Supplementary data for Nokelainen et al. 2019 Functional Ecology

Title: Improved camouflage through ontogenetic colour change confers reduced detection risk in shore crabs

Authors:

Ossi Nokelainen[1,2*], Ruth Maynes[1], Sara Mynott[1], Natasha Price[1] & Martin Stevens[1*]

[1]Centre for Ecology and Conservation, College of Life and Environmental Science, University of Exeter, TR10 9FE Penryn, UK

[2]Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, 40014 University of Jyväskylä, Finland

[*] Authors for correspondence (ossi.nokelainen@jyu.fi, martin.stevens@exeter.ac.uk)

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SUMMARY

Many animals change appearance with age but the reasons why are rarely tested. Common shore crabs (*Carcinus maenas*), for example, are known for their ability to change colour over time. Young crabs show remarkable variation in coloration and it has been suggested that their variable appearance may help them to hide from predators in the habitats they use. However, as crabs grow they become more mobile and adult crabs, in contrast, are known to possess a more uniform coloration. This creates a problem: how to remain hidden in habitats that are variable and very different in appearance?

To answer this, we first reared young shore crabs of two shades, pale or dark, on two background types resembling different habitats for 10 weeks. We predicted that crabs would adopt a coloration that would improve their match to the background, but instead they all developed a dark green or brown appearance. Next, we undertook an experiment at the Natural History Museum London during the Colour and Vision exhibition, where visitors searched for crabs representing natural colour variation from different habitats. Remarkably, crabs were not hardest to find against their original habitat, but instead the dark green or ‘mudflat crabs’ were hardest to detect against all backgrounds.

These findings suggest that shore crabs change into similar colour with age, which appears to be a good general solution to match several habitat types and therefore to hide from predators. We conclude that the evolution of camouflage can be better understood by recognizing that the optimal appearance to hide may change over the lifespan of many animals.

METHODS

Colour change experiment

The experiment was conducted at the University of Exeter, Penryn Campus, Cornwall between February and May 2016. Individual crabs used for the common garden experiment were collected from the Gyllyngvase beach (coordinates in decimal degrees: 50.141888, -5.063811), Cornwall, UK, during February 2016. Shore crabs are located in a wide range of habitat and substrate types around the shore, each with different appearances, including estuaries, mud flats, sandy beaches, shingle, pebbles, mussel beds, and rocky coastline (Edwards 1958, Crothers 1968, Brian et al. 2006, Todd et al. 2006, 2012, Stevens et al. 2014b). The collection methods largely follow established protocols (Stevens et al. 2014b, Nokelainen et al. 2017a). Briefly, the crabs were collected by hand during low tide alongside the beach from approximately 50 meters length and thus our sampling included crabs from different substrates. Crabs were transported from nearby tidal pools into the laboratory immediately after capture. Crabs entering the experiment were all of similar size, approximately 15 mm carapace width. After collection, crabs were photographed and divided into experimental groups based on their carapace lightness in a randomized block design (i.e. crabs with contrasting lightness were equally represented in treatment groups, see further). Crabs were photographed once a week and after moulting. Shore crabs are not a protected species and all work was conducted under approval from the University of Exeter Biosciences ethics committee (applications 2013/75 and 2014/556). The field locations are publicly accessible; no further permits were needed.

First we study if juvenile shore crabs adjust their appearance (i.e. including both colour and pattern) within and over successive moults in order to increase their

resemblance to heterogeneous substrates (unlike our previous work, which has tended to focus on more simplified uniform backgrounds; Stevens et al. 2014a, Stevens 2016). Experimental animals were divided into four treatment groups using a 2 x 2 factorial set up with crabs of two shades (pale, dark) on two naturalistic background types (i.e. rock pool and mudflat – Fig. 1). Carapace brightness was used to divide crabs in two distinct groups. Group discreteness was further validated based on the camera-obtained spectral data (see below; ANOVA for carapace brightness between dark and pale treatment groups, $N = 60$, $F = 34.15$, $df = 1$, $p < 0.001$). Beginning with two unambiguous groups allowed us to control for the extensive phenotypic variation of juvenile crabs.

We chose background types in which to rear crabs that represent two common natural extremes: relatively homogeneous mudflat and, more heterogeneous rock pool backgrounds. We replicated these backgrounds using standard aquarium gravel (UNIPAC) after subjective evaluation of their general properties of colour and pattern from photographs. 'Mudflat' background was a mixture of brown and green (i.e. representing brown mud and green algae) aquarium gravel (1:1 ratio), whereas 'rock pool' background was a mixture of black, grey, white and purple aquarium gravel (with equal ratios). We deliberately chose not to use actual natural substrates as this may contain chemical cues of predators or other stimuli that may influence crab development and that may also differ in texture / size as well as colour pattern, thereby hindering full control over the experiment. Using artificial gravel also enabled greater standardisation of background samples among individuals. We compared the match of our artificial backgrounds to natural ones using calibrated photographic data (see below). Similarity of the backgrounds in a trichromatic RGB colour space was calculated based on reflectance data for brightness (i.e. average reflectance across all colour channels; R+G+B / 3) and

hue (i.e. red divided by blue channel). Artificial backgrounds represented similar albeit not perfectly matching natural variation of colourful tidal environments (Fig. S1). In particular, the artificial backgrounds most effectively matched the brightness of their natural counterparts. In nature, rock pools harbour a great range of chromatic variability, both within and among patches, including pink-coloured elements such as red encrusting coralline algae and also have blue-coloured elements such as mussels. Mudflats instead are characterised by brown tones of wet soil and gravel and get mixed by green brown and red algae. Therefore, although our artificial substrates are not a perfect match to the natural substrates, they are broadly representative, and crucially, the appearance of the mudflat and rock pool treatments is very different.

Altogether, we reared 60 crabs (17 in ‘dark-mud’ treatment, 16 in ‘dark-rock’ treatment, 13 in ‘pale-mud’ treatment and 14 in ‘pale-rock’ treatment) in customized aquarium tanks (90 x 45 cm in area) for 10 weeks. Each tank was divided into 24 similar sections (11 x 15 cm). The section walls were glued using adhesive silicon glue and walls contained a mesh-covered hole ensuring water circulation through the system. Tanks were filled with dechlorinated tap water mixed with artificial sea salt (Aquarium Systems Instant Ocean Salt, Swell UK Ltd., UK) to simulate natural seawater, which was tested with a refractometer (D&D's Refractometer, Swell UK Ltd., UK) to ensure salinity of 30 ppt. The water was passed through a filtration system (Eheim classic 350 EHEIM GmBH & Co. KG, Deizisau, Germany) and cooler (D&D DC300 aquarium cooler 300w cooling power, Swell UK Ltd., UK), keeping the water both clean and at a constant temperature. Temperature was set to 16°C to mimic local sea temperature at the time of collection. Two sections were not used to accommodate crabs, but instead housed the inputs and outputs of the filtration system to allow for maximum water flow through each section of

the tank. An air stone (Aquarline High Output Air Compressor, 2880 Litre/Hour) was accompanied with the filter output section to allow as much oxygen to flow through the tank as possible. We used two daylight lamps and one near UV lamp (Grobeam600 Ultima and AquaBeam 600 Ultima MW, Tropical Marine Centre UK) to simulate natural light conditions, which were controlled by a timer to establish a constant light cycle (12:12 L/D-cycle). Crabs were fed daily with standard marine crustacean aquarium food. Water was changed, filters checked and tanks cleaned weekly to maintain living conditions of crabs. Some crabs did not survive through 10-week-experiment. However, mortality was not significantly different with regards to background type or crab initial shade, nor there was difference in moulting rates between the treatments.

Photography and vision modelling

Photography, initial image calibration and analysis broadly followed previously used methods (Stevens et al. 2014a). Full details are given in supplementary material (Table S1). Briefly, imaging was undertaken with a Samsung NX1000 digital camera converted to full spectrum with no quartz filter to enable UV sensitivity, and fitted with a Nikon EL 80 mm lens. For the human visible photos, we placed a UV and infrared (IR) blocking filter in front of the lens, which transmits wavelengths only between 400 – 680 nm (Baader UV/IR Cut Filter). For the UV images, a UV pass and IR blocking filter was used (Baader U filter), which transmits between 320-380 nm. Grey reflectance standards, which reflect light equally at 7% and 93% between 300 and 750 nm, were used.

For each image we measured the entire dorsal side of the crab carapace to obtain colour and pattern information. We analysed the data both with normalised camera responses and fish vision modelled data (see below). For reflectance data (i.e. colour), we

used normalised camera responses of brightness, red, green, blue and UV channel. The pattern analysis technique (a 'granularity' analysis) involved decomposing an image into a series of different spatial frequencies ('granularity bands') using Fourier analysis and band pass filtering, followed by determining the relative contribution of different marking sizes to the overall pattern (Barbosa et al. 2008, Hanlon et al. 2009, Stoddard and Stevens 2010). For the pattern data (see further details in supplementary Table S1), we used maximum power (i.e. pattern dominance – the energy at the spatial frequency with the highest pixel energy), proportional power (i.e. pattern diversity – maximum or peak energy value divided by the summed energy), total power (i.e. overall contrast or amplitude – the energy summed across all scales) and mean power (i.e. average contrast across the spectrum). Pattern analysis was conducted in custom files for Image J (Troscianko and Stevens 2015).

To examine the level of background match, we calculated how changes in the crab carapace influenced their level of match to the experimental backgrounds. To do so, we used a receptor noise limited visual discrimination model (Vorobyev et al. 1998), which is based on differences in colour or luminance based on photon catch values. For calculations, all crabs were photographed weekly over the course of the experiment. Also, the backgrounds (i.e. aquarium gravel mixtures from the slots individual crabs were kept on) were photographed. Thus, difference metrics (see below) were calculated between crab carapace and the very background each crab was reared on matching the size of the entire slot (c. 10 cm in diameter). We used a fish vision model based on the longwave (LW) and shortwave (SW) visual sensitivity of the pollack (*Pollachius pollachius*) (Shand et al. 1988). A Weber fraction value of 0.05 was used for the most abundant cone type with receptor cone ratios of SW 168 and LW 339 (Govardovskii et al. 2000). The

receptor noise model yield values in 'just noticeable differences' (JNDs), whereby differences between 1 and 3 are interpreted that two stimuli are unlikely to be discriminated by an observer (and hence indicate a good background match). Larger values than this are increasingly likely to be discriminable, whereas values lower than this (<1 JND) should be virtually indistinguishable (Kelber et al. 2003, Siddiqi et al. 2004, Olsson et al. 2015). Caution must be used in interpretation of JNDs, because the method is sensitive to estimates of receptor noise, light conditions and animal cognition. As such, we follow past work and use a slightly broader region of uncertainty in discrimination thresholds (1-3 JNDs), but ultimately the key consideration is that smaller JND values should equate to better camouflage match.

Visual predation computer detection experiment

To test camouflage efficacy of different crab phenotypes in varied backgrounds, we made a predation game where human participants searched for crabs of various sizes presented on a touch screen. Our main questions were: does the visual complexity of the background make it harder to find the prey, and are crabs hardest to find against their local habitat type (i.e. consistent with a background-specific camouflage hypothesis)?

To obtain crab and background images for the game, we sampled crabs from nine locations around Cornwall in the southwest UK and photographed them. These intertidal sites represent backgrounds of different visual complexity (with higher complexity involving substrates of many textures, contrasts, colours, shapes, and different-sized granules). Here, rock pools represent subjectively the most visually complex (A-C), mussel beds medium (D-F), and mudflats the simplest (G-I) sites. Sites were: A) Falmouth (all coordinates in decimal degrees, 50.141888, -5.063811) on the south coast,

comprising a stretch of shoreline collectively encompassing Castle and Gyllyngvase beaches. Sites hold rock pools with rocky crevices with stony or gravel substrates in the pools and, lower down on the shore, increasing abundance of seaweed. B) Summers beach at St. Mawes (50.157095, -5.017370), on the south coast comprising rock pools, gravel, and some low seaweed cover adjacent to a pebbled beach. C) Flushing (50.162191, -5.066843), on the south coast comprising rock pools, gravel, and seaweed cover. D) Godrevy Point (50.249499, -5.320966) on the north coast, which primarily consists of exposed rocky outcrops with mussel beds. E) Polzeath (50.576169, -4.920206), on the north coast of Cornwall, comprising mostly mussel bed cover adjacent to a beach. F) Mawgan-Porth (50.466705, -5.041101), on the north coast of Cornwall, comprising mostly mussel bed cover and pools adjacent to a beach. G) Helford Passage (50.098763, -5.132556), an estuarine location on the south coast has a large mudflat area as well as tiered craggy rock pools. H) Penryn (50.166956, -5.082634), mostly mudflats with a covering of green algae. I) Hayle (50.188010, -5.428120), on the north coast of Cornwall, an estuarine location has a large mudflat area.

For the game, crabs as well as the natural backgrounds from the field sites were photographed using the methods described above. Briefly, we used calibrated Samsung NX1000 equipped with Nikon EL-80 mm Nikkor and Nikon D7000 camera with a 60 mm Coastal Optics lens. The crabs were detached from the background using GIMP2 image manipulation software and the background images were cropped to 16:9 aspect ratios for the touch screen game. Crabs were scaled into the same pixel/mm aspect ratio to show crabs against the background images in natural size with respect to the background scale. Due to the number of crab images needed, custom software was designed (called 'autocrab') to automate the process of background subtraction. This

software allowed users to step through hundreds of images, automatically loading, thresholding and flood filling background areas, saving them with an appropriate transparency channel in the correct format and resolution needed for the game. This created usable crab images for 80% of the photographs very easily, with some additional cleaning up required for the rest using GIMP2 image manipulation software (https://zenodo.org/record/1101057). DOI for the source code: 10.5281/zenodo.1099634.

The experiment was a part of the Colour and Vision exhibition at the Natural History Museum of London (NHM), UK during autumn 2016. It followed the same general design of a previous online citizen science detection experiment to find hidden birds (Troscianko et al. 2017). Naturally, humans are not prime predators of crabs, but using this technique we were able to test visual detection under standardised conditions (see Discussion). Participants were visitors to the exhibition, that clicked on a screen to accept their participation in the game and the use of their data. Readers may play the game at http://crabgame.fo.am/. However, the data presented here only used the data collected at NHM. We collected basic player information, including player age and whether they had played the game before, but no personal information and participants were free to quit the game at any time. There were two versions of the game, comprising displays that broadly simulated the information to a dichromatic observer (e.g. dichromatic combined red and green layers; simulating fish vision) and trichromatic (e.g. human) observer (Troscianko et al. 2017). However, we did not find significant difference in how quickly people found the prey in these two versions of the game, and so we do not focus on these versions here. Prior to playing, the participants were asked to give their age group (<10, 10–15, 16–35, 36–50, >50, in order to control for any age effects), to state whether they had played the game before (to control for the multiple

attempts, here we used only first plays), and to choose whether they would like to play as a simulated dichromat ("fish", pollack vision) or a trichromat (human) vision. Participants were informed to click on the crab in each image as soon as they saw them. When participants successfully clicked on the target, their capture time was recorded (to the closest millisecond). The location of the target was made random in each slide without touching the edges of the screen. Participants were given 30 seconds to find the target in each slide. If they found the crab on time it was included as 'hit'. If they failed to find the crab within time limit their data were considered as 'miss', they were given a 'time-is-up-message' and the target crab was highlighted on a screen after which the player could move onto the next slide. A total of 20 slides were presented in each game trial. Each person saw a set number of random slides per treatment combination (i.e. a randomised block design). At the end mean capture time was displayed and a summary of results were shown.

To investigate colour and luminance discrimination values in the citizen science game, we also used the Vorobyev & Osorio (1998) receptor noise limited vision model. For this, we used colour and luminance contrasts based on human vision to predict crab camouflage to humans in the experiment. We used human longwave (LW), mediumwave (MW), and shortwave (SW) sensitivity data and Weber fractions after Hofer et al. 2005: LW 0.020, MW 0.028, SW 0.066 with receptor cone ratios LW 0.629, MW 0.214, and SW 0.057 for the human vision chromatic contrast, and 0.1 for luminance contrast (based on the human achromatic channel of LW+MW). Unfortunately, we could not analyse the appearance of the crabs and images as displayed to participants *in situ* on screen that the NHM London provided for the exhibition. Thus, for detectability comparisons we used a subset of crabs presented against experimental backgrounds of each treatment group

resulting in following comparisons in our 3x3 factorial set up: mudflat crab against mudflat (n = 99), mudflat crab against mussel bed (n = 110), mudflat crab against rockpool (n = 88); mussel bed crab against mudflat (n = 108), mussel bed crab against mussel bed (n = 99), mussel bed crab against rockpool (n = 96); rockpool crabs against mudflat (n = 108), rockpool crab against mussel bed (n = 120) and rockpool crabs against rockpool (n = 96). Note that here we have not analysed pattern match of crabs to each background, which requires a number of approaches, and visual detection will depend not just on colour and luminance match but also on pattern.

Statistical analyses

We used linear mixed effects analyses (LMER) to analyse developmental of background matching through ontogeny common garden data. For colour and pattern characterization we first used principal components analysis. We did this in order to reduce data dimensionality, because we wanted to integrate all colour as well as pattern metrics into single dependent variables for the analyses. For reflectance data (colour), we used normalised camera responses of brightness, red, green, blue and UV, which yielded one component (PC_{colour}) explaining 93% of the variance with an Eigenvalue 4.65. For pattern data, we used maximum power, proportional power, sum power and mean power, which yielded one component ($PC_{pattern}$) explaining 82% of the variance with an Eigenvalue 3.26. We also calculated colour and luminance JNDs (i.e. just noticeable differences using a fish vision model, see above).

To analyse colour change experiment data, PC_{colour}, $PC_{pattern}$, chromatic JND match and luminance JND match were used separately as dependent variables. Crab initial appearance, background, week and their interactions were set as fixed factors. Tank

and crab ID were set as random factors. Similarly, we analysed the following additional colour and pattern metrics for the supplementary material: luminance, hue, pattern diversity, pattern contrast, and marking size (see Table S2, Table S3). Model simplification here and on further analyses was conducted according to the lowest AIC (Akaike Information Criterion) value when necessary to improve the model fit (i.e. to test if removing term of interest does not significantly impair the model fit), although full models often held the best fit to the data. Results remained similar if a traditional maximum likelihood test to compare a full model with a simplified model without the combination of interest (i.e. using backward stepwise protocol with significant departures from chi-square distribution) was applied.

To analyse computer-based predation experiment data, we first tested whether finding crabs is more difficult against certain backgrounds using GLMM (generalized linear mixed modelling). The success of finding the crab correctly on time (hit, miss) was set as a binomial dependent variable. Similarly, we ran another analysis using LMER where we used search time as a dependent variable. In both of these analyses crab habitat, photo habitat, vision system (tri/di-chromatic; this however was omitted from the final models) and their interactions were set as fixed factors. Crab size was set as a random covariate. Also, the game ID was set as a random factor to account for games with different players and settings. Similarly, we ran two LMER analyses to analyse crab detectability, using luminance and chromatic match (separately) as dependent variables and crab ID as random factor. All analyses were done with IBM SPSS Statistics (v22) and program R (3.2.1).

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