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Colour plasticity and background matching in a threespine stickleback species pair

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Examining differences in colour plasticity between closely-related species in relation to the heterogeneity of background colours found in their respective habitats may offer important insight into how cryptic colour change evolves in natural populations. In the present study, we examined whether nonbreeding dorsal body coloration has diverged between sympatric species of stickleback along with changes in habitat-specific background colours. The small, limnetic species primarily occupies the pelagic zone and the large, benthic species inhabits the littoral zone. We placed benthic and limnetic sticklebacks against extremes of habitat background colours and measured their degree of background matching and colour plasticity. Benthics matched the littoral background colour more closely than did the limnetics, although there was no difference between species in their resemblance to the pelagic background colour. Benthics were able to resemble both background colours by exhibiting greater directional colour plasticity in their dorsal body coloration than limnetics, which may be an adaptive response to the greater spectral heterogeneity of the littoral zone. The present study highlights how habitat-specific spectral characteristics may shape cryptic coloration differences between stickleback species. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **102**, 902–914.

ADDITIONAL KEYWORDS: cryptic coloration – divergent natural selection – environmental heterogeneity – *Gasterosteus aculeatus* – pigmentation.

INTRODUCTION

The selective advantages of differences in cryptic coloration among animal species have received significant attention (Endler, 1978; Ruxton, Sherratt & Speed, 2004; Stuart-Fox & Moussalli, 2009). In particular, when the colour patterns of closely-related species or subspecies are compared, the results can provide valuable clues about how populations adapt to their respective habitats (Macedonia, 2001; Hoekstra, Drumm & Nachman, 2004; Rosenblum, 2006). For example, examining differences in colour plasticity between closely-related species in relation to the heterogeneity of background colours found in their respective habitats may offer important insight into how cryptic colour change evolves in natural populations (Doughty & Reznick, 2004; Schlichting, 2004).

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Threespine sticklebacks (*Gasterosteus aculeatus*) are an ideal and powerful system for investigating selection on colour patterns because wild populations vary in pigmentation and much is already known about their evolution and ecology. In small lakes (Paxton and Priest) of southwestern British Columbia, the benthic species of two sympatric pairs of stickleback have reduced skin pigmentation relative to the limnetic species, and this interspecific difference is caused by alleles of the *Kit ligand* (*Kitlg*) gene (Miller *et al*., 2007). Despite the recent and ongoing genetic findings, however, the adaptive significance of these pigmentation patterns is unknown in threespine sticklebacks and warrants further attention.

One possible driver of phenotypic pigmentation differentiation is habitat-specific background colour. In habitats with high levels of predation, cryptic body coloration in the form of background matching is

common among prey species and has been studied extensively (Endler, 1978; Ruxton *et al*., 2004). Background matching is a prey's visual resemblance to the background (in colour, brightness, and pattern) and is typically an adaptation to decrease the risk of detection by predators (Endler, 1978). There are numerous examples of habitat-specific body colour variation in natural populations across a wide variety of taxa, including turtles (McGaugh, 2008), freshwater fish (Whiteley *et al*., 2009), frogs (Wente & Phillips, 2003), salamanders (Storfer *et al*., 1999), snails (Reed & Janzen, 1999), isopods (Hargeby, Johansson & Ahnesjö, 2004), lizards (Macedonia, 2001; Stuart-Fox *et al*., 2004; Rosenblum, 2006), and mice (Dice & Blossom, 1937). Such studies suggest that the habitat-specific variation in colour is primarily a result of selection by visual predators, leading to adaptive background matching.

In the present study, we tested the hypothesis that the observed pigmentation differences between individuals of the benthic and limnetic species of stickleback during the nonbreeding season are the result of habitat-specific background matching. Each stickleback species pair in southwestern British Columbia evolved independently from the marine stickleback 10 000–12 000 years ago subsequent to the retreat of the glaciers (McPhail, 1994) and, in each lake, the benthic and limnetic species differ in their ecology and morphology. The small, limnetic species feeds primarily on zooplankton in the pelagic (open-water) zone, and the large, benthic species feeds mainly on invertebrates in the littoral zone (Schluter & McPhail, 1992). In addition, limnetics and benthics are exposed to differential predation regimes: limnetics are likely preyed upon primarily by diving birds and predatory fish (Reimchen, 1994), and benthics by large invertebrate predators (Reimchen, 1980, 1994). The two habitats (littoral and pelagic zone) also differ in colour (Boughman, 2001). During the nonbreeding season, the benthic species inhabits a light environment that is more variable in background colour and shifted to longer wavelengths (i.e. less blue) than the limnetic species' habitat (Fig. 1). We investigated how the Paxton Lake species may have adapted to their respective habitats by examining whether the dorsal body colour of each species is better matched to an extreme sample of its own habitat colour than is the body colour of the other species.

Similar to many fish species, sticklebacks have some capacity to lighten or darken their dorsal body colour according to their background (Hogben & Landgrebe, 1940; Huntingford & Coyle, 2007; J. M. Clarke, pers. observ.). Chromatophore motility is a common component of rapid (physiological) colour change in fish (Fujii, 2000). On the basis of this information, it is worth testing whether natural populations of stickleback have evolved the capacity to change colour over a short period of time to adjust to their current background. Depending on the number of different backgrounds that a prey is seen against, the best strategy for coloration may not be one that provides crypsis against one background but, instead, one that provides crypsis against a variety of backgrounds (Ruxton *et al*., 2004). With colour change, an individual has the ability to respond to a spectrally heterogeneous environment by expressing a phenotype that is optimally cryptic against a suite of background colours (Wente & Phillips, 2003). We investigated whether the more variable colour background of the littoral habitat may have favoured greater colour plasticity in the benthic species relative to the limnetic species.

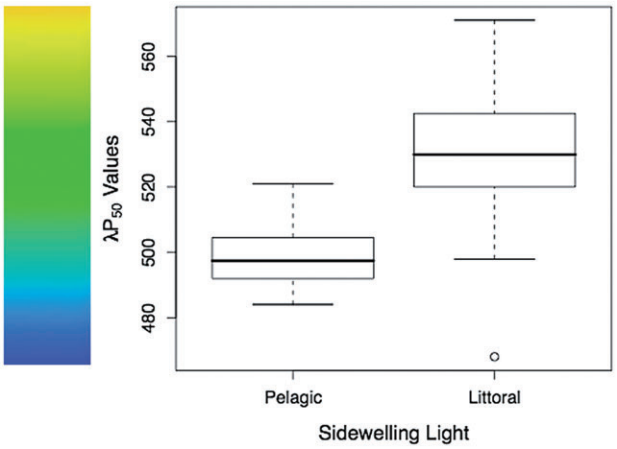

Figure 1. The distribution of sidewelling λP_{50} values from the pelagic and littoral zone in Paxton Lake measured at 10-cm depth in June 2008. The λP_{50} value is the wavelength that halves the area under the irradiance spectrum (300–700 nm) and is an index of the dominant (median) water colour. The box plots depict the values of the λP_{50} at 20 locations in the pelagic habitat and 23 locations in the littoral habitat.

MATERIAL AND METHODS

Measurement of lake water colour

We measured the background colour of the water in Paxton Lake in both the littoral zone and the pelagic zone to quantify colour differences between these two habitats. In June 2008, the sidewelling light was measured (as relative irradiance) in three areas of the lake: open water (pelagic zone), a depth of 1 m (littoral zone), and a depth of 0.3 m (littoral zone). The irradiance spectrum sums the radiances from all light sources (Endler, 1990), so that underwater sidewelling irradiance provides a measure of the background colour of the habitat that the fish is seen against

(Cummings, 2004). We used sidewelling light because it incorporates components of the upwelling and downwelling light fields, as well as any vegetation colour, in addition to sidewelling light (Cummings, 2004), making it suitable for a general measure of overall background irradiance viewed from any angle. Forty-three sampling locations were chosen randomly across the lake and comprised twenty open water locations (pelagic), twenty 0.1-m depth locations (littoral) and three 0.3-m depth locations (littoral).

At each of these 43 sampling locations, we measured sidewelling irradiance at depths (in order of measurement) of: 10 cm, 30 cm, 50 cm, 1 m, and 2 m. For sampling locations less than 2 m deep (all littoral zone samples), irradiance was measured at as many of these depths as possible. Irradiance was measured using an Ocean Optics USB2000 spectrometer and a 200 μm ultraviolet (UV)-visible fiber-optic cable attached to a CC-3-UV cosine corrector (acting as an irradiance probe which collected light from a 180° field of view), all of which were connected to a laptop computer running OOIBASE32 spectrum-analyzing software (Ocean Optics). We modified the end of the cable into a 90° angle so that the cosine corrector was oriented parallel to the lake's surface. We lowered the weighted probe vertically into the water at each sampling location and recorded the irradiances at the predetermined depths using OOIBASE32. The direction of the probe was random with respect to the shoreline. All of these measurements were taken on the same day within a 4-h period during the late morning and early afternoon. There was very little variation in cloud cover during the time of measurement, such that the lighting conditions were consistent across measurements.

We then calculated the λP_{50} value of each sidewelling irradiance spectrum recorded by OOIBASE32. λP_{50} is the wavelength that divides the area under the irradiance curve in half and is considered to be the dominant wavelength of the sidewelling spectrum (Boughman, 2001; Albert, Millar & Schluter, 2007).

Background matching experiment

To determine whether Paxton Lake sticklebacks match their respective habitat colours and have the ability to change colour based on their current background, we performed an experiment during the non-breeding season that involved placing individual fish against extremes of a 'littoral' colour background and a 'pelagic' colour background in an alternating fashion. Using digital photography, we photographed each fish from above against one of the backgrounds after a predetermined acclimation period, and then transferred to a different coloured background (if they were against a 'littoral' background, first they were transferred to a 'pelagic' background, and vice versa). After an identical acclimation period against this new background, digital photographs were taken of the fish once again. Using a pixel sampling technique that measured the RGB (Red, Green, Blue) colour scores of the dorsal body region of each fish (from a dorsal perspective) and its background, we were able to examine the degree of human-visible background matching in the benthic and limnetic sticklebacks. Based on observations (J. M. Clarke & D. Schluter, pers. observ.), it is evident that colour plasticity is very common on the dorsal region of these fish. This body area thus provided an excellent platform upon which to examine the hypothesis that fish colour varies with habitat-specific background colour. Below, we describe, in greater detail, the steps taken to perform this background matching experiment.

The use of digital photography for measuring animal colour has become increasingly common in recent years (Gerald *et al*., 2001; Stevens *et al*., 2007; Bergman & Beehner, 2008). Although spectrophotometry is considered to be the most reliable and objective method for measuring colour and can measure wavelengths beyond the human-visible spectrum (e.g. UV), it is not ideal for all types of animal colour measurements (Gerald *et al*., 2001; Bergman & Beehner, 2008). Spectrophotometry is limited to small, localized points of measurement, which do not adequately capture heterogeneity across the colour patch (Stevens *et al*., 2007). In addition, this method typically requires handling the animal in some fashion because the probe needs to be extremely close to or touching the subject (Stevens *et al*., 2007). Not surprisingly, this can disrupt the natural behaviour of the animal being measured (Stevens *et al*., 2007). As a result, digital photography was used in the present study because it allowed us to sample colour (via pixels) from a much larger body region at a single moment in time with minimal direct interference to the animals' behaviour.

Selection of representative habitat colours

To experimentally test habitat background matching, we selected extreme representative colours from the littoral habitat and pelagic habitat. This was performed by selecting pixels from photographs taken of each habitat (or habitat-specific vegetation) from Paxton Lake. To represent the pelagic zone, a photograph was taken of the surface water in the middle of the lake in full sunlight during the mid-afternoon; to represent the littoral zone, stonewort (*Chara* spp.) was extracted from the experimental ponds at the University of British Columbia and photographed outdoors in an empty aquarium, also in full sunlight during the mid-afternoon (see Supporting

information, Fig. S1). *Chara* dominates the vegetated littoral zone of Paxton Lake and serves as an important foraging microhabitat for the benthic species (J. M. Clarke & D. Schluter, pers. observ.). Both of these representative photographs were taken using a Nikon D1H digital SLR camera set to 'Daylight' white balance and saved as uncompressed Tagged Image File Format (TIFF) files, *sensu* Stevens *et al*. (2007).

We imported these photographs into Adobe Photoshop CS3 Extended, where they were converted to greyscale, with each pixel in the photograph being represented by a greyscale value between 0 and 255 (0 = black, 255 = white). The photographs were then converted to a text file, such that each pixel was replaced by its integer greyscale value. A frequency distribution of the greyscale values for each of the photographs was then created, providing a graphical representation of the pixel brightness distribution in the photograph.

When choosing the representative colour from each photograph, two primary criteria formed the basis for our selection process: (1) the colour should be of a brightness value that is relatively common in the photograph (to avoid the inclusion of rare white or black pixels) and (2) the colours from the two habitats should be highly contrasted from each other in brightness. The littoral zone has a higher mean greyscale brightness than the pelagic zone, so we took further extremes in this same direction when selecting the representative colours for each habitat (i.e. pelagic = darker; littoral = brighter). As a result, 'background matching' in the present study refers to an individual's ability to match an extreme sample of the habitat colour. On the basis of these criteria, 20 random pixels were chosen from the 5–10th percentiles of the brightest pixels in the littoral zone photo, and 20 random pixels were chosen from the 5–10th percentiles of the darkest pixels in the pelagic zone photograph. We avoided the brightest and darkest 0–5% tails of the pixel distributions to prevent the inclusion of colours that do not accurately represent the habitats. One pixel was then randomly chosen to be the representative extreme littoral zone colour (RGB = 168, 179, 74). Similarly, one pixel was randomly chosen as the representative pelagic zone colour (RGB = 25, 35, 44). However, all 20 of the littoral pixels had very similar RGB values, as did all 20 of the pelagic pixels. The representative pixel colours chosen were also familiar colours from the Paxton Lake habitats (Fig. 1): a green–yellow for the littoral zone and a dark blue for the pelagic zone.

These two colours were then printed onto paper using a high-quality laser jet printer. On the basis of these colour samples, paint was created that faithfully recreated these habitat colours. The paint used in the present study was a waterborne, 100% acrylic, interior-exterior 'EcoLogic' brand (#70654) paint with eggshell finish (Cloverdale Paint) and was not harmful to the fish. Although printer ink colours and paint colours are tailored to human vision, the selected colours used in the present study provided us with a good representation of the extremes of the respective habitat spectra in a controlled experimental setting. Choosing extreme colours from each habitat presumably challenged each species' background matching abilities to a point beyond which they regularly experience in their natural environments, creating a situation where any differences in background matching between benthics and limnetics could be more easily detected. One drawback of this approach is that it limits the extent to which the results can be directly extrapolated to an ecological/adaptive context. Despite this constraint, however, the experiment still provides a crucial first step in assessing interspecies differences in background matching and colour change in sticklebacks.

Fish collection

For this experiment, we used juvenile benthic and limnetic sticklebacks (less than 6 months old) sampled from the experimental pond facility at the University of British Columbia. Juveniles were used so that colour would not be confounded by sexual display. These fish were spawned and hatched in the experimental ponds and were the offspring of parents taken directly from Paxton Lake, Texada Island, British Columbia, in the spring of 2008 (the 'benthic' and 'limnetic' ponds were both stocked with 20 adult sticklebacks during this time). Each pond is 25×15 m^2 and contains a shallow (littoral) zone at one end, and a 6 m deep (pelagic) zone at the other end. The shallow littoral zone contains *Chara* vegetation and a layer of sand and limestone gravel extracted from surface mines near Paxton Lake. In August 2008, we sampled juvenile fish from both the 'benthic' pond and the 'limnetic' pond.

As we collected fish from the ponds, we separated them into labelled buckets based on their common-garden source pond ('benthic' or 'limnetic'). The fish were processed immediately after collection.

Experimental set-up and procedure

We cut forty-eight transparent plastic cups to a height of 5 cm and painted them on the inside (to actively expose the fish to the background colour) using either the littoral or pelagic paint colour described above. An array of 24 painted cups (six rows of four) was placed in equally sized circular holes within a cardboard frame with the cups arranged in an alternating littoral/pelagic pattern such that 12 littoral and 12

pelagic cups were used (see Supporting information, Fig. S1). In the second frame, we placed 12 littoral cups and 12 pelagic cups in a similar alternating pattern as the first frame but in reverse sequence of colours. An X-Rite/GretagMacbeth Mini ColorChecker chart (X-Rite Inc.) was positioned beside the plastic cup in each shot and thus appeared in every photograph taken (see Supporting information, Fig. S2). The ColorChecker chart consists of an array of 24 coloured squares (including a six-step greyscale), and served as a technical reference for both greyscale and colour. In this manner, we were able to normalize all photographs to a standard incident spectrum (Bergman & Beehner, 2008).

We then placed each fish in a cup with a small amount of water from the source buckets (approximately 2 cm deep). This was performed in a staggered fashion such that every fish was in a cup for 15 min before being photographed. During this acclimation period, human disturbances were kept to a minimum. We chose 15 min as the acclimation time because real-time photography (every 20 s) over a 20-min test period revealed that this was an adequate length of time to observe physiological colour changes in these fish (see Time Course Experiment, below)

We took two photographs of each fish using manual focus. Photographs were taken outdoors under natural light conditions using the Nikon D1H digital SLR camera set to 'Shadow' white balance and were saved as uncompressed TIFF files. After the first photograph was taken, the lens was unfocused and refocused on the same fish for the second photograph. Each photograph captured the stickleback in the cup, the ColorChecker chart, and the identification tag (Fig. S2).

Immediately after being photographed, we transferred each fish to a cup of the opposite colour in Frame 2. As in Frame 1, each fish was given 15 min to acclimate to its new background before being photographed. In total, we used 96 fish in this experiment (48 benthics and 48 limnetics), with four photographs taken of each fish (two against the littoral background and two against the pelagic background).

To determine whether coloured reflections from the sides of the painted cups had any effect on the observed dorsal body colour of the fish, we placed a single piece of white chalk (acting as a white standard) under water in each of the cups and photographed it in the same manner as the fish.

Sampling of pixels

One photograph was selected for each fish on each background (littoral and pelagic) and used for subsequent pixel sampling. Our selection was based on the criteria: (1) the fish was not obscured by any shadows in the cup, (2) the fish was not obscured by glare on the water surface, and (3) the fish was in focus.

After we standardized the best quality photographs for colour and brightness (for a more detailed description of this procedure, see Supporting information, Appendix S1), a circular sample of pixels along the dorsal region of each fish directly between the pectoral fins was selected using the circular marquee tool provided by Adobe Photoshop CS3 Extended. The size and position of the circle was determined by the width of the fish between the pectoral fins such that the edges of the circle reached the edges of the fish (at the point where the pectoral fins meet the body). A second circle of pixels having an area equal to 75% that of the first circle was then centred within the first circle, extracted, and saved to a file. This reduced circle ensured that reflections on the extreme outer edges of the fish were not sampled. The number of pixels sampled on a fish was in the range 421–2828, depending on the size of the fish and magnification of the photograph. We then used a circle of pixels of equivalent reduced area to sample the background colour of each fish's cup. The circle was positioned over an area of uniform colour of the cup as close to the fish's body as possible. This sample of pixels was also saved in a file. We measured the average R, G and B colour scores of both the dorsal sample and the background sample using the 'Eyedropper' tool with the '101 by 101 average' setting in Adobe Photoshop. Pixels were sampled from the white chalk standard in the same fashion to investigate any possible cup reflection effects on the dorsal body colour of the fish.

Time course experiment

To further investigate the change in colour exhibited by these fish, an additional background matching trial was performed that more closely examined this colour change as it was progressing in real-time. As in the previous experiment, digital photography was used to capture the dorsal body coloration of the fish in each background but, in this case, a photograph was taken every 20 s as the fish was acclimating to its current background instead of after the acclimation period.

Two limnetics and two benthics were sampled from the same experimental ponds described above and were held for a short period in white buckets, sorted by source pond. As in the previous experiment, 2 cm of water was placed in the plastic cups immediately before the trial. Two fish were sampled at a time. The first fish was placed in a cup and time '0' began as soon as the first photograph was taken. The second fish was then placed in an adjacent cup and time '0' for this fish began 10 s after time '0' for the first fish,

at which time a photograph was taken. Subsequent photographs were taken of each fish every 20 s for 20 min. This alternated between the two fish such that a photograph was taken every 10 s. The photographs were taken in exactly the same way as described above in the previous experiment and all shots included the ColourChecker card and an identification tag.

After the 20-min time period, the fish were then transferred to the oppositely coloured cup and time '0' for the first fish in this new background began as soon as the first photograph was taken. Photography then proceeded in the same fashion as described for the first background. In total, 120 high-quality photos were taken of each fish (60 per background) and all photos were standardized using PICTURE WINDOW PRO, version 4.0 (Digital Light & Color) and pixels were sampled from each photograph in Adobe Photoshop as described earlier. On the basis of the RGB scores across a 20-min time series, the dynamics of colour change could then be examined more closely in these fish.

Statistical analysis

Our analysis focused on RGB ratios rather than the absolute colours (R, G, B) because the value in each colour channel is only informative relative to the values in the other channels (Bergman & Beehner, 2008). We focused on the R : B and G : B ratios because there was almost no difference between the habitats in the R : G ratio.

We measured background matching by taking the difference between the dorsal colour sample and the local background sample, which corrected for any slight variation in illumination between images. The degree of colour change (plasticity) was measured as the difference in dorsal colour between the two different backgrounds (littoral minus pelagic). In addition to plasticity, we also measured the consistency of colour change for each species. Consistency (an intraclass correlation coefficient) provides an estimate of the similarity of dorsal body colour change among individuals of the same species (Shrout & Fleiss, 1979).

On the basis of the pixel samples from the white chalk photos, we found a small effect of cup reflection on dorsal coloration. Between backgrounds, the R : B and G : B ratios of the white chalk exhibited a mean change of 0.15 and 0.09, respectively. In comparison, benthics exhibited a mean change of 0.96 and 0.55 in these ratios between backgrounds, whereas limnetics exhibited a mean change of 0.55 and 0.33. Rather than correct for this effect, we emphasize comparisons between benthics and limnetics in the present study because both species experienced the same colour-reflection effect on each background. All statistical analyses were performed in R, version 2.6.0 (R Development Core Team, 2007).

RESULTS

Lake water colour

In Paxton Lake, the dominant wavelength of the pelagic zone was blue, whereas the littoral zone exhibited a dominant wavelength that was more greenish–yellow (Fig. 1). At a lake depth of 10 cm, the dominant wavelength of sidewelling light was shorter (more blue) in the pelagic zone (mean ± SE : $\lambda P_{50} = 498.7 \pm 2.24$) than in the littoral zone (529.9 ± 4.65) ($t_{41} = 5.77$, $P < 0.00001$; Fig. 1). Additionally, the littoral zone had more variance in λP_{50} values than the pelagic zone at 10-cm depth ($F_{19,22} = 0.203$, $P = 0.0009$), revealing that the littoral zone is more spectrally heterogeneous than the pelagic zone in Paxton Lake. These trends remained the same at additional depths. Even though the λP_{50} values of both habitats increased with depth as a result of an expected redshift in wavelength (Boughman, 2001; Albert *et al*., 2007), the means remained significantly different at each measured depth, as did the variance. This strongly suggests that the littoral zone and pelagic zone have different background colours, with the littoral zone exhibiting more variation in colour than the pelagic zone, regardless of depth (up to 50 cm).

Background matching

Benthics and limnetics matched the colour of the pelagic background equally well (Table 1). Benthics and limnetics did not differ significantly in their deviation from the pelagic background with respect to either the R : B or G : B ratio (Table 1). However, benthics matched the littoral background better than did the limnetics (Table 1). Limnetics deviated significantly more than benthics from the littoral background colour R : B and G : B ratios (Table 1).

Colour plasticity and consistency

Degree of plasticity was measured as the difference in dorsal body colour between the littoral background and the pelagic background. Here, we have defined 'directional plasticity' as colour change that is directed towards the colour of the current background (i.e. colour change that exhibits a positive plasticity score; positive slopes in Fig. 2). Both species showed some ability to change colour, although benthics exhibited significantly greater directional plasticity than the limnetics in the R : B and G : B ratios (R : B: $t_{58} = 5.86$, $P < 0.00001$; G : B: $t_{58} = 4.81$, $P = 0.00001$) (Fig. 2, Table 2).

Table 1. Mean dorsal body colour deviations from the two backgrounds (pelagic and littoral; degrees of freedom = 62 and 86, respectively)

	Mean ± SE deviation from pelagic background			Mean ± SE deviation from littoral background		
	Benthic	Limnetic	*P*	Benthic	Limnetic	*P*
R : B	0.53 ± 0.03	0.53 ± 0.04	0.934	0.92 ± 0.07	1.34 ± 0.09	**0.0004**
G : B	0.31 ± 0.02	0.33 ± 0.03	0.570	0.89 ± 0.05	1.08 ± 0.06	**0.012**

Values in shown bold indicate statistical significance.

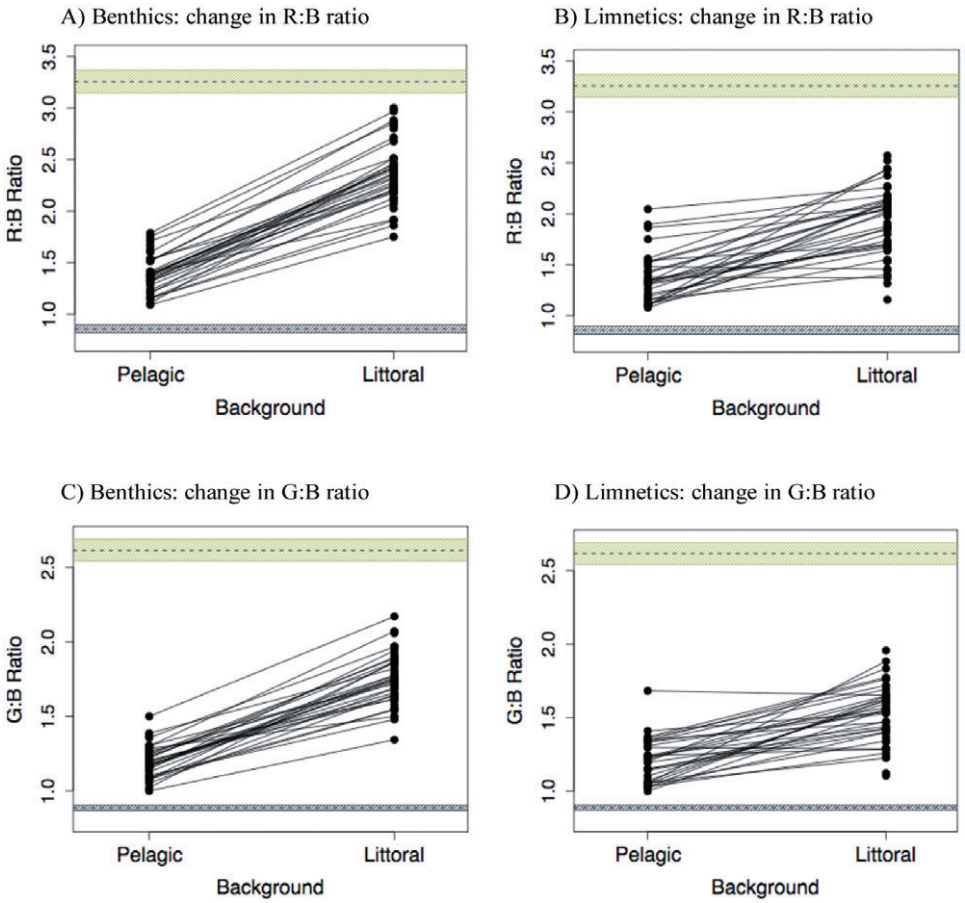

Figure 2. Change in dorsal body colour between the pelagic and littoral background. Individuals are depicted by a line connecting their colour in the pelagic background to their colour in the littoral background (when possible) in the R : B (A, B) and G : B (C, D) ratios. Benthics are represented on the left (A, C) and limnetics on the right (B, D). The mean and 95% confidence interval for the littoral background ratios are illustrated by a dashed line and pale green band, respectively. Similarly, the mean and 95% confidence interval for the pelagic background ratios are represented by a dashed line and a pale blue band, respectively.

Benthics also exhibited greater consistency in this colour change than limnetics for both colour ratios considered (Table 2). Consistency is an intraclass correlation coefficient that measures the similarity of dorsal body colour change among individuals of the same species.

Time course experiment

As shown in Figures 3, 4, both species have the ability to rapidly change their dorsal body coloration, although there were clear differences in this response between benthics and limnetics. In benthics, the most

rapid rate of colour change occurred within the first 2–2.5 min of being introduced into a new background, regardless of the colour of the background. This change exhibited by the benthics was directed towards the colour of the background ('directional plasticity'), suggesting that benthics were adjusting their dorsal body coloration to match the current background colour. After this initial and rapid response, the rate of colour change plateaued and a continued, gradual change towards the background colour was exhibited.

Table 2. Plasticity and consistency values for benthics and limnetics

	Benthics ($N = 30$)	Limnetics ($N = 30$)
Plasticity (mean ± SE difference between backgrounds)		
R : B	0.96 ± 0.04	0.55 ± 0.06
G : B	0.55 ± 0.03	0.33 ± 0.04
Consistency (intraclass correlation coefficient)		
R : B	0.65	0.28
G : B	0.50	0.25

Plasticity is the mean difference in the colour ratio between backgrounds. Consistency is the intraclass correlation coefficient for the change in colour ratio between backgrounds and measures the similarity of dorsal body colour change among individuals of the same species.

Limnetics displayed strikingly different patterns of colour change over time from the benthics and between backgrounds. Despite the fact that limnetics have the ability to change their dorsal body coloration, there did not appear to be a directed change towards the background colour such as that exhibited by the benthics. As shown in Figure 4, the dorsal body coloration of limnetics against the littoral background changed over time in an oscillatory fashion and did not ultimately result in a noticeable directed change towards the background colour by the end of the 20-min test period. In the pelagic background, the colour change exhibited by the limnetics was more comparable to that of benthics because there were dramatically smaller fluctuations over the time course (Fig. 3). Unlike the benthics, however, there

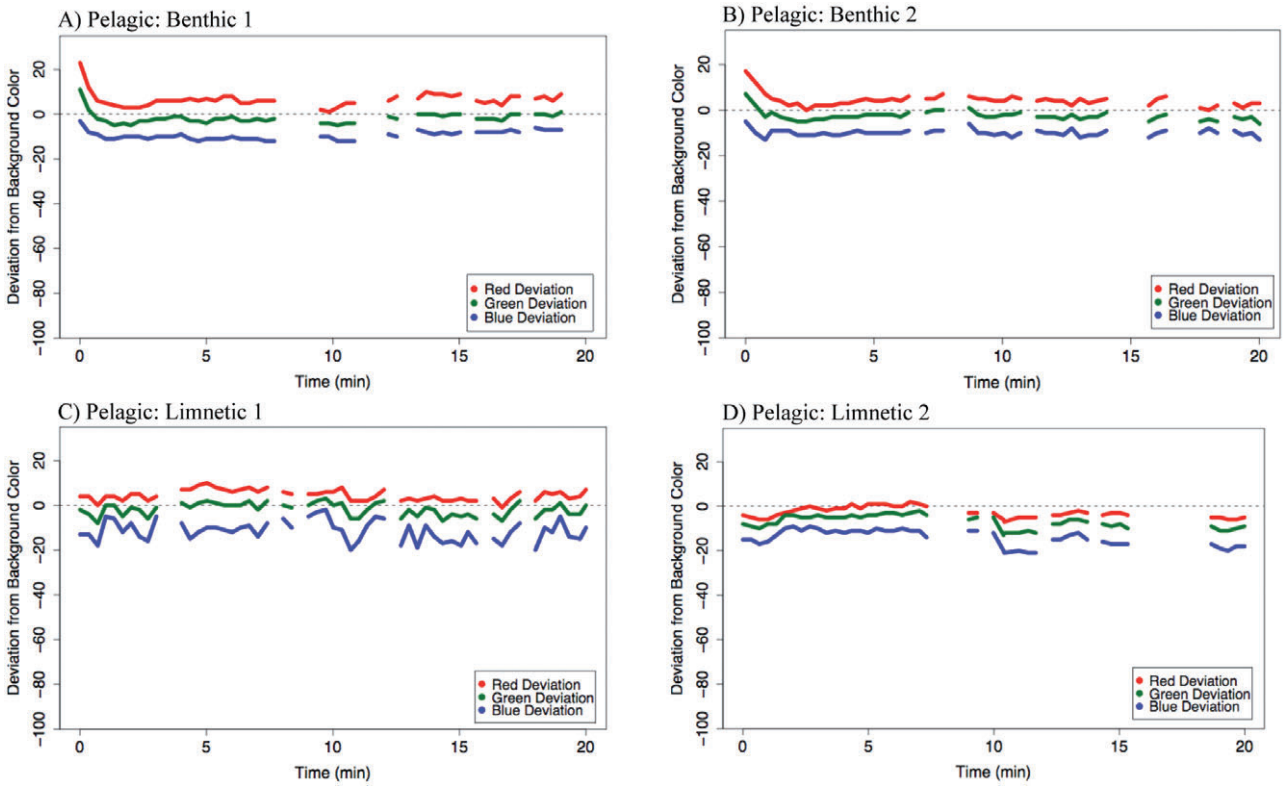

Figure 3. Time series depicting the change in dorsal body coloration of benthic individuals (A, B) and limnetic individuals (C, D) against the pelagic background. At each time interval, the body colour deviation from the pelagic background colour is illustrated by a red, green, and blue line, which represent the degree to which the body colour of the fish deviates from the red, green, and blue channels of the background colour, respectively. Gaps in the time series indicate the fish was not sampled as a result of poor photographic quality.

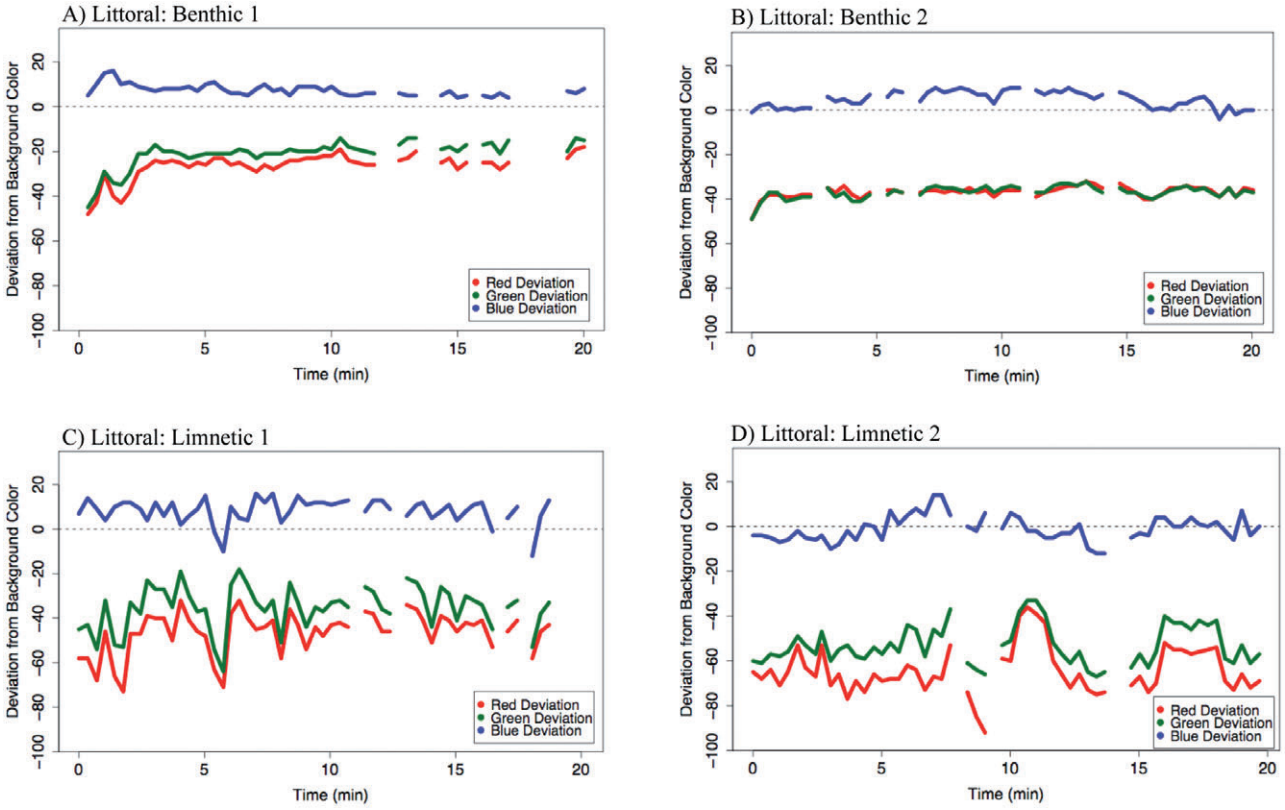

Figure 4. Time series depicting the change in dorsal body coloration of benthic individuals (A, B) and limnetic individuals (C, D) against the littoral background. At each time interval, the body colour deviation from the littoral background colour is illustrated by a red, green, and blue line, which represent the degree to which the body colour of the fish deviates from the red, green, and blue channels of the background colour, respectively. Gaps in the time series indicate the fish was not sampled as a result of poor photographic quality.

was still no recognizable directed change towards the colour of this background.

DISCUSSION

In the present study, we examined whether dorsal body coloration has diverged between the benthic and limnetic species of stickleback in association with different background colours in their habitats, and whether colour plasticity plays a role in this coloration pattern. The results revealed that the dorsal body colour of benthics matched the littoral background colour more closely than did the dorsal body colour of limnetics, suggesting that, in their own habitat (littoral zone), benthics are more cryptically coloured than the limnetic species. There was no difference between species, however, in the resemblance of their dorsal body coloration to the pelagic background (limnetics' habitat). This is because benthics exhibited greater directional colour plasticity than the limnetic species, which enabled them to resemble both backgrounds. Benthics also displayed greater consistency than limnetics in their colour changes between habitats. These results suggest that the greater degree of directional colour plasticity and consistency observed in the benthics may be an adaptive response to the greater spectral heterogeneity of the littoral zone.

In the littoral background, benthics exhibited a more green–yellow dorsal body colour than did the limnetics (Fig. 2), suggesting that benthics may have adapted to the colour of the littoral zone by chromatic background matching. A stickleback possessing body coloration that contrasts sharply with the colour of its habitat background will presumably have lower fitness (as a result of predation by visual predators) than a stickleback that is chromatically matched to its background (Endler, 1978). As a result, the green–yellow background irradiance of the littoral zone may have favoured background matching in the benthic species.

The greater degree of directional plasticity observed in the benthics may be an adaptation to the greater spectral heterogeneity of the littoral zone (Fig. 1). A

similar trend was found by Cox *et al*. (2009) in which various 'nonbenthic' fish species of a freshwater spring exhibited reduced directional plasticity relative to the benthic species, which lives in a more spectrally heterogeneous environment. Environmental heterogeneity is assumed to select for adaptive plasticity (Buskirk, 2002; Doughty & Reznick, 2004; Schlichting, 2004). The result obtained in the present study is the first key step towards demonstrating this strategy as an adaptation (Berrigan & Scheiner, 2004; Doughty & Reznick, 2004). Doughty & Reznick (2004) outline six indirect lines of evidence to support the hypothesis that plasticity is an adaptation: (1) the production of different phenotypes in different developmental environments; (2) environmental heterogeneity; (3) reversal of fitness of alternative phenotypes in different environments; (4) cue reliability; (5) genetic variation for plasticity that evolves in response to selection; and (6) comparative evidence that plasticity is correlated with environmental heterogeneity. We show that stickleback meet three of these criteria directly (1, 2, and 6) and two indirectly (4 and 5).

Benthics produced alternative phenotypes in different environments (Fig. 2), fulfilling criterion 1. Second, the sidewelling irradiance measurements from Paxton Lake provide strong evidence that the littoral zone is spatially heterogeneous in background colour compared to the pelagic zone (Fig. 1), fulfilling criterion 2. The reversal of fitness of alternative phenotypes in different environments (criterion 3) was not tested in the present study.

According to the fourth criterion, reliable cues that predict the future selective environment are necessary for adaptive plasticity to evolve. In fish, the cue for rapid colour change is light information collected by the eye from the environment, which is then processed by the retina and higher regions of the central nervous system (Fujii, 2000). Benthics in the present study likely used light information from the background as a cue to change colour because the only feature that differed between the littoral and pelagic treatment was the background colour of the cups.

Documenting the presence of genetic variation in plastic traits is the fifth criterion, although this was not directly examined in the present study. However, as described by Doughty & Reznick (2004), differences in plasticity among species provide evidence for genetic variation in plasticity, indirectly fulfilling criterion 5. For example, a correlation between phenotypic plasticity and ecological variation among species suggests that genetic variation for plasticity was 'shaped' by selection in each species' habitat (Doughty & Reznick, 2004).

The sixth and final line of evidence is that plastic phenotype expression across species is correlated with environmental heterogeneity (Doughty & Reznick, 2004), which is supported by our findings. Specifically, benthics exhibited greater directional phenotypic plasticity of body coloration than the limnetics and utilize a habitat that is more spectrally heterogeneous than that of the limnetics.

Although limnetics exhibited reduced directional plasticity compared to benthics, it is evident that they still have the capacity for rapid and substantial colour change in general (Figs. 3, 4). This suggests that colour plasticity in these fish may be an ancestral trait and that more controlled ('directional') plasticity may be a derived characteristic. Similarly, the general result that both species appear to match the pelagic background better than the littoral background suggests that background matching against a littoral-like habitat may be a derived trait as well because a pelagic-like habitat is considered ancestral in these fish (Bell, 1976; McPhail, 1994). Another possibility suggested by these results is that the limnetic response may be a different cryptic strategy altogether. For example, limnetics utilize the pelagic (open-water) zone where light 'flickers' rapidly through the water column, particularly near the surface (McFarland & Loew, 1983). A colour response that simply exhibits rapid changes without clear directionality may provide adequate crypsis in such an environment. Furthermore, the larger amplitudes of the colour change response exhibited by limnetics relative to benthics (Figs. 3, 4) could indicate a cryptic strategy to match the fluctuating light intensity in the pelagic zone. Future studies that examine the dynamic lighting conditions of each habitat in greater detail will offer more insight into these possibilities.

Fully understanding the evolution of colour differences between the limnetic and benthic species may require an examination of dorsal colours at the physiological level before an adaptive explanation can be assigned (Grether, Kolluru & Nersissian, 2004). For example, the findings reported in the present study may result from a difference between species in the physiological constraints of colour change. Fish have three different types of chromatophores in their skin: melanophores (containing melanins), iridophores (containing crystalline platelets) and xanthophores/erythrophores (containing carotenoids and pteridines) (Grether *et al*., 2004). Because changes in any one component can drastically alter the colour produced, different morphs or species may exhibit variation in the types and/or numbers of chromatophores that they possess (Grether *et al*., 2004). The density of chromatophores in the skin of poikilotherms may constrain colour change ability, and this relationship may differ between closely-related species. In a study investigating colour change in

sister salamander species, Garcia & Sih (2003) suggested that the reduced capacity of the light coloured *Ambystoma texanum* to change colour (relative to the dark coloured *Ambystoma barbouri*) was a result of *A. texanum* having a lower density of melanophores overall. In Tularosa Basin lizards, however, the opposite relationship appears to exist. That is, the darker coloured lizards were those that had a reduced capacity for colour change, rather than the lighter coloured lizards (Rosenblum, 2005). In the present study, the limnetics are darker and likely have a greater density of melanophores in their skin than benthics (Miller *et al*., 2007). Although the darker limnetics had a reduced capacity for 'directional' colour change relative to benthics, they were entirely capable of fluctuating their colours in a 'non-directional' fashion (Figs. 3, 4), suggesting that any existing relationship between colour change ability and chromatophore density in sticklebacks is not clear. A detailed physiological investigation of stickleback chromatophores would help disentangle how these colour responses evolved and diverged in this species pair.

In summary, the results obtained in the present study suggest that the observed dorsal body colour differences between the benthic and limnetic species may be a result of background matching, and that the greater directional plasticity of benthics may be a result of the greater environmental heterogeneity of the littoral zone. Selection studies that directly test the adaptive significance of dorsal body coloration in this species pair using live predators will provide critical insight into this result, as will studies that examine the parallel evolution of cryptic coloration in other stickleback species pairs, as well as those that examine other body regions and colour patterns. In addition, a genetic mapping study is required to identify the genes that may underlie the observed benthic–limnetic difference in dorsal body coloration, and a more comprehensive investigation of stickleback chromatophores would be extremely valuable for assessing the underlying physiology of this intriguing disparity between stickleback species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Photographs of the open water of Paxton Lake (A) and *chara* vegetation (B) used to sample representative pixels for the pelagic zone colour and littoral zone colour, respectively. C, one of the two frames used in the background matching experiment; 24 coloured cups (12 pelagic and 12 littoral) were used in each frame.

Figure S2. Examples of standardized photographs from the background matching experiment. The same benthic individual is shown against the pelagic (A) and littoral (B) backgrounds, respectively. Similarly, the same limnetic individual is shown against the pelagic (C) and littoral (D) backgrounds, respectively.
Appendix S1. Standardization of photographs.