity levels between blue and brown "morphs" of *F. virilis* but did not report the reproductive form of the crayfish in that study, referring only to the experiments as occurring during the "summer." Thus, the animals used in that study could have been a mix of reproductive and non-reproductive form individuals, which would complicate any comparisons of behaviors between different color forms.

Color changes may also result from extended captivity or from diet. Although Thacker et al. (1993) reported no observable color effects related to diet in their study, at least one other investigation has shown that individuals of the marbled crayfish *P. fallax* f. *virginalis* fed a commercial diet in a laboratory setting experience differential color changes depending on the astaxanthin content of the diet (Kaldre et al. 2015). In the current study, the effects of extended captivity, including diet or light regime, may explain some of the observed color changes. For example, this seems likely to explain the observed changes in value, which increased slightly but consistently over all measured body regions and showed similar inter-individual variability over the entire study period. The observed changes in hue and saturation could also be related to laboratory conditions, but the high intra- and inter-individual variability are not consistent with a laboratory effect where all crayfish were subject to the same housing and diet conditions.

In conclusion, this investigation sheds new light on the phenomenon of color variability in crayfish and suggests interesting new lines of investigation. It remains unclear whether inter-individual color variability represents a genetic difference within populations or if the different color forms that exist during the reproductive season reflect, for example, condition or other physiological differences between individuals. Future investigations should also focus on elucidating whether color variation may be a target of adaptive evolution, with chela coloration possibly functioning in intraspecific communication. Given the wide variability in color and color pattern both within and between crayfish species, there are plentiful opportunities for future research.

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Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

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