

## Differential predation alters pigmentation in threespine stickleback (*Gasterosteus aculeatus*)

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### Abstract

Animal pigmentation plays a key role in many biological interactions, including courtship and predator avoidance. Sympatric benthic and limnetic ecotypes of threespine stickleback (*Gasterosteus aculeatus*) exhibit divergent pigment patterns. To test whether differential predation by cutthroat trout contributes to the differences in pigmentation seen between the ecotypes, we used a within-generation selection experiment on F<sub>2</sub> benthic–limnetic hybrids. After 10 months of differential selection, we compared the pigmentation of fish under trout predation to control fish not exposed to trout predation. We found that stickleback exhibited more lateral barring in ponds with trout predation. Ponds with trout were also less turbid, and a greater degree of barring was negatively correlated with the magnitude of turbidity across pond replicates. A more benthic diet, a proxy for habitat use, was also correlated with greater lateral barring and green dorsal pigmentation. These patterns suggest that differential exposure to cutthroat trout predation may explain the divergence in body pigmentation between benthic and limnetic ecotypes.

### Introduction

Colouration and pigmentation patterns have long been considered important traits in animals (Dice & Blossom, 1937; Endler, 1978), as these traits are well known to mediate intra- and interspecific interactions. In many species, different patches of colour across an animal's body enable an individual to distinguish its own species from another and among the individuals of its own species. For example, male nuptial colouration influences reproductive outcomes in many taxa; often females prefer brightly coloured males over dull ones (Ciccotto & Mendelson, 2016), and colouration can indicate quality or reproductive status (Houde, 1987). Colouration can also be important for mediating the outcome of interspecific interactions such as predation (Godin & McDonough, 2003). Body colouration is often used for

camouflage, where species have adapted to their environment in such a way that they are matched to their surroundings and can avoid detection by a predator (Endler, 1978; Slagsvold & Dale, 1995; Sherratt *et al.*, 2004).

Pigment and structural traits that function in predator avoidance are predicted to be favoured by natural selection in the presence of visual predators, while conspicuous visual signals, such as bright nuptial colours that attract mates, are thought to be selected against when visual predators are present (Endler, 1983). A cost of conspicuous male ornamentation has been shown in guppies, where fish under higher predation pressures have evolved duller colouration (Godin & McDonough, 2003). However, it remains unclear how often bright colouration is disfavoured and cryptic colouration favoured. Here, we sought to test whether two nonreproductive pigment traits and one nuptial pigment trait were favoured or disfavoured in the presence of predators and whether pigment traits evolve independently. To determine the effect of predation-based natural selection on pigment traits, we conducted

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a manipulative experiment using hybrid benthic–limnetic threespine stickleback that varied in pigmentation. By manipulating the presence or absence of a visual predator, we could make progress in identifying the mechanisms driving the evolution of pigmentation. We also used the experimental design to assess how habitat use and turbidity influence pigmentation.

Sympatric benthic and limnetic threespine stickleback (*Gasterosteus aculeatus*) are an excellent system to examine the interaction between pigmentation and predation-based natural selection. Benthic and limnetic stickleback exhibit divergent pigmentation for two male nuptial traits, a red throat patch and blue iris; for both traits, male limnetics are generally brighter and more colourful than male benthics (Boughman *et al.*, 2005; Albert *et al.*, 2007). Year round, there is variation between the species in body colouration and lateral barring (black vertical stripes) (Clarke & Schluter, 2011; Greenwood *et al.*, 2011). Benthic and limnetic stickleback also experience differential predation (Schluter & McPhail, 1992); benthic fish are primarily preyed upon by invertebrate predators, whereas limnetic fish are primarily preyed upon by cutthroat trout (*Oncorhynchus clarkii*) (Schluter & McPhail, 1992).

The two different suites of predators that each species is exposed to have distinct prey detection methods. Cutthroat trout use vision as a core sensory system for prey detection; trout are known to rely heavily on visual cues during pursuit of their prey (Vogel & Beauchamp, 1999), and hunting success declines with increasing turbidity (Vogel & Beauchamp, 1999; Mazur & Beauchamp, 2003). Cutthroat trout are predicted to be tetrachromatic (Bowmaker & Kunz, 1987; Rennison *et al.*, 2012) and thus should be able to detect a wide variety of wavelengths and discriminate among a multitude of colours. In contrast, the invertebrate predators of threespine stickleback are largely ambush predators and are less dependent on visual cues for prey detection (Foster *et al.*, 1988). Thus, exposure to these distinct predators could contribute to divergence in the colouration and patterning of benthic and limnetic species.

Differences between benthic and limnetic stickleback in nonreproductive colouration and patterning have been hypothesized to be important for camouflage in the presence of vertebrate predators (Clarke & Schluter, 2011; Greenwood *et al.*, 2011), but this has not been directly tested. The dorsal colouration of benthic stickleback is more closely matched (i.e. has less contrast) to the littoral background, than that of limnetic stickleback (Clarke & Schluter, 2011); this suggests that within the littoral habitat, the green dorsal colouration of benthics may be more cryptic than the limnetic colouration. Neither species shows significant pigment matching to the pelagic background (Clarke & Schluter, 2011). The lateral barring exhibited by stickleback may play a role in predation avoidance either through

background matching in a spatially complex environment (Josef *et al.*, 2012), as disruptive colouration (Cut-hill *et al.*, 2005), or through motion dazzle camouflage (when high-contrast geometric patterns interrupt the motion detection systems of a visual predator) (Thayer, 1909). A variety of factors have been hypothesized to underlie reduced nuptial colouration in some stickleback populations, including differential predation pressure (Semler, 1971), increased turbidity and carotenoid deficiency (Reimchen, 1989), yet direct tests of these hypotheses have been lacking.

To determine the effect of differential predation on pigmentation traits, we used hybrid F<sub>2</sub> benthic–limnetic stickleback in a selection experiment conducted under seminatural conditions in artificial ponds. Four experimental ponds were exposed to cutthroat trout predation, and four ponds were kept as trout-free controls. After 10 months of differential predation, differences in colour and the degree of lateral barring were estimated. Based on the observed matching between benthic colouration (green dorsal pigmentation) and the littoral habitat (Clarke & Schluter, 2011), we predicted that green pigmentation would be favoured in the trout predation treatment where background matching may be more beneficial. The hypothesized role of lateral barring in predation avoidance led us to predict that barring should be more common in the presence of vertebrate predation. Based on previous work suggesting that bright nuptial colouration is selected against in the presence of predation (e.g. Semler, 1971; Endler, 1978), we predicted that the bright blue eye displayed by many reproductive males would be disfavoured in the trout predation treatment.

The visual environment under which pigment signals are viewed is an important determinate of whether a signal appears to be cryptic or conspicuous (Hemmings, 1965); this is because visibility depends on the contrast between a signal, the background it is viewed upon and any medium between the two objects (Hemmings, 1965). Increased turbidity is one factor that can reduce the visibility of visual displays and signals by diminishing the contrast between an object and the background; this is due to the scattering of light and through an overall reduction in light penetration within the water column (Lythgoe, 1979; Utne-Palm, 2002). As a result, under turbid conditions, signals that would have high contrast and appear bright in clear water may appear less conspicuous. Previous work has shown that increased turbidity leads to a reduction in bright nuptial colouration (Reimchen, 1989; Seehausen *et al.*, 1997) and reduced reliance on colour-based signals during courtship (Luyten & Liley, 1985; Seehausen *et al.*, 1997; Engström-Öst & Candolin, 2006).

To further explore our hypothesis that lateral barring and dorsal pigmentation could be beneficial for predation avoidance (camouflage), we considered the effect of turbidity and diet (a proxy for habitat usage) on the

magnitude of divergence in colour pigmentation and patterning. In this experiment, it was previously shown that the addition of cutthroat trout led to a shift in stickleback habitat use and diet, which also affected the turbidity of the ponds (Rudman *et al.*, 2016). The shift in turbidity was the result of a trophic cascade: in ponds with trout, the stickleback foraged more on benthic invertebrates, which released zooplankton from predation and decreased phytoplankton abundance, thereby decreasing turbidity (Rudman *et al.*, 2016). The opposite was seen in control ponds, where stickleback foraged more heavily on zooplankton. Given the observed differences in turbidity between the treatments (Rudman *et al.*, 2016) and the known effect of increased turbidity on the visibility of pigment patterns and bright colouration (e.g. Hemmings, 1965), we predicted that under turbid conditions, the utility of bright nuptial colouration in mate displays would be reduced and the necessity for pigmentation that aids in camouflage would be lessened. To determine whether habitat use affected colouration, we examined the relationship between diet (estimated by stomach contents) and pigmentation. We considered the proportion of zooplankton vs. benthic invertebrates in the diet, as this would indicate where fish most often foraged. Again, considering the observed matching between green dorsal pigmentation and the littoral habitat (Clarke & Schluter, 2011), we predicted that increased green pigmentation would be favoured by individuals that more frequently exploited the littoral habitat.

## Materials and methods

### Experimental design

In spring 2011, four benthic females were artificially crossed with four limnetic male threespine stickleback from Paxton Lake (Texada Island, British Columbia) to create four  $F_1$  benthic–limnetic hybrid families. These  $F_1$  hybrid offspring were reared under common laboratory conditions in 100-L tanks for 1 year. In spring 2012, these  $F_1$  fish were introduced into eight seminatural experimental ponds located on the University of British Columbia campus. Each  $F_1$  hybrid family was randomly split in half and introduced into a pair of ponds. See Fig. S1 for a depiction of the experimental design. Each pond within a pair received the same number of individuals. However, different pond pairs received different numbers of individuals depending on the original  $F_1$  family size (between 23 and 31 individuals were added per pond). The experimental ponds were 15 × 25 m in size with a maximal depth of 6 m (see Arnegard *et al.*, 2014 for further details on the pond structure). Each pond contained a natural assemblage of food resources and vegetation. Prior to fish introduction, the eight ponds were paired based on count surveys of macrophyte coverage, phytoplankton,

zooplankton and insect abundance. In spring 2012, the  $F_1$  fish reproduced naturally within the ponds to create the focal  $F_2$  hybrid generation. In September 2012, two coastal cutthroat trout (*Oncorhynchus clarkii*) were introduced into one randomly chosen pond within each pond pair. The majority of the  $F_1$  fish died following the 2012 breeding season; however, due to size differences between 2-year-old  $F_1$  fish and the approximately 1-year-old  $F_2$  cohort, we are confident that any rare  $F_1$  survivors did not contribute to the sample of fish we phenotyped for the analysis.

At the beginning of the experiment, on average, there were 1834  $F_2$  fish per pond, with no significant difference in the population size of fish in trout treatment pond vs. control ponds (Rudman *et al.*, 2016). After 7 months of the experiment, and immediately prior to the breeding season (and phenotyping time point), the stickleback population size in control ponds had been reduced on average by 25%, in contrast there was an average 65% reduction in population size for trout treatment ponds (Rudman *et al.*, 2016). This differential mortality between treatments, combined with observed predation events, provides strong evidence that the trout were active predators over the course of the experiment.

The purpose of using hybrids in the experiment was to increase the genetic variation available for selection to act upon.  $F_2$  hybrids specifically were instrumental in this study as they had experienced two generations of recombination, which allowed unlinked traits to segregate independently. By establishing the ponds using full-sibbling  $F_1$  crosses, that were intermediate in the pigmentation phenotypes and heterozygous at loci that are differentiated between their pure benthic and limnetic parents, we were able to generate  $F_2$  individuals that exhibited phenotypic variation. Previous benthic–limnetic  $F_2$  crosses (e.g. Arnegard *et al.*, 2014; Conte *et al.*, 2015) have shown that there are individuals produced in an  $F_2$  cross that are very benthic in their phenotype, that are very limnetic and that have phenotypes intermediate relative to either pure ecotype (with an approximately normal distribution of phenotypes). The  $F_1$  experimental design ensured that starting frequencies of each phenotype would be very similar between treatment and control ponds within a pair.

### Pond sampling

In May and June of 2013 (after 9–10 months of natural selection), adult (~1 year old) reproductively mature  $F_2$  stickleback were caught using a combination of unbaited minnow traps, open water seining and dip netting. One hundred  $F_2$  individuals were randomly subsampled from all of the captured individuals from each pond (800 individuals total) and were retained for phenotyping before being returned to the pond of origin.

### Phenotyping of pigmentation traits

The F<sub>2</sub> individuals were photographed using a Nikon D300 camera with a 60-mm macro lens (Nikon, Melville, NY, USA). The photographs were illuminated with ambient light, the camera flash and an external ring flash. The camera settings were ISO 200, automatic white balance, 2.5-s exposure and F22. Prior to analysis, a white balance was applied in Photoshop (Adobe Creative Suite 5 and 6) to all pictures. Quantitative analysis was carried out in ImageJ (<https://imagej.nih.gov/ij/download.html>) with the additional Color\_Histogram.jar plugin (<https://imagej.nih.gov/ij/plugins/color-histogram.html>). From the pictures, eye colouration, dorsal colouration and the degree of lateral barring were measured.

The degree of barring along the lateral flank was determined by estimating the absolute differences between light and dark patches. This was done by selecting two squares 20 × 20 pixels in size, with one square placed on a dark patch, and the second one was placed on the brighter area between two of the vertical bars. When an individual did not have any barring, we selected two squares at the average distance found between vertical bars when present. From these two squares, the colour mode (a value of brightness and intensity between 0 and 255 where 0 is black and 255 is white) was recorded. We then calculated the absolute difference in mode between squares; more pronounced barring yielded a higher absolute difference in brightness. To evaluate dorsal colouration, we selected an area of 20 × 150 pixels in length and placed it directly above the pectoral fin joint for consistency. Within this area, the mean green pixel number (dorsal greenness) was estimated.

To estimate the blue colouration of the iris, a segmented line of 15 pixel width was captured around the pupil, and the mean number of blue and red pixels was extracted from the area. The segmented line was used to standardize the area surveyed and minimize effects of light reflection off of the top of the eye. Male eye blueness was estimated by dividing the mean blue pixel number by the mean red pixel number. To consider whether male nuptial colouration had diverged in the experiment, it was necessary to classify individuals as reproductive males. From photographs, the sex and reproductive state of some individuals could unequivocally be determined, and for other individuals, this was less certain. To identify all individuals that were reproductive males, we plotted red pigmentation against blue pigmentation (both colours are indicative of male reproductive state) and then used Gaussian mixture modelling for model-based clustering, using the *mclust* package (Fraley *et al.*, 2012). Using this method, we could identify two trait clusters that differentiated the previously sexed individuals. We then used these clus-

ters to classify the individuals of unknown or ambiguous sex/reproductive state; there were 163 individuals classified as reproductive males and 639 as females or nonreproductive males. We then proceeded with the nuptial colouration analysis only considering the 163 individuals putatively classified as reproductive males.

We did not evaluate red throat colour in males, which is an important mate choice cue in some populations of threespine stickleback (e.g. Bakker & Mundwiler, 1992). Our reasoning for omitting red throat pigmentation was that only a small proportion of males in either treatment group exhibited the trait. We are not sure why red throats were rare among our pond fish, one possible explanation is parasites; parasitic infections have been shown to contribute to reduced red pigmentation in sticklebacks (Bolnick *et al.*, 2015).

### Ecological data

Water turbidity was assessed in April 2013 (the month preceding the pigmentation phenotyping) by measuring phytoplankton abundance using spectrofluorometry ~10 cm below surface. The data were then converted into  $\mu\text{g l}^{-1}$  phytoplankton by applying a laboratory standard calibration curve (see Rudman *et al.*, 2016 for full details). To quantify diet, 10 fish were collected in December 2012 from each pond using a combination of dip netting and seining. Fish were euthanized and preserved in 95% ethanol. Prey items in the stomach were identified to the lowest feasible taxonomic unit, and the length of each item was measured using an ocular micrometre (see Rudman *et al.*, 2016 for full details). We then used these taxonomic classification data to quantify the proportion of the diet that was comprised of zooplankton. It should be noted that colour measurements and stomach content data were not collected from the same individuals.

### Statistical analyses

All analyses were carried out in R (R Development Core Team 2017) and R Studio version 3.2.3 (R Studio Team 2015). To determine whether trout predation influenced pigmentation, we used a paired *t*-test (two-sided with a null of zero); this allowed us to determine whether the control and predation ponds differed significantly in each pigmentation trait (i.e. there were three tests run, one for each trait). In the analysis, control and treatment ponds were paired by F<sub>1</sub> family. Ponds were used as our level of replication; thus, our test statistics are based on three degrees of freedom. To look for an association between ecological data (diet and water turbidity) and pigment traits, we estimated correlation coefficients using Pearson's product-moment correlations.

## Results

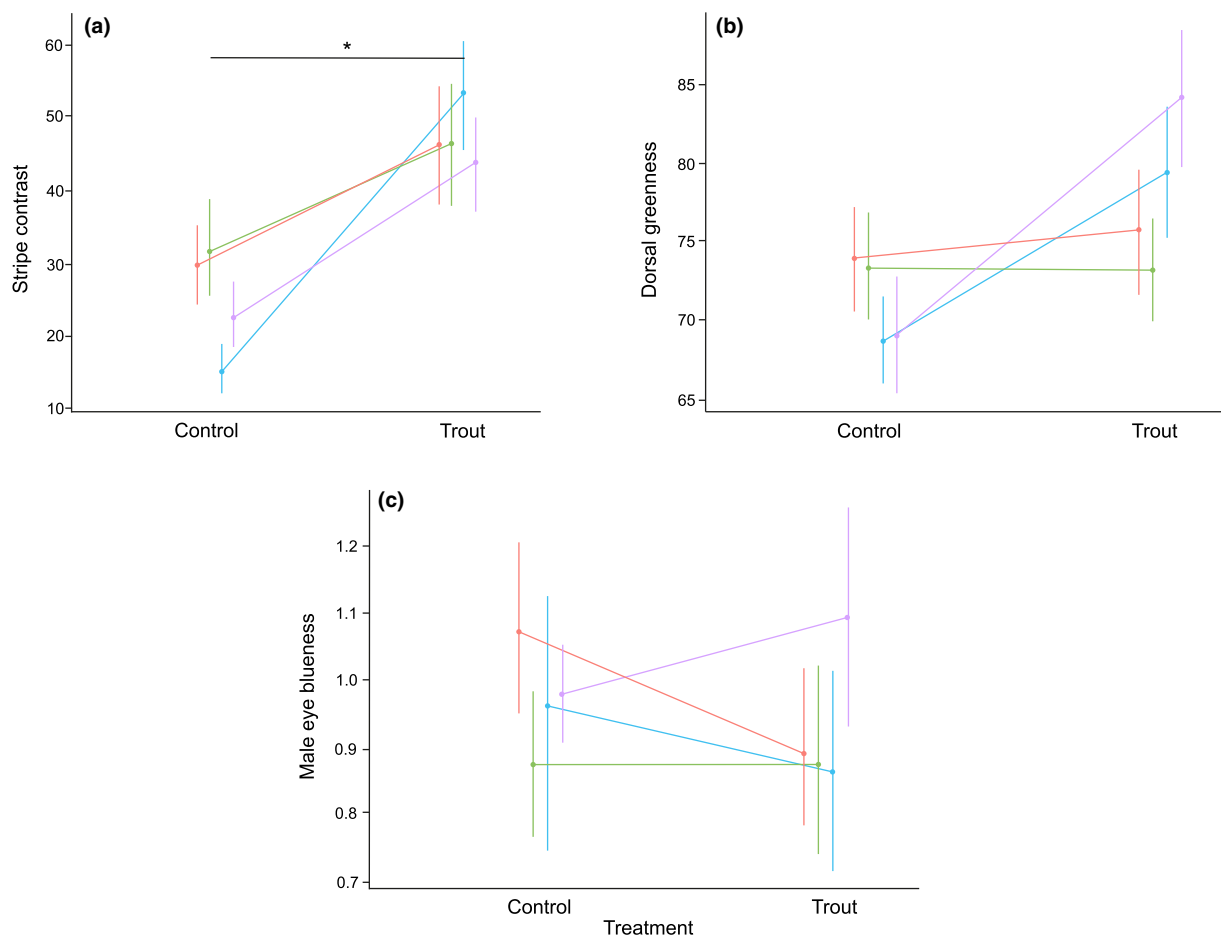
There was a greater degree of barring along the lateral flank of stickleback from trout predation ponds relative to those from the paired control ponds (Fig. 1a; mean treatment effect = 22.55,  $t_3 = 4.24$ ,  $P = 0.024$ , 95% CI: 5.64–39.46). Across the replicate pond pairs, there was no significant treatment effect on the greenness of dorsal pigmentation (Fig. 1b; mean = 6.85,  $t_3 = 1.90$ ,  $P = 0.15$ , 95% CI: –4.62 to 18.32), although there was a significant effect in two of the four pairs (Fig. 1b). There was a nonsignificant trend of a reduced blue eye colouration in reproductive males, with an effect seen in two predation treatment ponds (Fig. 1c; mean = –0.14,  $t_3 = -2.59$ ,  $P = 0.08$ , 95% CI: –0.31 to 0.03).

Among the ponds of both treatments, there was a significant negative relationship between the degree of barring and the proportion of zooplankton in the diet (Fig. 2a;  $r = -0.764$ ,  $t_6 = -2.90$ ,  $P = 0.027$ , 95% CI: –0.96 to –0.13). The extent of barring was also

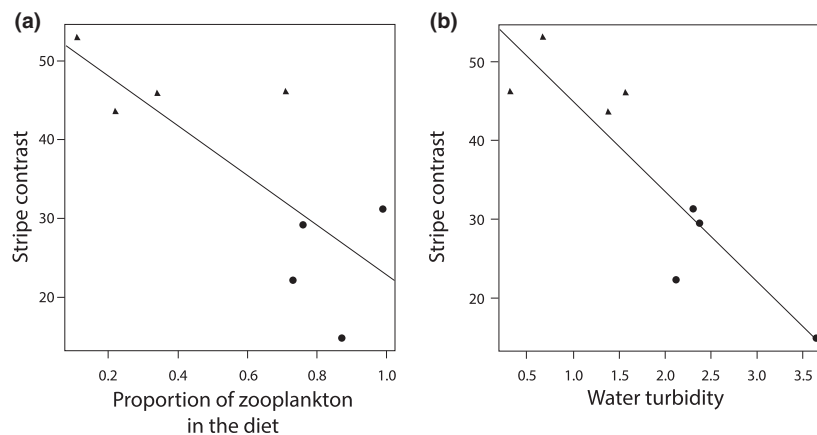
negatively correlated with water turbidity (Fig. 2b;  $r = -0.903$ ,  $t_6 = -5.16$ ,  $P = 0.0025$ , 95% CI: –0.98 to –0.55). Thus, fish with a greater degree of barring were found in ponds with lower turbidity and were less likely to consume zooplankton, *that is* a more benthic habitat usage.

There was a significant negative correlation between the proportion of zooplankton in the diet and dorsal greenness (Fig. 3a;  $r = -0.803$ ,  $t_6 = -3.30$ ,  $P = 0.016$ , 95% CI: –0.96 to –0.23), suggesting that fish with greener backs were more common in ponds where fish consumed less zooplankton. However, there was no significant correlation between the dorsal greenness and water turbidity. (Fig. 3b;  $r = -0.56$ ,  $t_6 = -1.66$ ,  $P = 0.15$ , 95% CI: –0.907 to 0.2387). There was also no correlation between dorsal greenness and the degree of barring ( $r = -0.045$ ,  $P > 0.05$ ), which suggests the two pigmentation traits were evolving independently in the F<sub>2</sub> hybrids.

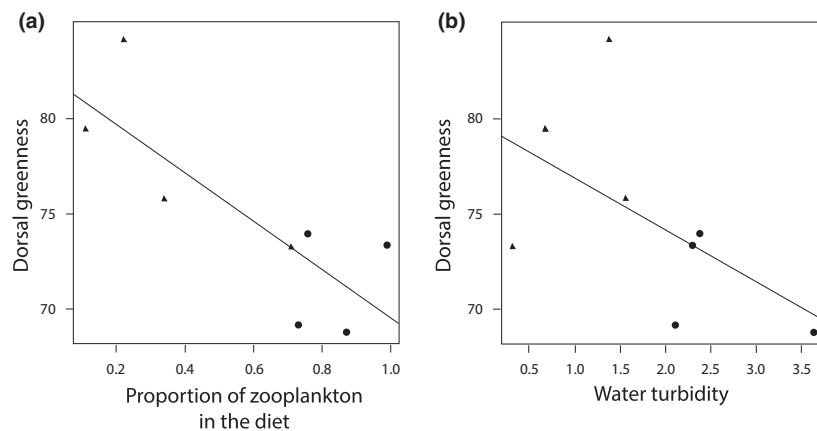
The environmental factors of diet and turbidity did not explain patterns of divergence in eye colour; there was



**Fig. 1** Effect of trout predation on four pigmentation traits. (a) Barring on the lateral flank (mode difference). (b) Dorsal greenness (mean green pixels). (c) Male eye blueness (ratio of blue to red pixels). Colour is consistent across panels and indicates ponds derived from the same F<sub>1</sub> family (paired ponds). \* indicates a significant treatment effect.



**Fig. 2** Relationship between barring on the lateral flank (mode difference) and (a) proportion of zooplankton in the diet and (b) water turbidity ( $\mu\text{g l}^{-1}$  phytoplankton). In both panels, triangles indicate trout treatment ponds and circles indicate control ponds.



**Fig. 3** Relationship between dorsal greenness (mean green pixels) and (a) the proportion of zooplankton in the diet and (b) water turbidity ( $\mu\text{g l}^{-1}$  phytoplankton). In both panels, triangles indicate trout treatment ponds and circles indicate control ponds.

no significant correlation between the eye colouration and water turbidity or stomach content (turbidity  $r = 0.26$ ,  $t_6 = 0.6$ ,  $P = 0.57$ , 95% CI:  $-0.61$  to  $0.85$ ; diet  $r = -0.19$ ,  $t_6 = -0.42$ ,  $P = 0.69$ , 95% CI:  $-0.82$  to  $0.66$ ). There was also no significant correlation between the degree of barring and eye blueness ( $r = -0.01$ ,  $P = 0.91$ ). There was a weak positive correlation between eye blueness and dorsal greenness (Fig. S2;  $r = 0.35$ ,  $t_{161} = 4.68$ ,  $P < 0.001$ , 95% CI:  $0.2$ – $0.47$ ), individuals with greener backs tended to have bluer eyes.

## Discussion

### Evidence that predation causes selection on colour

The aim of our study was to determine whether pigment traits in threespine sticklebacks shifted in response to the introduction of a vertebrate predator.

To accomplish this, we used an experiment that manipulated the presence of cutthroat trout, which are thought to differentially encounter wild benthic and limnetic stickleback. The traits we focused on were lateral barring and dorsal pigmentation in males and females, and blue nuptial eye colouration in reproductive males. We found repeated differentiation in the two nonreproductive pigmentation traits, but not in the blue eye pigmentation. It seems plausible that these nonreproductive pigmentation traits aided in predation avoidance through crypsis. Yet, the precise mechanisms by which increased lateral barring and perhaps increased green pigmentation provide a selective advantage remain to be determined.

In the wild, benthic and limnetic stickleback differ in their pigmentation patterns (Boughman *et al.*, 2005; Albert *et al.*, 2007; Clarke & Schluter, 2011; Greenwood *et al.*, 2011) and their exposure to cutthroat trout

(Schluter & McPhail, 1992). In the experiment, fish were significantly more barred in the predator treatment ponds relative to paired control ponds. Despite a trend, there was no significant difference in dorsal greenness between the trout and control treatments. These results suggest that the presence of cutthroat trout may directly or indirectly select for increased pigmentation (particularly increased lateral barring). In the wild, differential exposure of benthic and limnetic stickleback to cutthroat trout may be a key mechanism underlying the divergence of pigmentation between these ecotypes. This corresponds with previous work which has shown that predation plays an important role in explaining differences in pigmentation between species and populations (e.g. Endler, 1991; Stuart-Fox *et al.*, 2004).

Experimental fish were not reared in a common garden after exposure to trout. As a result, we cannot definitively say whether the shifts in pigmentation we observe are due to genetic changes or a result of phenotypic plasticity. Previous work on the lateral bar trait in freshwater threespine stickleback has identified quantitative trait loci explaining over 30% of the variance (Greenwood *et al.*, 2011), suggesting that it is heritable to some degree. The heritability of green dorsal pigmentation remains to be determined. It is also important to keep in mind that there may have been covariance in additional unmeasured traits, such as body shape which has been hypothesized to affect the probability of escape from predation (Walker, 1997), so we cannot rule out a correlated response.

### Crypsis as the mechanism behind the observed colour change

Evidence from a variety of taxa suggests that crypsis plays a substantial role in the evolution of colour variation between populations and species (Endler, 1978). There are three types of pigmentation thought to be useful for avoiding detection by predators. The first is object mimicry, resemblance to a common object in the environment (such as a leaf or twig) (Allen & Cooper, 1985). Object mimicry is not likely to be the mechanism that stickleback would be utilized when considering the pigmentation traits examined in this study and will not be discussed here further. The second is background matching (Endler, 1984), when an animal takes on colouration useful in blending into the local background. The third is disruptive colouration (Cott, 1940), which is when dark pigment elements make the detection of body shape more difficult. The latter two mechanisms could plausibly contribute to the observed shifts in pigment phenotype between the treatments during the experiment.

Background matching is an important mechanism of predation avoidance in a variety of taxa (Stevens & Merilaita, 2011). In benthic stickleback, background

matching has been suggested to underlie the advantage of green dorsal pigmentation in the littoral environment (Clarke & Schluter, 2011). Our findings suggest that background matching may indeed provide a selective advantage for stickleback in the presence of predators. In a few cases, disruptive colouration has been found to increase survival in the presence of visual predators (e.g. Schaefer & Stobbe, 2006; Stevens & Cut-hill, 2006), and in others, a lack of support for this mechanism has been found (e.g. Silberglied *et al.*, 1980). Disruptive colouration, such as striping or barring, is thought to be particularly useful for generalist taxa, as they may encounter more variable visual backgrounds (Ruxton *et al.*, 2004; Sherratt *et al.*, 2005). Our results suggest that lateral barring in threespine stickleback may be another example of the advantage of such disruptive pigmentation; although direct tests of this will be required to confirm whether this is indeed the mechanism by which lateral barring confers an advantage in this species.

### The role of predators in shaping nuptial colouration

Nuptial colouration is often thought to be costly (Andersson, 1994); bright colours in the presence of predators may bring unwanted attention and thus be disfavoured in high predation environments (Zuk & Kolluru, 1998). In a variety of taxa, it has been shown that predators lead to duller nuptial colouration (e.g. Godin & McDonough, 2003; Husak *et al.*, 2006; Giery & Layman, 2015). We found no significant difference in male eye colouration between treatments. It is possible that nuptial colouration in sticklebacks is unaffected by predation. However, we cannot rule out the possibility that we failed to detect differences due to misclassification of reproductive status (and perhaps sex) or due to changes in the effect over the course of the breeding season. Additionally, nuptial colouration may also have been more strongly affected if the experiment were conducted over a longer time period as was done in guppies (Godin & McDonough, 2003).

### Correlations between colour pigments and the light environment

Colouration is a visual signal which strongly depends on light transmission and visibility in the water (Wilkins *et al.*, 2016) and the background upon which signals are viewed (Abrahams & Kattenfeld, 1997). Ponds containing trout were less turbid (Rudman *et al.*, 2016) than control ponds. In the low turbidity trout predation ponds, visibility would be high; as a result, fish that were greener and/or barred would likely exhibit reduced contrast against background light. Under these conditions, reduced contrast against the background would potentially aid in predation avoidance. Consistent with this, we found that fish with lateral barring

and green dorsal pigmentation were favoured in the presence of cutthroat trout (although not significantly for green pigmentation) and that turbidity was strongly negatively correlated with both pigmentation traits.

In a variety of fish species, increased turbidity has been shown to have important implications for the expression of pigmentation traits (Reimchen, 1989; Seehausen *et al.*, 1997), predation risk (Utne-Palm, 2002) and mate choice (Luyten & Liley, 1985; Seehausen *et al.*, 1997; Engström-Öst & Candolin, 2006). Human activities such as logging and farming have been shown to cause eutrophication (Sharpley *et al.*, 2003), which in turn can lead to increased turbidity. In this experiment, we show that turbidity associated with a trophic cascade can also directly or indirectly affect pigmentation traits. This suggests that changes in the composition of a local fish community can have broad-reaching phenotypic effects that include pigmentation, and such shifts in pigmentation could have important secondary effects on predation risk and mate choice.

Green dorsal pigmentation in wild benthic stickleback is well matched to the littoral habitat (Clarke & Schluter, 2011). Fish in trout predation ponds exhibited a more benthic diet (lower proportion of zooplankton in the diet) and presumably fed more often in the littoral habitat (Rudman *et al.*, 2016). If background matching is important for benthic stickleback, it would be predicted that fish that spent more time in the littoral habitat would have more benthic-like pigmentation (increased barring and/or green dorsal pigmentation). This is indeed what we find to be the case; there was a significant positive association between benthic diet, a proxy for littoral habitat use (Wund *et al.*, 2012), and both pigmentation traits. Unfortunately, because turbidity and habitat use (diet) covary in our study, we are unable to distinguish whether one or both environmental factors mediated the proposed cryptic effects we found here.

### Correlations among traits

To determine whether the pigmentation traits could change independently of one another, we analysed the correlations between them. We found that lateral barring and dorsal pigmentation were uncorrelated and thus likely to evolve independently. However, there was a weak correlation between blueness of the eye and dorsal greenness. It is possible that this association constrained the divergence of these traits and could explain the weaker pattern of differentiation between treatments for dorsal pigmentation. Given that this experiment used  $F_2$  hybrids, we do not have the resolution needed to determine whether this association is due to tight genetic linkage (which may have varied among  $F_1$  families) or due to the pleiotropic effects of a locus on both traits. Alternatively, if these traits are costly to produce or maintain covariance could be explained if both traits were to some degree condition

dependent (i.e. high condition individuals were able to produce and maintain a bright blue eye and green dorsal pigmentation). Further analyses must be conducted to distinguish between these options.

### Conclusion

Using a controlled manipulative experiment, we show that lateral barring (and perhaps green dorsal pigmentation) is favoured in the presence of trout (and/or disfavoured in the absence of trout). We suggest the shift in lateral barring is likely adaptive as it arises across independent replicates. Differential predation did not have the same effect on blue eye pigmentation, a male nuptial trait that varies in the wild. Lateral barring and dorsal pigmentation were associated with littoral habitat use and decreased turbidity, which suggests that crypsis may be the key mechanism mediating the observed shifts. These findings suggest that cutthroat trout predation may be a factor contributing to the divergence of pigmentation between benthic and limnetic stickleback ecotypes.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

**Figure S1** Experimental set-up. Blue circles represent the four initial F1 families that were split into a trout pond (T) and a control pond (C). 100 F2 individuals were photographed and analysed from each pond.

**Figure S2** Relationship between dorsal greenness (mean green pixels) and male eye blueness (ratio of blue to red pixels).

Data deposited at Dryad: <https://doi.org/10.5061/dryad.ct19ms9>

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