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Colour variation in signal crayfish *Pacifastacus leniusculus*

Roberto SACCHI*, Stefania CANCIAN, Daniela GHIA, Gianluca FEA, Alan COLADONATO

Dipartimento di Scienze della Terra e dell'Ambiente, Università degli Studi di Pavia, Viale Taramelli 24, Pavia, I-27100, Italy

*Address correspondence to Roberto Sacchi. E-mail: roberto.sacchi@unipv.it

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Abstract

External colouration in animals depends on the interaction of several different factors including the genetics and epigenetics processes that underlie the colour expression, the mechanisms of colour perception, and the general mechanisms controlling colour evolution and function. Among all, camouflage from predators and conspicuousness are of particular interest since pose animal to choose between opposite adjustment in colouration. The external colouration of crustaceans is mainly due to the accumulation of carotenoids in the exoskeleton and the epidermal layer, and the trade-off between camouflage and communication had lead to a variety of responses, involving signal partitioning, spectral sensibility, changing colouration or signalling behaviour. Here, we used digital images to explore intrapopulation variability of the external colouration of *Pacifastacus leniusculus* among body regions within an individual and between sexes. We found that i) ventral colouration of claws are more saturated and brilliant than upperparts, ii) males express a more saturated and brightness colouration than females, especially on the lower portion of claws, iii) colour intensity and brightness increases with size differently in different body regions, and iv) brightness is more variable in males than in females. All the above patterns support the hypothesis that colour in this species could be the result of a compromise between camouflage from predators and conspicuousness for communication. The results of this study suggest that carotenoid might have something to do with intraspecific communication and perform more complex functions than that of a simple pigment.

Keywords: external colouration, crayfish, individual variation, sexual dimorphism, HSV

The study of animal colouration is an interdisciplinary sector in great growth that tries to explain the different ways in which animals develop the colours, the genetics and epigenetics processes that underlie the colour expression, the mechanisms of colour perception, and the general mechanisms controlling colour evolution and function (Cuthill et al. 2017). A lot of effort has been directed to these mechanisms, with the understanding that animal colouration represents a compromise between pressures that may conflict one another, rather than the result of a single key factor (Endler 1981, Stuar-Fox and Moussalli 2009, Cuthill et al. 2017). Camouflage from predators and conspicuousness for communication requires opposite adjustment in colouration, as crypsis leads to reduce the contrast between colour and background, whereas signals for communication promote bright and flashy colours, increasing the contrast with the background (Franklin et al. 2020). This trade-off between has several solutions both animals, including i) signal partitioning, in which body regions visible to predators are used for camouflage and body regions hidden from potential predators are used for signalling (Stuart-Fox and Ord 2004; Sacchi et al. 2007), ii) colours that are not visible to

predators but only to conspecifics, like UV or polarized light (Cummings et al 2003; Siebeck et al. 2010, Abramjan et al. 2020), iii) spatial resolution of colouration in a way that colour is conspicuous only at a short distance, while at distance colours blend matching the background (Barnett er al. 2018), and iv) changing colourations that become cryptic or conspicuous when necessary (Stuart-Fox et al. 2008), or v) altering signalling behaviours when predators are present (Endler 1987).

Crustaceans show a huge variety of colour patterns that are used both for camouflage to predators (Stevens et al. 2013; Stevens et al. 2014; Nokelainen et al. 2017) and communication with conspecifics (Detto et al. 2008). As in other taxa, even in crustaceans, the trade-off between camouflage and communication had lead to a variety of responses. For example, stomatopods employ signals that can be hidden from predators and use primary body colouration for camouflage (Gagnon et al. 2015, Franklin et al. 2020), male crabs of genus *Uca* possess a larger coloured claw, which waves in species-specific undulations during courtship (Zeil and Zanker 1997), and several crabs display prominent colour patterns on the carapace and claw that allow recognition and mating attraction (Cummings et al. 2008). Changing in camouflage requirements as well as in behavioural pattern causes ontogenetic colour changes and this kind of response is quite common in crustaceans. For example, the isopods (*Idotea montereyensis*) change from red to green to match the background as they change the habitat from red to green algae (Lee 1966). Similarly, the shore crabs (*Carcinus maenus*) keep white spots on the carapace for camouflage in mussel beds, but lose them when grow and move into the algae (Todd et al. 2006). Although a lot of effort has been devoted to the analysis of the relationship between colouration and background (e.g., Thacker et al. 1993, Franklin et al. 2020), the variability in the colour pattern within a population, including sexual dimorphic colourations and individual variation, has been often neglected in crustaceans. When sexually dimorphic colourations are found, they are usually interpreted in terms of adaptation for predator avoidance (e.g. *Idotea baltica*, Jormalainen et al.1995; *Austinixa aidae*, Peiró et al. 2013). However, in some cases, sexually dimorphic colourations cannot be explained in terms of camouflage, such as the soft red patch in the crayfish male *Cherax quadricarinatus*, which possibly transmits information about the gender, size, and quality of its owner (Karplus et al. 2003). Intra-individual variability in colouration in crustaceans has been attributed to a range of factors including diet (Harpaz et al. 1988; Kaldre et al. 2015), background substrate colour (Beingesser and Copp 1985; Thacker et al. 1993), light condition (Thacker et al. 1993), genetic variation (Dunham et al. 1979; Walker et al. 2000) and ontogeny (Beingesser and Copp 1985; Hartzell 2017). An additional factor increasing variability in colour patterns is the ability of some crustaceans, and more in general arthropods, to individually recognize conspecifics through their morphological traits including colour (Vannini and Gherardi 1981; Tibbets 2004; Detto et al. 2006; Van der Velden et al. 2008; Detto et al. 2008). In order to fully understand the function of variation in the colour pattern of crustaceans, it is necessary to first describe the naturally occurring diversity among individuals at a population level (Bauer 1981, Detto et al. 2008).

Among crustaceans, freshwater crayfish form a group in which the knowledge on the meaning of colour and colour pattern is still largely incomplete, also because colours quickly disappear within a few days of being preserved in fixatives such as formalin or alcohol (reviewed in Schuster 2020). Variation in colour patterns among crayfish results from contrasting colour in a variety of structure in different body regions including antennae, rostral margins, leg joints, spines, claws, carapace, telson and uropodes, just to name a few (but see Schuster 2020 for detail and pictures). Freshwater crayfish show a wide intra-population variability in colouration in terms of saturation, hues, and brightness (Schster 2020), which has been the focus of numerous studies, especially resulting in a description of causative mechanisms of colourations, i.e. pigments and their distribution in the skin, rather than an analysis of the environmental

pressure maintaining colour variation at population levels. For example, Beingesser and Copp (1985) reported that young *Procambarus clarkii* are green and match the background with physiological colour changes, but shift to a morphologically fixed red colouration when adults. Hartzel (2017) found that intraspecific colour variation in the *Cambarus bartonii* and *Faxonius obscurus* does not vary due to an ontogenetic shift in colouration, and hypothesized that some underlying genetic factors may be most likely responsible for the observed patterns in both crayfish species. Kaldre et al. (2015) supplied adult *Procambarus fallax* f. *virginalis* with different food and found a positive correlation between feed quality and the intensity of carapace colouration, but did not find any effect of feed quality on survival. Thacker et al. (1993) studied colour variation in *Orconectes virilis* both in field and laboratory conditions and showed that adults can change colour, brown morphs becoming blue and vice versa.

The signal crayfish *Pacifastacus leniusculus* is native to northwest North America, but, due to introductions by humans, is now the most widely spread alien crayfish species in Europe (Kouba et al. 2014). Crayfish of this species are bluish-brown to reddish-brown, occasionally light- to dark-brown; underside of claws is red and the white-turquoise oval patch on the upper side of the claws is unique (Souty-Grosset et al. 2006). Signal crayfish have a predominantly nocturnal behaviour (Styrishave et al. 2007; Johnson et al. 2014), but they remain relatively active during daylight hours, showing an adaptation to day-activity until to about 34% of the total activity time (Lozan, 2000). The species is able to a subtle deep visual detection at least against predators (Blake and Hart 1993). This paper we used digital image analysis to quantitatively describe colour variability among individuals within a population and among different body regions within an individual as well, taking into account the effects of sexes and ontogeny (size).

Materials and Methods

We collected signal crayfish during autumn 2018 from two stretches in the Valla stream (Northern Italy) of approximately 200 m long. Crayfish were searched by night when they are more active and outside the covers, collected by hand and placed into containers with stream water. All individuals that have just undergone a moult were excluded from the sample. After capture, each individual was sexed and was measured to record the cephalothorax length (CL, from the tip of the rostrum to the posterior median edge of the cephalothorax) using a digital calliper (accuracy 0.1mm); then, crayfish have been taken into the laboratory of the University of Pavia and photographed for the colour measure in ten different body regions using a Nikon D50 camera at a 1.2-million-pixel resolution, equipped with a Nikkor 60 mm AF-S Micro lens, and fixed on a stand at a distance of 18 cm. For each individual, we took high-resolution digital images of 10 different body parts (Fig.1), including thorax (TRX), rostrum (RST), left and right upper claws (UCLWdx, UCLWsx), left and right lower claws (LCLWdx, LCLWdx), left and right upper oval patch (UOPdx, UOPsx), and left and right lower oval patch (LOPdx, LOPsx). Each portion was photographed adjacent to a GretagMacBeth Mini ColorChecker chart (24 colour references, 5.7 cm × 8.25 cm) in a 44 × 44 cm lightbox illuminated with two daylight 22 W circular neon tubes (Reporter 55100 Studio-kit). Overall, the sample included 152 individuals, 100 males and 52 females. Males were on average 85.9 ± 17.3 mm long (range: 40.8 – 120.8 mm) with a cephalothorax of 43.8 ± 9.5 mm (range: 19.8 – 67.0 mm), while females were 80.8 ± 13.7 mm long (range: 61.0 – 114.1 mm) with a cephalothorax of 39.3 ± 6.5 mm (range: 29.3 – 55.0 mm).

Colour measures

Images were analysed in the RGB colour system following the method proposed by Bergmann and Beehner (2008) and Sacchi *et al.* (2013). Firstly, we used the Camera plug-in for Adobe Photoshop CS3 to create a new colour profile that adjusted the colour in the photographs (in the jpeg format) to the known colour levels in each square of the ColorChecker chart. Then, for each image, we selected the 'region of interest' (ROI) using the 'lazoo' tool, and pixels corresponding to point of reflected light were removed using the package "magick" in R (Ooms 2018). After that, we analysed on average 27554 ± 14082 pixels for image, ranging from 10478 to 46079 of LOPdx and TRX respectively. Finally, the RGB colour values were rearranged in the Hue, Saturation, and Value (HSV) system. In this system the cubic geometry of the RGB colour space is rearranged in a cylindrical-coordinate system in which the hue is the angle around the vertical axis and corresponds to the colour lights, the saturation is the distance from the axis, and the brightness corresponds to the distance along the axis.

We were not able to achieve HSV values for all portions of the 152 individuals since some individuals lacked claws or had damaged rostrum. Therefore, the final sample included 1420 images, of which 152 TRX, 149 RST, 141 UCLWdx, 140 UCLWsx, 142 LCLWdx, 136 LCLWdx, 141 UOPdx, 141 UOPsx, 142 LOPdx, and 136 LOPsx.

Statistical analysis

Colour variation in sampled crayfish was assessed in a two-step analysis: firstly, we generate the individuals' frequency distributions of hue, saturation, and brightness by using values computed on the whole samples of pixel analysed for each individual; secondly, we used those distributions to compute single HSV values for each individual, and use it in statistical analyses through random intercept Linear mixed Models (LMM). Frequency distributions of the HSV values were obtained by processing data of each image with a kernel density function (bandwitH = 0.02) and normalization to one. In this way, we obtained a probability density distribution of the three colour measures for each body region within an individual. These distributions were averaged to estimate the mean density distributions (± 95% CI) for the HSV values in each body region, separately for males and females. Since all distribution were bell-shaped (see results), we extracted the distribution modes of hue, saturation and brightness as descriptors for the external colouration of crayfish, and we used them in the statistical analyses. LMMs to analyse intra- and inter-individual variation of crayfish' external colouration included the three-way interaction sex × body region× size as the fixed effect. The size was expressed as the standardized CL of individuals and was used a proxy for individual age (Flint 1975). The individual entered the model as the random effect (random intercept) to account for unexplained variation at the individual level (σ^2_{ind}), after we controlled for the explanatory variables. The dependent variables were the H, S and V modes of each individual, as estimated by the peak of the density distributions. So three different models were run, one for each colour measure. The initial model was simplified using backward elimination of the not-significant terms (likelihood-ratio χ^2 test, Zuur et al. 2009), and we checked residuals of the initial model for normality and homoscedasticity (Zuur et al. 2009). Finally, pseudo-R^2 accounting for the variance explained by the model (fixed and random e□ects combined) and for the fixed e□ects alone were computed according to Nakagawa and Schielzeth (2013). In order to test if males showed a larger among-individual variance than females, we used the F-test for homogeneity of variances on the HSV values predicted by the LMM models obtained at the end of the simplification procedure. Analyses were performed in R ver. 3.2.4 (R core Team 2016) using the packages lme4 (Bates et al. 2015), LmerTest (Kuznetsova et al. 2017), and merTools (Jared and Frederick 2019) to generate models and perform statistical tests, whereas the package MuMIn (Barton 2019) was used to compute the pseudo-R^2. Otherwise stated, data reported are means ± standard errors.

Results

The density distribution curves of the HSV values computed over the 10 body regions in males and females (Figs. 2–4) revealed a substantial variation in external colouration of crayfish as much between sexes as among portions within the same individual. The mean density distributions of hue values had a bell-shaped appearance in all analysed portions in both sexes (Figure 2), ranging from red-orange (H = 0.05) to lemon-yellow (H = 0.20), with a peak near to orange-yellow (H: 0.12 – 0.15). Males appeared to have a redder shade than females, and the same was much more evident for lower portions with respect to upper portions in both sexes. Mean density distributions of saturation (S values, Figure 3) differed much more between sexes and body regions than hue values did. Colour was more saturated in males than females, and the same occurred in lower portions with respect to upper portions of the same individual. Finally, the mean density distribution of brightness (V values, Figure 4) confirmed differences previously observed: males had more brilliant aspect, as well as lower with respect to upper portions within an individual.

Analyses with LMMs supported statistically all the observed pattern, as for each one of the three colour measures we found a significant effect of the interactions body part × sex and body part × size (Table 1, and Table S1 for means and standard errors). Models were able to explain a large proportion of the total variance of colour variation: fixed effects captured 36.9%, 61.0%, and 77.2% of the variation in H, S, and V values respectively. These values raised to 81.9%, 70.5%, and 83.9% respectively when we included the proportion of variance associated with the random effect.

The body part × sex interaction suggested that i) the colour of the exoskeleton differed among body regions within an individual, but with different patterns in males and females, and ii) the colour of the exoskeleton differed between males and females but only limitedly to some body regions (all statistics for pairwise comparisons of HSV of body regions within and between sexes are reported in Supplementary Tables S2, S3, and S4). In both males and females, the colour of lower claws and oval patches was significantly redder, more saturated, and more brilliant than in all other body regions (Figure S8). Still in both sexes, the rostrum was significantly more orange (higher hue), more saturated but less brilliant than thorax, upper claws and oval patches (Figure S8). The colour of both upper claws and oval patches of males was redder and more saturated than the colour of thorax, whereas the brightness was similar (Figure S8). In females, the colour was more saturated in upper claws and oval patches than in thorax, but no difference occurred for hue and brightness (Figure S8). Finally, upper oval patches were redder and showed a more saturated colouration than upper claws in both sexes (Figure S8).

Males and females did not differ for hue values in all body portions but rostrum, which had lower values (i.e. was redder) in males (Figure S8). Saturation of colour in upper and lower claws was significantly higher in males than in females, and the same was true for lower oval patches (Figure S8). Finally, the colour was more brilliant in males than in females in lower claws and lower oval patches (Figure S8).

The body part × size interaction revealed ontogenetic effects on the expression of colour in the exoskeleton of *P. leniusculus*, but with different patterns in the different body regions. So, differences among body regions in the external colouration within individual varied according to the body size (Figure 5, all statistics for pairwise comparisons of slopes among body regions within an individual are reported in Supplementary Tables S5, S6, and S7).

The hue value did not have a consistent pattern of variation among body regions, as it slightly decreased, remained unchanged, or even slightly raised with increasing individual size (Figure 5). However, none of the slopes significantly differed from zero (Table S5). Despite this, pairwise differences in some cases became significantly more pronounced

in larger individuals as the results of the opposite tendencies observed thorax and rostrum with respect to claws and, especially, oval patches (Figure 5). Notably, the difference between thorax and rostrum respect to upper oval patches significantly increased by 4-7 times in larger individuals, whereas the opposite occurred with respect to lower oval patches, which gives the difference to significantly decrease by 1.5 – 2 times (Figure 5). Thorax and rostrum besides shared the same pattern of variation.

Saturation significantly increased with growing crayfish size in all body regions, but upper oval patches. The effect was statistically significant for thorax, left upper claw, lower claws, and left lower oval patch, while in the remnant parts the relationship was marginally significant ($0.063 \leq P \leq 0.087$, Table S5). As a consequence, colouration in upper oval patched was significantly less saturated with respect to all other body regions in larger individuals. Furthermore, smaller individuals had a thorax significantly less saturated then rostrum, and this difference disappeared in larger individuals (Figure 5, Table S5).

Thorax, left lower claw and left oval patch were significantly more brilliant in larger than smaller individuals (Table S6), while all other body regions did not vary with increasing size (Figure 5). As a consequence, the differences between thorax and rostrum, upper claws and upper oval patches were significantly amplified in larger crayfish (Table S6).

Random effects were highly significant in each of the three models (H: $LR\chi^2 = 1264$, $df = 1$, $P < 0.001$; S: $LR\chi^2 = 177.21$, $df = 1$, $P < 0.001$, V: $LR\chi^2 = 248.9$, $df = 1$, $P < 0.001$), suggesting that a large portion of the variance unexplained by sex, portion, and size (i.e, the fixed effects) depended on some unconsidered features of the individuals, especially for the hue. These proportions were 70.7%, 23.4%, and 28.8% for H, S, and V respectively.

Finally, the colour appeared to be more variable in males rather than females, notably for S and V measures (Figure S9). This ratio ranged from 0.88 to 1.05 for the hue and did not significantly deviate from one in any body region. The ratio for the saturation was larger than one in all body regions, varying from 1.18 to 1.46, even none of the ten values still deviated significantly from one (but deviation was marginally significant for the thorax: $F_{99} = 1.462$, $P = 0.13$). In the case of brightness, the ratios varied from 1.85 to 2.25 and all were significantly higher than one ($F > 1.852$, $P < 0.021$).

Discussion

Our data shows that Signal crayfish possess an extremely variable pattern of colouration within the same population, and such a variability relies on sex and size. Colour variability even occurs within body regions of the same individual, notably between upper and lower portions of the body, and also among dorsal ones. Lower claws and oval patches had a redder hue, with a much more saturated and brilliant colouration that both upper claws and oval patches. We also found significant differences between thorax and rostrum, especially in colour saturation and brightness. Furthermore, we found ontogenetic patterns of colour expression specific to different body regions, resulting in shaper colour difference among body regions with increasing body size. The ontogenetic pattern was noteworthy in upper claws and oval patches, which showed a much larger increase in saturation and brightnessthan other body regions.

Patterns of intra- and inter-individual phenotypic differences might yield insights into the processed, namely selective pressures, which have governed its evolution. In particular, colour patterns can make an individual

conspicuous or cryptic, depending on the prevalent function of colouration, where duller colourations can be easily explained through natural selection for predator avoidance, while brilliant and full of pigment colouration may be evolved as signals for intraspecific communication. Intra-population variation of the exoskeleton in freshwater crayfish has been constantly interpreted as an adaptation to the background of environmental light to improve mimicry and reduce predation risk (Kent 1901, Thacker et al. 1993, Nokelainen et al. 2017). Our data for thorax and upper claws were consistent with this general idea, but the pattern of variation for both saturation and brightness of the redder portion, especially in males, were not. The specific reason is that red pigmentations in crustaceans are primarily due to the accumulation of carotenoids (notably astaxanthin, but also lutein and zeaxanthin, Latscha 1989, Pan et al. 2001) into the exoskeleton and in the epidermal layer between the exoskeleton and abdominal muscle (Katayama et al. 1972; Tanaka et al.1976; Simpson et al. 1981; Sagi et al. 1995; Boonyaratpalin et al. 2001). Crustaceans, including crayfish, are incapable to synthesize carotenoids but need to acquire them through diet (Katayama et al. 1971). Carotenoids are among the most effective antioxidants, and their primary function in the organism is scavenging free radicals (Svensson and Wong, 2011). Since immune cells are highly sensitive to oxidative stress, carotenoids can enhance immunity (Svensson and Wong 2011), establishing the basis for a trade-off for carotenoids between colouration and immunity (Lozano 2001). Crayfish possess a complex visual system including well-developed compound eyes contains multiple lenses (Kubec et al. 2019), which makes them able to receive and analyse complex visual signals, and examples of communication though visual signals are not lacking (Bruski & Dunham 1987, Detto et al. 2006, Galeotti et al. 2006,2007, Rubolini et al. 2007, Van der Velden et al. 2008, Kubec et al. 2019). The fact that larger males in signal crayfish were redder than small ones, and in general males were redder than females makes plausible the hypothesis that conspicuous red colourations might be involved in intraspecific communication rather than in crypsis. This hypothesis is further supported by the fact that the most saturated and brilliant colouration located in the lower claws, which can be rapidly hidden in the presence of a predator. Such a kind of response is also consistent with the signal partitioning as a solution for the trade-off between camouflage from predators and conspicuousness for communication. Thus, the dorsal body pattern is likely to provide signal crayfish with camouflage from predators (namely fish), whereas and the portion of claws hidden from potential predators can be used for signalling to conspecifics.

An alternative explanation for the occurrence in signal crayfish of colour variation among body regions within individual is that the orange-red colour might be used as a way to communicate with conspecifics while avoiding detection from predators.. In the species'native range of North America, the main predators of signal crayfish include teleost fish like smallmouth bass *Micropterus dolomieu* (Pflug & Pauley 1984), brown trout *Salmo trutta* and mountain whitefish *Prosopium williamson* (Lewis 1999), northern pikeminnow *Ptychocheilus oregonensis* (Jeppson & Platts

1958, Lewis 1999) and European perch *Perca fluviatilis* (Appelperg & Odelstrom 1987). In general the crayfish are consumed during the major activity months (Stein 1977; Hepworth & Duffield 1987). The young crayfish, 0 and 1 years old, are the most frequently found in stomachs of predatory fish (Stein 1977). In general mammals and avian predators feed of crayfish too for example racoon *Procyon lotor*, *otter Lutra lutra* and mink *Mustela vison* (Hogger 1988). While among the avian predators there are heron *Ardea cinerea* and kingfisher *Alcedo atthis* All age classes of crawfish are preyed upon by mammals and birds but the diet is made up mostly by larger and heavier crayfish (Correia 2001) (. Mammals with nocturnal activity have reduced ability to detect colours, above all long-wavelength (notably, orange-red), due to the marked prevalence of rods on cones in the retina, and short-wavelength-sensitive (S-) cones on long-wavelength-sensitive (L-) cones (Peichl 2005). By contrast, the spectral sensitivity of crayfish peaked towards red, between 550-600 nm (reviewed in Schuster 2020), and did not correlate with the different habitat preferences among the species (Crandall & Cronin 1997). Therefore, by using orange-red colourations signal crayfish may produce signals conspicuous to conspecifics using a property of light that is invisible to predators, allowing them to avoid being vulnerable to predators. The hypothesis is not unrealistic since such a kind of adaptation has been already described in crustaceans (Cummings et al 2003; Siebeck et al. 2010, Gagnon et al. 2015, Abramjan et al. 2020). For example, the mantis shrimp *Gonodactylaceus falcatus* produces strongly circularly polarized body patterns, which are discriminated by conspecifics to avoid occupied burrows when seeking refuge but are not seen by potential predators (Gagnon et al. 2015).

A further result of this study was that the exoskeleton colour differed between sexes: males having a redder, more saturated and brilliant colouration while females a duller and drabber colouration. This difference varied among body regions, and, notably, was especially noticeable in lower claws and lower oval patches. Sexual dichromatism is normally thought to have evolved in response to selection pressures that differ between the sexes: males evolving bright colouration due to sexual selection for increased ornamentation (reviewed by Badyaev and Hill 2003) whereas females missing colourful pattern due to natural selection for cryptic colouration (Wallace, 1868; Soler and Moreno 2012). In birds, sexual dimorphism in carotenoid-based colourations have been shown to depend on intense sexual selection by female mate choice for honest signals (Badyaev and Hill 2000). Substantial variability of sexual dichromatism depends on the balance between the exaggerated trait in males and the environmental conditions affecting trait expression (Badyaev and Hill 2003). Accordingly, we found sexual dichromatism vary between the upper and lower portion of claws, and was at the best in the lower portion, where the cost due to predation risk can be minimized. Support for a possible role of sexual selection in maintaining sexual dichromatism comes from the finding that inter-individual variation of colour brightness was significantly larger in males with respect to females. Notably, just lower claws and

lower oval patches were most variable among males than females. However, colour dichromatism may arise in some other way than sexual selection, including thermoregulation, biases in the visual system, species identification, interspecific communication, which with a correlative approach such that we used in this study cannot be disentangled. In conclusion, carotenoids shaping the colouration of the exoskeleton of freshwater crayfish are well suited for testing ideas on colour signals and their potential role in conveying information in these species.

In conclusion, crayfish colour patterns have not been widely studied to better understand their meaning and adaptive function in crayfish, and only in recent years, the patterns of variation in the external colouration at both inter- and intraspecific level and have begun to be analysed not only for camouflage, but also in the light of possible role in intraspecific communication and, more in general, crayfish behaviour. Next steps will necessarily pass through experimental investigations to try to test hypotheses of communicative function of crayfish colouration and assess how colour affects male-male competition or mate choice by females, or if juvenile colouration confers some sort of protection from predators because juveniles are subject to higher predator risk than adults.

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References

Appelberg M, Odelström T, 1988. Interaction between European perch *Perca fluviatilis* and juvenile *Pacifastacus leniusculus* (Dana) in a pond ecosystem. *Fresh Crayfish* **7:** 37−45.

Badyaev AV, Hill GE, 2000. Evolution of sexual dichromatism: Contribution of carotenoid- versus melanin-based coloration. *Biol J Linn Soc* **69**:153–172.

Badyaev AV, Hill GE, 2003. Avian sexual dichromatism in relation to phylogeny and ecology. *Annu Rev Ecol Evol Syst* **34**:27–49.

Bauer RT, 1981. Colour patterns of the shrimps *Heptacarpus pictus* and *H. paludicola* (Caridea: Hippolytidae). *Mar Biol* **64**: 141–152.

Barnett JB, Cuthill IC, Scott-Samuel NE, 2018. Distance-dependent aposematism and camouflage in the cinnabar moth caterpillar (*Tyria jacobaeae*, Erebidae). *R Soc Open Sci* 5: 171396.

Barton K, 2019. MuMIn: Multi-Model Inference. R package version 1.43.6. https://CRAN.R-project.org/package=MuMIn.

Bates D, Maechler M, Bolker B, Walker S, 2015. Fitting Linear Mixed-Effects Models Using lme4. *J Stat Soft* **67**:1-48.

Beingesser KR, Copp NH, 1985. Differential diurnal distribution of *Procambarus clarkii* (Girard) juveniles and adults and possible adaptive value of color differences between them (Decapoda, Astacidea. *Crustaceana* **49**:164–172.

Bergman TJ, Beehner JC, 2008. A simple method for measuring colour in wild animals: Validation and use on chest patch colour in geladas (*Theropithecus gelada*). *Biol J Linn Soc* **94**:231–240.

Blake MA, Hart PJB, 1993. The behavioural responses of juvenile signal crayfish *Pacifastacus leniusculus* to stimuli from perch and eels. *Freshw Biol* **29**:89–97.

Boonyaratpalin M, Thongrod S, Supamattaya K, Britton G, Schilipalius LE, 2001. Effects of b-carotene source, *Dunaliella salina*, and astaxanthin on pigmentation, growth, survival and health of *Penaeus mondon*. *Aquac Res Oxf* **32**:182–190.

Bruski CA, Dunham DW, 1987. The importance of vision in agonistic communication of the crayfish *Orconectes rusticus*. I: an Analysis of Bout Dynamics. *Behaviour* **103**, 83–107.

Correia AM, 2001. Seasonal and interspecific evaluation of predation by mammals and birds on the introduced red swamp crayfish *Procambarus clarkii* (Crustacea, Cambaridae) in a freshwater marsh (Portugal). *J Zool* **255**: 533–541.

Crandall KA, Cronin TW, 1997. The molecular evolution of visual pigments of freshwater crayfishes (Decapoda: Cambaridae). *J Mol Evol* **45**: 524–534.

Cummings ME, Jordao JM, Cronin TW, Oliveira RF, 2008. Visual ecology of the fiddler crab *Uca tangeri*: effects of sex, viewer and background on conspicuousness. *Anim Behav* **75**:175–188.

Cummings ME, Rosenthal GG, Ryan MJ, 2003. A private ultraviolet channel in visual communication *Proc Biol Sci* **270**: 897-904.

Detto T, Backwell PRY, Hemmi JM, Zeil J, 2006. Visually mediated species and neighbour recognition in fiddler crabs (*Uca mjoebergi* and *Uca capricomis*). *Proc R Soc B* **273**: 1661–1666.

Dunham DW, Jordan SD, Niglas M, 1979. A new color morph of the crayfish *Orconectes propinquus* (Girard). *Am Midl Nat* **102**:384–387.

Endler JA, 1981 An overview of the relationships between mimicry and crypsis. *Biol J Linn Soc* **16**: 25–2.

Endler JA 1987, Predation, light intensity and courtship behaviour in *Poecilia reticulata* (Pisces: Poeciliidae). *Anim Behav* **35**, 1376–1385

Flint RW, 1975. Growth in a population of the crayfish *Pacifastdcus Leniusculus* ftom a subalpine lacustrine environment. *J Fish Res Board Can* **32**: 2433-2440.

Galeotti P, Rubolini D, Fea G, Ghia D, Nardi PA et al., 2006. Female freshwater crayfish adjust egg and clutch size in relation to multiple male traits. *Proc R Soc London Ser B* **273**:1105–1110.

Galeotti P, Pupin F, Rubolini D, Sacchi R, Nardi PA et al., 2007. Effects of female mating status on copulation behaviour and sperm expenditure in the freshwater crayfish *Austropotamobius italicus*. *Behav Ecol Sociobiol* **61**:711–718.

Harpaz S, Rise M, Arad S, Gur N, 1988. *Aquaculture Nutrition.* Blackwell Science.

Hartzell SM, 2017. Ontogenetic color change in the crayfish *Cambarus b. bartonii* and *Faxonius obscurus*: a test of Ortmann's hypotheses. *Fresh Crayfish* **23**:59–63.

Hepworth DK, Duffield DJ, 1987. Interactions between an exotic crayfish and stocked rainbow trout in Newcastle Reservoir, Utah North Am. *J Fish Manage* **7**: 554-561.

Hogger JB, 1988. Ecology, Population Biology, and Behaviour. *Freshw Crayfish* **7:** 139–145.

Jared EK, Frederick C, 2019. *merTools: Tools for Analyzing Mixed Effect Regression Models.* R package version 0.5.0. https://CRAN.R-project.org/package=merTools

Jeppson PW, Platts WS, 1959. Ecology and control of the Columbia squawfish in northern Idaho lakes. *Trans Am Fish Soc* **88**: 197-202.

Johnson MF, Rice SP, Reid I, 2014. The activity of signal crayfish *Pacifastacus leniusculus* in relation to thermal and hydraulic dynamics of an alluvial stream, UK. *Hydrobiologia* **724**: 41–54

Jormalainen V, Merilaita S, Tuomi J, 1995. Differential predation on sexes affects color polymorphism of isopod *Idotea baltica* (Pallas). *Biol J Linn Soc* **55**: 45–68.

Kaldre K, Haugjärv K, Liiva M, Gross R, 2015. The effect of two different feeds on growth, carapace colour, maturation and mortality in marbled crayfish (*Procambarus fallax* f. *virginalis*). *Aquac Int* **23**:185–194.

Katayama T, Hirata K, Chichester CO, 1971. The biosinthesis of Axn-IV. The carotenoids in the prawn *Peaneus japonicus* Bate (pat.I). *Bull Jpn Soc Sci Fish* **37**:614–620.

Katayama T, Kitama T, Chichester CO, 1972. The biosynthesis of astaxanthin in the prawn *Penaeus japonicus* Bate (Part II). *Int J Biochem* **3**:363–368.

Kent WJ, 1901. The colors of the crayfish. *Am Na* **35**: 933–936

Kouba A, Petrusek A, Kozák P, 2014. Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl Manag Aquat Ecosyst* 413:05

Kubec J, Kouba A, Buric M, 2019. Communication, behaviour, and decision making in crayfish: A review. *Zool Anz* **278**:28–37.

Kuznetsova A, Brockhoff BB, Christensen RHB, 2017. "lmerTest Package: Tests in Linear Mixed Effects Models." *J Stay Soft* **82**:1–26.

Latscha T, 1989. The role of astaxanthin in shrimp pigmentation. *Adv Tropical Aquacul* **9**: 319–325.

Lee WL, 1966. Colour change and the ecology of the marine isopod *Idothea* (Pentidotea) montereyensis Maloney, 1933. *Ecology* **47**: 930–941.

Lewis SD, 1999. Abundance, Activity, and diet of littoral fish in Lake Billy Chinook, Lake Simtustus, and Reregulating Reservoir, Oregon, 1997–1999. Portland General Electric, Portland, Oregon pp 35.

Lozan JL, 2000. On the threat to the European crayfish: a contribution with the study of the activity behaviour of four crayfish species (Decapoda: Astacidae). *Limnologica* **30**:156–161

Lozano GA, 2001. Carotenoids, immunity, and sexual selection: comparing apples and oranges? *Am Nat* **158**:200–203.

Nakagawa S, Schielzeth H, 2013. A general and simplemethod for obtaining R^2 from generalized linear mixed-effects models. *Met Ecol Evol* **4**:133–142.

Nokelainen O, Hubbard N, Lown AE, Wood LE, Stevens M, 2017. Through predators' eyes: phenotype-environment associations in shore crab coloration at different spatial scales. *Biol J Linn Soc* **122**:738–751.

Ooms J, 2018. *magick: Advanced graphics and image-processing in R*. R package version 2.0. https://CRAN.R-project.org/package=magick.

Pan C-H, Chien Y H, Cheng J-H, 2001. Effects of light regime, algae in the water, and dietary astaxanthin on pigmentation, growth, and survival of black tiger prawn *Penaeus monodon* post larvae. *Zool Studies* **40**:371–382.

Peichl L, 2005. Diversity of mammalian photoreceptor properties: adaptations to habitat and lifestyle? *Anat Rec A Discov Mol Cell Evol Biol* **287**: 1001-1012.

Peiró DF, Baeza JA, Mantelatto FL, 2003. Host-use pattern and sexual dimorphism reveals the mating system of the symbiotic pea crab *Austinixa aidae* (Crustacea: Brachyura: Pinnotheridae). *J Mar Biol Ass UK* **93**: 715-723.

Pflug DE, Pauley GB, 1984. Biology of smallmouth bass *Micropterus dolomieu* in Lake Sammamish, Washington.

Northwest Sci **58**: 118-129.

R Core Team, 2014. R: *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

Sacchi R, Pellitteri-Rosa D, Bellati A, Di Paoli A, Ghitti M et al., 2013. Colour variation in the polymorphic common wall lizard *Podarcis muralis*: An analysis using the RGB colour system. *Zool Anz* **252**:431–439.

Stein RA, 1977. Selective predation, optimal foraging, and the predator-prey interaction between fish and crayfish. *Ecology* **58**: 1237–1253

Rubolini D, Galeotti P, Pupin F, Sacchi R, Nardi PA et al., 2007. Repeated matings and sperm depletion in the freshwater crayfish *Austropotamobius italicus*. *Fresh Biol* **52**:1898–1906.

Sagi A, Rise M, Kahalaila I, Arad S, 1995. Carotenoids and their derivatives in organs of the maturing female crayfish *Cherax quadricarinatus*. *Comp Biochem Physiol* **112B**:309–313.

Schuster G, 2020. Review of crayfish color patterns in the Family Cambaridae (Astacoidea), with discussion of their possible importance. *Zootaxa* **4755**: 63–98.

Simpson KL, Katayama T, Chichester CO, 1981. *Carotenoids in fish feeds*. In: Bauernfeind JC ed. *Carotenoids as Colourants and Vitamin a Precursors*. New York: Academic Press, 463–530.

Soler J, Moreno J, 2012. Evolution of sexual dichromatism in relation to nesting habits in European passerines: a test of Wallace's hypothesis. *J Evol Biol* **25**:1614–1622.

Souty-Grosset C, Holdich DM, Noël PY, Reynolds JD, Haffner P, 2006. *Atlas of Crayfishin Europe*. Paris: Muséum National d'Histoire naturelle .

Stevens M, Lown AE, Wood LE, 2014. Color change and camouflage in juvenile shore crabs *Carcinus maenas*. *Front Ecol Evol* **2**:14–18.

Stevens M, Rong CP, Todd PA, 2013. Colour change and camouflage in the horned ghost crab *Ocypode ceratophthalmus*. *Biol J Linn Soc* **109**:257–270.

Stuart-Fox DM, Moussalli A, Marshall NJ, Owens IP, 2003 Conspicuous males suffer higher predation risk: visual modelling and experimental evidence from lizards. *Anim Behav* **66**: 541–550

Stuart-Fox D, Moussalli A, Whiting MJ, 2008. Predator-specific camouflage in chameleons. *Biol Let* **4**: 326–329

Styrishave B, Bojsen BH, Witthøfft H, Andersen O, 2007. Diurnal variations in physiology and behaviour of the noble crayfish *Astacus astacus* and the signal crayfish *Pacifastacus leniusculus*. *Marine Fresh Behav Physiol* **40**: 63-77

Svensson EI, Wong BBM, 2011. Carotenoid-based signals in behavioural ecology: a review. *Behaviour* **148**:131–189.

Tanaka Y, Matsuguchi H, Katayama T, Simpson KL, Chichester CO, 1976. The biosynthesis of astaxanthin- XVI. The carotenoids in Crustacea. *Comp Biochem Physiol* **54B**:391–393.

Thacker RW, Hazlett BA, Esman LA, Stafford CP, Keller T, 1993. Color morphs of the crayfish *Orconectes virilis*. *Am Midl Nat* **129**:182.

Tibbetts EA, 2004. Complex social behaviour can select for variability in visual features: a case study in *Polistes* wasps. *Proc Roy Soc B* **271**: 1955–1960.

Todd PA, Briers RA, Ladle RJ, Middleton F, 2006. Phenotype-environment matching in the shore crab *Carcinus maenas*. *Mar Biol* **148**: 1357–1367.

Van der Velden J, Zheng Y, Patullo BW, Macmillan DL, 2008. Crayfish recognize the faces of fight opponents. *PLoS ONE* **3**:e1695.

Vannini M, Gherardi F, 1981. Dominance and individual recognition in *Potamon fluviatile* (Decapoda, Brachyura):

possible role of visual cues. *Mar Behav Physiol* **8**: 13–20

Walker ML, Austin CM, Meewan M, 2000. Evidence for the inheritance of a blue variant of the Australian fresh-water crayfish *Cherax destructor* (Decapoda: Parastacidae) as an autosomal recessive. *J Crustac Biol* **20**:25–30.

Wallace AR, 1868. A theory of birds' nests: showing the relation of certain sexual differences of colour in birds to their mode of nidification. *J Travel Nat Hist* **1**:73– 89.

Zeil J, Zanker JM, 1997. A glimpse into crabworld. *Vis Res* **37**:3417–3426.

Zuur AF, Ieno EN,Walker NJ, Saveliev AA, Smith G, 2009. *Mixed Effects Models and Extension in Ecology with R*. Berlin, Heidelberg: Springer.

Table 1. Minimum significant LMMs for the effect of sex, body region and size on the colour of the exoskeleton of *Paciastacus leniusculus* measured in the HSV system.

Model	***F***	***df***	***P***
Hue			
Sex	0.175	1, 246	0.68
Body region	300.215	9, 1241	< 0.001
Size	0.038	1,152	0.84
Sex × Body region	3.927	9,1242	< 0.001
Size × Body region	3.680	9,1242	< 0.001
Saturation			
Sex	7.468	1, 163	0.007
Body region	276.321	9, 1246	< 0.001
Size	14.703	1, 152	< 0.001
Sex × Body region	2.633	9, 1247	0.005
Size × Body region	2.342	9, 1250	0.013
Value			
Sex	16.743	1, 169	< 0.001
Body region	637.485	1, 1247	< 0.001
Size	9.568	1,154	0.002
Sex × Body region	4.482	9, 1248	< 0.001
Size × Body region	2.685	9, 1251	< 0.001

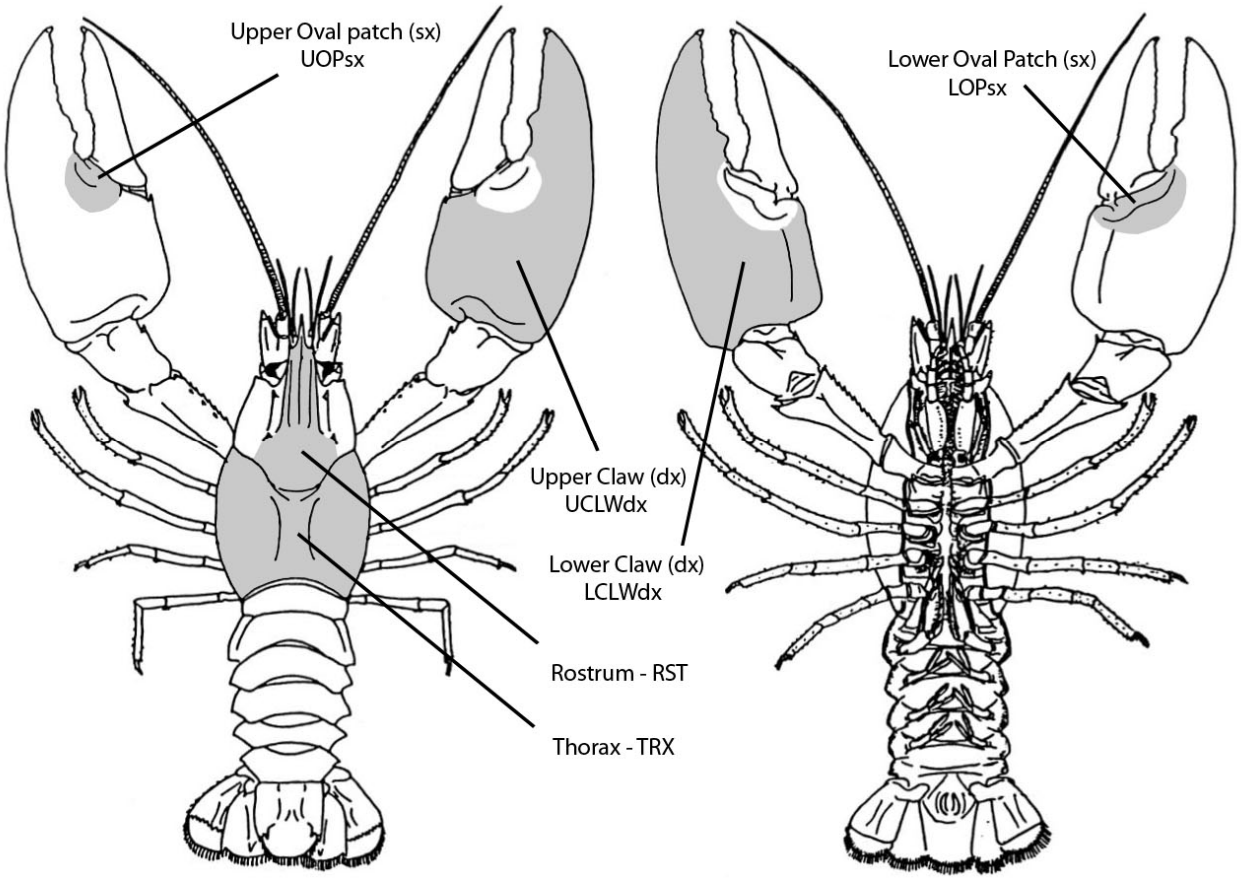

Figure 1. Localization and codes of the ten body region we used to characterize hue, saturation and brightness in males and females of signal crayfish.

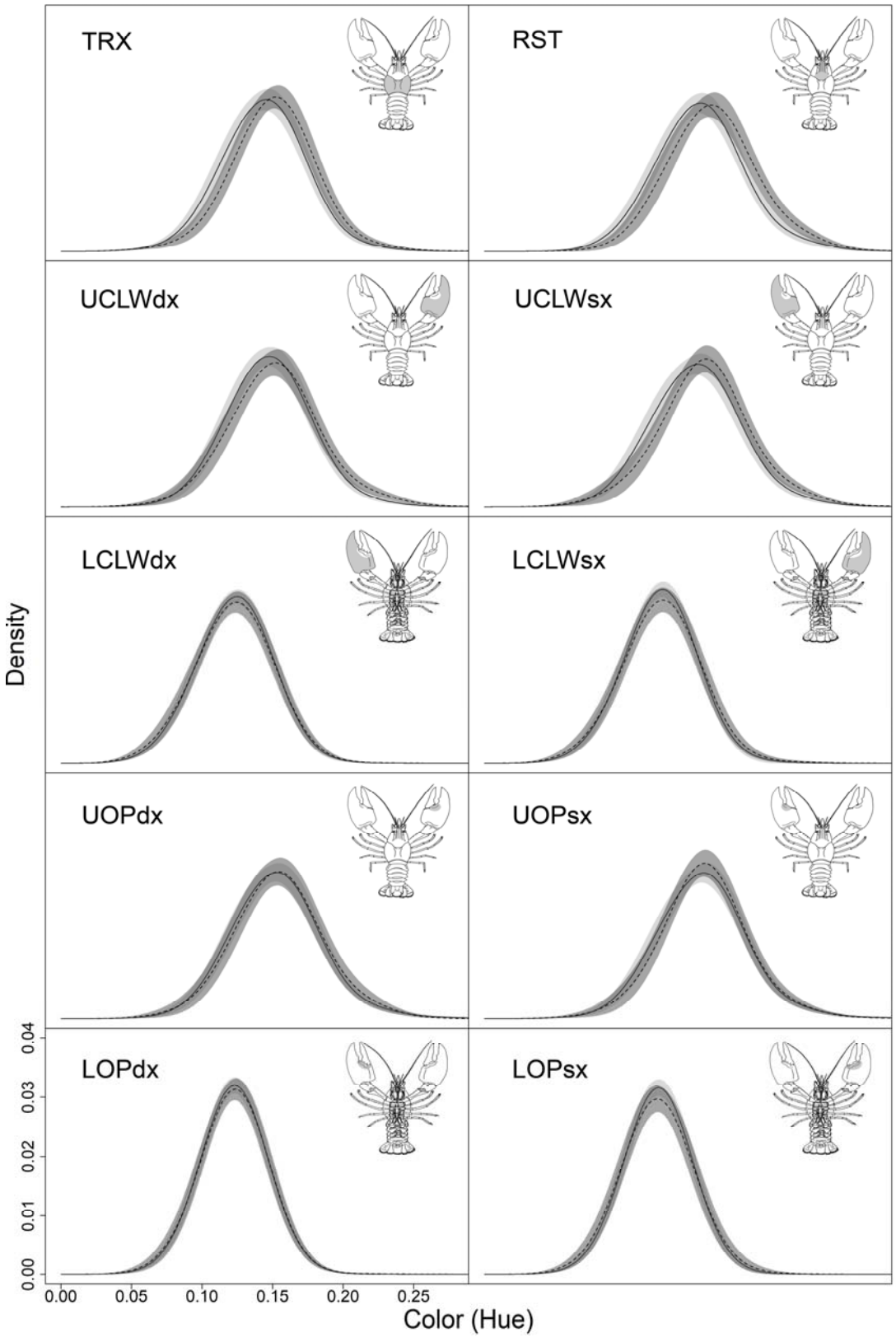

Figure 2. Distribution frequency of hue for different portions of the body and claws of male (solid line and light gray area) and female (dashed line and dark gray area) signal crayfish. Lines are means and area represent 95%CI.

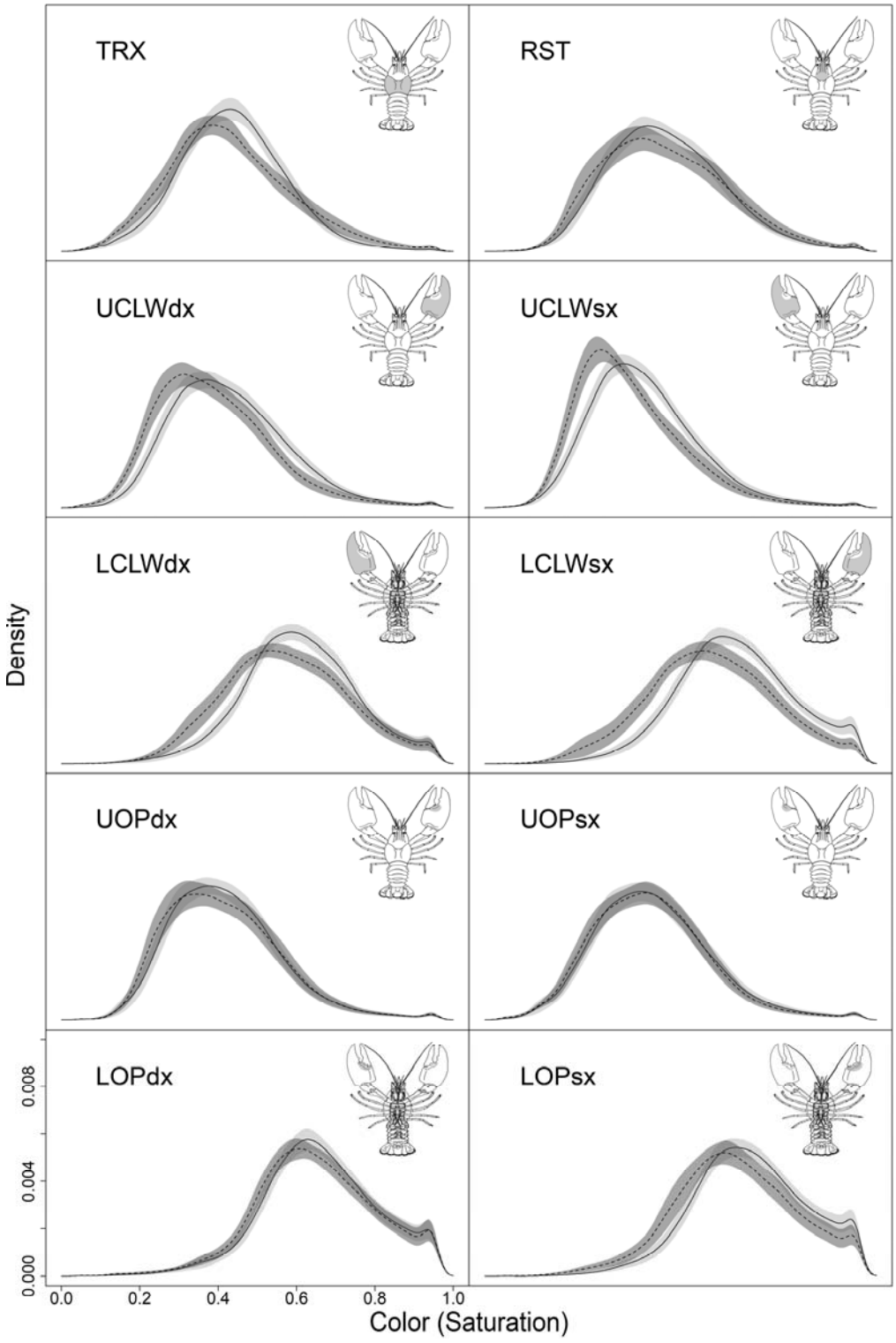

Figure 3. Distribution frequency of hue for different portions of the body and claws of male (solid line and light gray area) and female (dashed line and dark gray area) signal crayfish. Lines are means and area represent 95%CI.

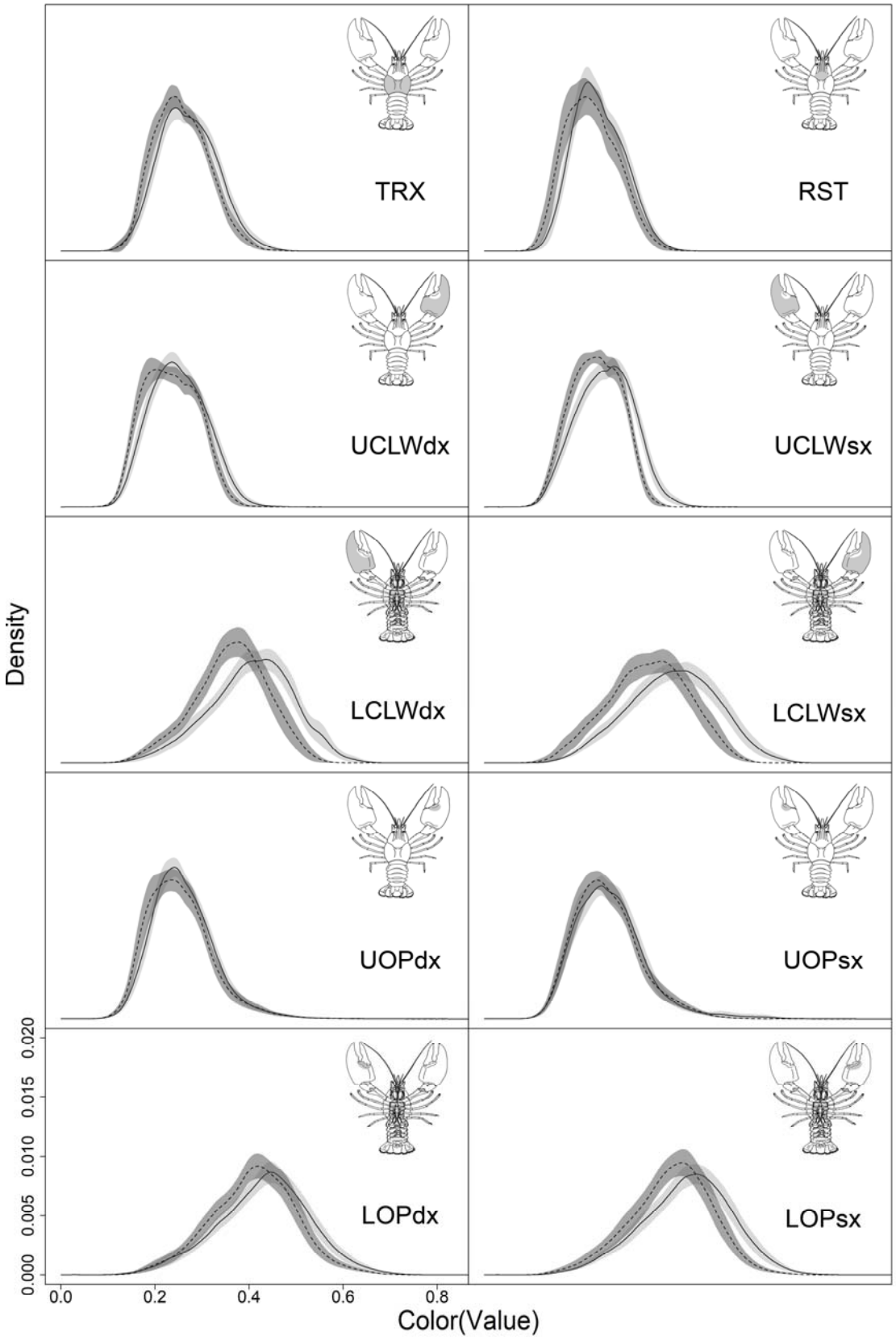

Figure 4. Distribution frequency of brightness for different portions of the body and claws of male (solid line and light gray area) and female (dashed line and dark gray area) signal crayfish. Lines are means and area represent 95%CI.

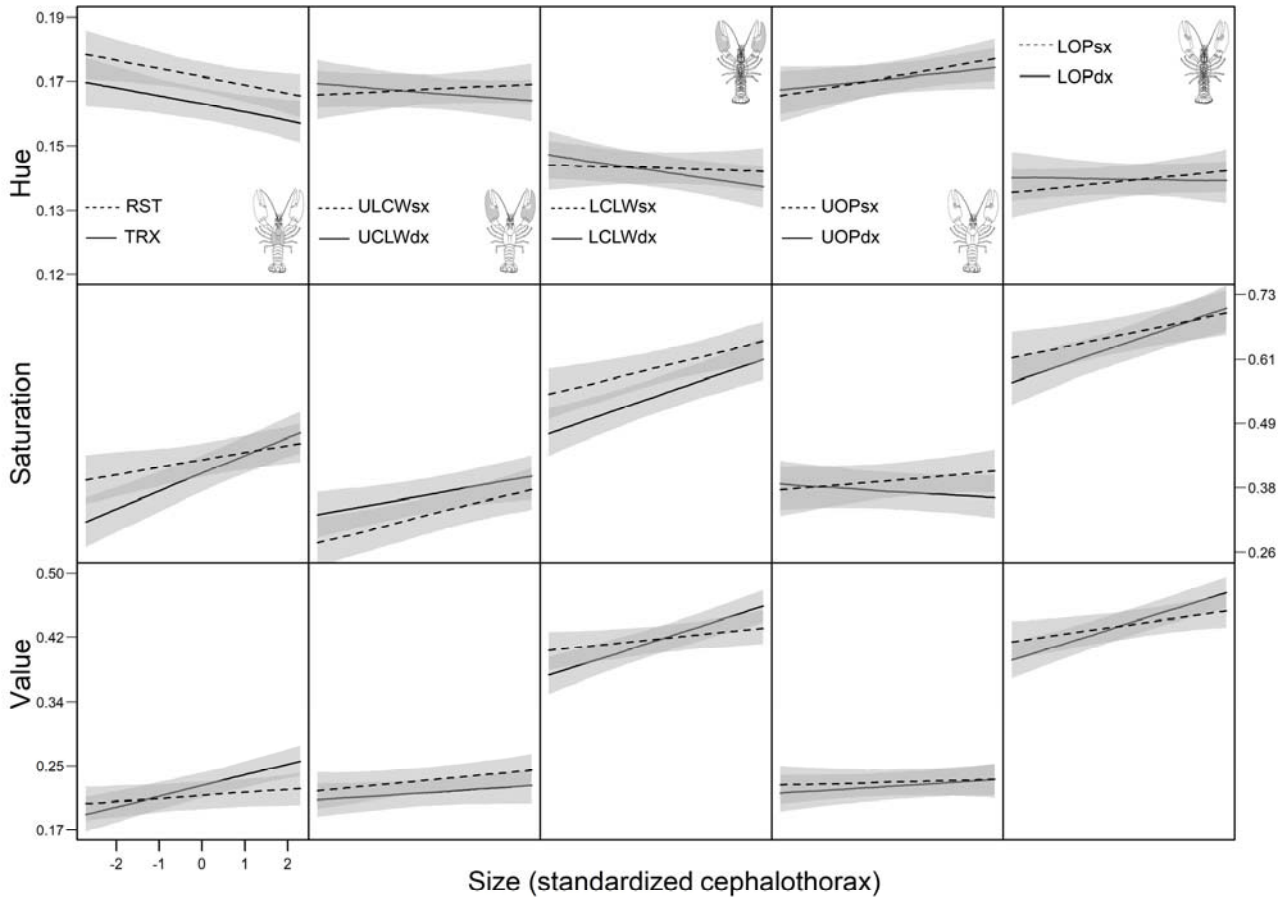

Figure 5. Relationships between body size and the three components of external colouration (hue, saturation and brightness) in different portions of the body and claws of signal crayfish. Lines are means and area represent 95%CI.