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

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We thank the two anonymous reviewers and the associate editor for their thoughtful comments. Below you will find each of the comments made by the reviewers (in bold) and the action that we have taken to satisfy any concerns (marked by >).

Reviewer 1:

The manuscript reports a study that investigates the effects of predation on specific colour pattern elements in threespine stickleback. The authors use first and second generation hybrids between two ecotypes (benthic and limnetic) in predator and non-predator treatments. The results show that predation had an effect on the striped colour pattern element, with a higher contrast of stripes on individuals in the predator treatment.

Overall, I found the manuscript to be interesting and largely well written. I have a few concerns which I have detailed below in the order in which they appear in the manuscript.

1) With regards to the use of the term 'cryptic colouration' early in the introduction (line 41). Initially there is no justification for the stripe/green colouration to be assigned as 'cryptic'. A justification comes later in the introduction (line 90) but this two points need to be more strongly reconciled for the introduction to make sense to the reader. I see no mention of how these colours might be perceived by the predators which is the basis of the manuscript. In order to understand how predation is acting on visual signals it is standard to incorporate the visual parameters of the trout (or a close relative). In this case, I am willing to accept that the colours being investigated are at least detectable by the predators, however, his needs to be stated in the manuscript (preferably in the introduction, by stating something like 'our study does not determine how this colouration is perceived by the predator, but it is reasonable to assume that these colours can be seen by the predator based on.....').

>The words cryptic and conspicuous have been removed from the early part of the manuscript's introduction and downplayed throughout the manuscript.

>There is strong evidence to suggest cutthroat trout are able to perceive a wide variety of wavelengths of light (and correspondingly colours). Close relatives of the cutthroat trout (e.g. rainbow trout and most species of anadromous salmon) have been shown to possess (and express) five or six opsin genes which allow the perception of most wavelengths of light (Rennison et al., 2012). Correspondingly, these species are predicted to be at least tetrachromatic. Visual cues are also known to be key for trout foraging (Mazur & Beauchamp, 2003). Stickleback express four opsin genes (Rennison *et al.,* 2015), thus trout would likely have as good or better wavelength discriminatory abilities. A statement indicating this has been incorporated in the introduction – Line 59 onwards.

2) There is no mention of the relative proportions of the phenotypes at the start of the experiment. The assumption is that all of the ponds started with the same abundance of each phenotype, but there is no mention of this in the text. It seems entirely possible that an unfortunate experimental bias with regards to the starting populations resulted in some of the effects seen. Were any steps taken to control the frequency of phenotypes in each pond pair? I can see how determining the frequency of the intermediate phenotypes would be difficult, but a line or two addressing this seems like a fundamental addition (as it is, the authors refer to 'a few' of each phenotype – line 120).

>Unfortunately, it is impossible to know the precise starting frequencies of the phenotypes because the traits we were interested were not expressed at the start of the experiment prior to selection (i.e. when the fish were fry or very young juveniles) and the nuptial coloration is only expressed during the breeding season; this is why we surveyed adults and made our collection in the breeding season. However, all of the F_1 fish were intermediate in these traits and were heterozygous for loci that are differentiated between their pure benthic and limnetic parents (*i.e.* at tens of thousands if not hundreds of thousands of loci – genome sequencing suggests that large portions of the benthic and limnetic genomes harbour fixed differences (Schluter unpublished)). Pigmentation traits are also likely to be highly polygenic - thus heavily skewed trait distributions would be unlikely to be generated from breeding events between heterozygous parents.

>Given that full-sibling F_1s were evenly distributed between a pair of ponds it is unlikely that there would arise significantly differential trait distributions between paired ponds. Since we had 4 replicate pairs of ponds it would be particularly unlikely that all treatment ponds would show the same random skew by chance. Many previous benthic-limnetic F_2 crosses (*e.g*. Arnegard *et al*, 2014; Conte *et al*., 2015) have shown that there are individuals produced that are very benthic in their phenotype, individuals that are very limnetic, and individuals that have phenotypes intermediate relative to either pure ecotype. A statement has been added to reflect this information – Lines 175 onwards

Additionally, the reader has no idea how big the starting populations, ('F_1 fish were left to reproduce naturally in the ponds..' – line 113) or the final populations, were in each pond. Because of this the authors are asking the readers to assume that predation actually occurred (rather than differential mortality or sampling biases for example).

>The population sizes of each pond were estimated through mark recapture at several points during the experiment (Rudman et al., 2016). On average there were 1834 fish per pond at the beginning of the experiment. There was not a significant difference in population size between the trout and control treatments. Just preceding the survey of colouration the control ponds had an average population size of 1262 fish, a ~25%

reduction in population size. In contrast the trout predation ponds had an average population of 710 fish, a ~65% reduction in population size. We believe that the stark difference in population declines between treatments combined with visual observations of successful predation events provide strong evidence for actual predation. Statements indicating the starting and pre-sampling population size, and evidence of predation have now been added – Line 163.

3) I found the first two paragraphs of the results section a little difficult to follow. As it is the reader has to jump between figures in order to follow the current format. Please can this be rectified by referring to each of the figures in the order that they appear.

>The results have now been reordered to make this more intuitive.

4) Eye blueness is discussed in the results and discussion. I would really like to see the author's predictions of the effects of predation on eye blueness included briefly in the introduction (if that is what is meant by 'nuptial colouration' in line 76 please can the authors be more specific).

>This has been clarified Lines 100-101 & 126-131

5) The discussion fails to put the study into a broader context regarding predation and colouration. This would be a nice addition to the manuscript, at the moment the discussion has too much of a narrow focus in my opinion. Also, how do differences in body shape between the limnetic and benthic phenotypes contribute to the results? This should be discussed as surely some phenotypes could escape predation more effectively.

>The breadth of discussion has been widened. We now discuss the implications of our turbidity findings with regards to more general shifts in fish community and discuss animal crypsis more generally. Since we didn't collect body shape data from these individuals we cannot say whether or how shape and colour co-varied. In general, benthic and limnetic fish have different body shapes and some of these differences are thought to be due to predation – limnetics are streamlined whereas benthics are thought to have tail build for quick burst swimming. A sentence has been added to the discussion addressing this Line 328.

Additional comments:

Line 34: Any colour trait (structural or pigmented) that contributes to crypsis can be favoured by natural selection (not just pigment based colour).

>The word structural has been added here to reflect this.

Line 40: Colour conspicuousness is a function of the visual system viewing it.

Here the authors imply that they are testing conspicuous colouration but immediately below the authors write about cryptic colouration. The introduction needs to talk about components of a colour pattern rather than assigning them to being cryptic or conspicuous without providing the justification.

T>he word crypsis and its discussion has been moved towards the end of the introduction and is now more explicitly and thoroughly addressed throughout the manuscript.

Line 41: I don't understand why the authors assume that pigment traits are cryptic. Whether a colour is cryptic or not really depends on who is viewing it. It would be better to talk about the potential function of colours rather than prematurely deciding that they are cryptic (after all, the authors state whether these colours are cryptic this has not been tested – starting line 63).

>As addressed above the word crypsis and its discussion has been moved towards the end of the introduction and is now more explicitly and thoroughly addressed.

Line 49: Please mention that these species are sympatric somewhere in the introduction. It is currently implies rather than stated.

>This has been added to line 48.

Line 73: The authors are not showing 'divergence' here, but rather differences.

>The word divergence has been changed to differences.

Line 74: I am missing the justification for these predictions. What makes stripes and green pigmentation cryptic in this system? Is it because of the microhabitat, the visual system of the predators, prey/predator behaviour? There are a number of reasons that colours can be considered conspicuous or cryptic and this is often dependent on the context under which they are viewed. The justification of the predictions needs to be developed further.

>Clearer and evidence-based predictions are now explicitly made – Lines: 93 – 102, 126-130).

Line 90: If this is the justification that I was missing in the previous comment please can the last two paragraphs of the introduction be rearranged so that the justification of the predictions and the predictions are more closely linked. Also, this statement needs a reference; what is the evidence that 'few patterns or colours would be conspicuous in highly turbidity environments..'?

>This is now more explicitly linked to the predictions as outlined above (Lines: 93 – 102, 126-130)

Line 96: This sentence reads as though the ponds would be greener and more striped.

>This sentence has been revised to reflect our intended meaning – that the *fish* in these ponds would be more striped and green.

Line 106: How were the families 'split'? Was it randomly? By phenotype? This is actually quite an important detail given that this step can strongly dictate the results.

>The F_1 fish were all intermediate in phenotype, thus the family was split in half randomly. A statement indicating this has been added – Line 146

Line 107: The ponds didn't receive the same number of individuals; they received between 23 and 31. Please remove 'the same number of individuals' from this sentence.

>Each pond *within* a pair received the same number of fish. The number of fish between pairs varied. The sentence has been revised to reflect this.

Line 113: Were F1 fish remove from the tanks? If so, this isn't clear. If not, then the authors are not testing the effects on the F2 generation alone as implied in the introduction (line 69).

>F_1 fish were not removed from the ponds. However, they had little or no impact on the experiment. This is because most stickleback live for only one year, correspondingly the vast majority of the F_1 fish died following the breeding season. Perhaps one or two F_1 fish survived the breeding season in each pond – in contrast there were ~1834 F_2 fish in each pond, thus any hypothetically surviving F_1 would comprise about 0.01% of the population. We are also confident that none of the fish phenotyped for colour or pigmentation were F_1 individuals. As any remaining F1s would be substantially larger than the 10-11 month old F_2 fish. A statement indicating this has been added – Line 158

Line 120: 'A few' isn't very scientific. If the authors have the number of individuals of the three phenotypes (benthic, limnetic and intermediate) they should put them here. If not then a sentence or two addressing how this was initially controlled should be added.

>The word few has been removed and as described above a statement addressing the initial distribution has been added.

Line 124: It would be useful to know how many individuals were in the tanks at the start of the experiment and at the sampling period. The strength of the predation (and that predation did occur) can be assessed by determining the difference between the population at the start and end of the experiment

(accounting for natural mortality which could be judged from the controls).

>As described above a statement addressing the initial and pre-sampling population size has been added.

Also the readers will want to know what fraction of the final population 100 individuals represents (presumably this is both the F1 and F2 generation?). Additionally, it seems important to know how these individuals were caught (given the differences in feeding behaviour and tendency to sort by depth) – could the sampling have been subject to any bias?

>As described above this was only the F2 generation. A statement on how we conducted pond sampling has been added – Line 186.

Line 125: Please be explicit in stating how many generations 9-10 months represents? It is implied that this represents a single generational time period, but it is not stated. Also, why was this time period chosen?

>Stickleback generally live one year, the fish sampled were about 1 year old. The reason for choosing this time point was that this is when the fish are reproductively mature and breeding, thus expressing the nuptial coloration and pigmentation patterns we were interested in. This information has been integrated at various points in the manuscript.

Line 136: I am curious as to whether the authors considered comparing the number of individuals with and without stripes between the treatments?

>We hadn't previously considered looking at the proportion of individuals with or without stripes, the reason is that you have to define a somewhat arbitrary cut-off differential pigmentation to determine whether an individual is or is not striped. However, we did try this during the revision and it yields the same results of our more quantitative measure. 31-41% of fish in predation ponds were striped compared to 6—20%. Thus, we have kept our original quantitative measure rather than a binary one.

Line 140: Contrast should be calculated with regards to the visual system that is viewing it. At the very least this should be acknowledged and it stated that contrast was determined by absolute differences between two colours.

>To avoid confusion associated with the word contrast in the visual perception literature we have replace our use of "stripe contrast" with "degree of lateral barring", which has been used previously (*e.g.* by Greenwood *et al.* 2011) to describe the horizontal stripes in threespine stickleback. We now also make it clear that the degree of barring was determined by estimating the absolute differences between the two colours. Line 209

Line 145: Why was the total iris area not used? Is the outer edge the most

colourful part? Please can a line be added explaining this?

>Eye size varies tremendously among individuals, this was a way to standardize the area surveyed. Additionally, in the photos there was often glare (reflection of the illumination) at the top of the iris, the standardization was also implemented to avoid the effect of this, as it varied from fish to fish. A statement indicating this has been added – Line 215

Line 157: Please include the sample sizes for the males classed as reproductive.

>There were 163 individuals designated as reproductive males. As statement indicating this has been added – Line 227

Line 176: I am not clear what data went into the one sample t-test. Was it the paired difference between the control and treatment ponds?

>Yes, the paired differences (which is the same as a paired t-test). A statement indicating this has been added – Line 254 onwards.

Line 203: There was a trend in two of the families, not four as is implied by this sentence.

>This has been revised.

Line 214: The assumption that the stripes are cryptic is again made here. The authors are testing whether stripes can be considered cryptic.

>The use of the word cryptic and the basis for our hypotheses have been revised throughout the manuscript.

With the positive correlation between eye blueness and greenness how are the authors sure that greenness isn't related to nuptial colour or reproductive status. Really it seems that the authors are investigating the functions of different aspects of a colour pattern rather than cryptic vs nuptial.

>Previous work (e.g. Clarke & Schluter, 2011) has clearly shown that both male and female fish exhibit green pigmentation (*i.e* unlike blue eyes it isn't sexually dimorphic) and that fish exhibit this trait outside of the breeding season. One explanation for the correlation could be that fish that are in good enough condition to maintain bright dorsal pigmentation can also maintain bright blue eye pigmentation (i.e. both traits are to some degree condition dependent). We agree these are different aspects of colour pattern and we now try not to contrast cryptic vs nuptial as much throughout the manuscript.

Line 246: If red pigmentation indicates reproductively active individuals, I don't understand how nuptial colour was measured in reproductively active males if

there weren't enough males with red pigmentation in the populations. I am clearly missing something! Whatever that is, please can it be added to the methods or discussion.

>Red is indeed one nuptial colour trait; however, it can be a relatively rare phenotype in some populations (Reimchen, 1989 Evolution) (and even expressed in females in other populations). In wild benthic and limnetic populations many male individuals in reproductive condition (i.e. exhibiting the pigmented eye, with mature testis and building nests) do not exhibit red coloration. Whether due to genetic or plastic (e.g. condition or parasite load (Bolnick et al., 2015 PloS One)) effects a relatively small fraction of the males in our experiment exhibited red throat pigmentation. Since so few individuals exhibited the trait in either treatment we choose not to conduct an analysis of this trait due to the small sample size. A section in the methods has been expanded to explain this more thoroughly. Lines 232 - 238

Reviewer: 2

Comments to the Author General comments:

In this interesting study, the authors explored whether differential predation by trout contributes to differences in pigmentation in sticklebacks. The authors used a within-generation selection experiment on F2 benthic-limnetic hybrids. After 10 months, they compared the pigmentation of fish under trout predation to control fish and found that stickleback were more striped in ponds with trout. Fish in ponds with trout foraged more on benthic invertebrates, which released zooplankton from predation and decreased phytoplankton abundance, which in turn decreased turbidity. The authors found that greater stripe contrast was negatively correlated with the magnitude of turbidity across pond replicates. A more benthic diet, which they used as a proxy for habitat use, was also correlated with greater stripe contrast and green dorsal pigmentation. These patterns suggest that differential exposure to predation, and the cascading effects on turbidity and habitat use, may explain divergence in cryptic body pigmentation between benthic and limnetic ecotypes.

The across-generation experimental approach is excellent, and the system is ideal for testing the hypotheses. Photographing 100 individuals per pond is also a strength of the work that allowed the authors to get precise estimates of their response variables. The manuscript is also well-written and the study appears to have been carried out well.

My primary concerns are: 1) I found the description of the statistical methods to be somewhat vague and confusing, and think that more detail of analyses is needed; 2) I wonder if the number of analyses could be reduced by using ANCOVA (see comment below); and 3) the discussion is almost entirely focused on sticklebacks, and for a broad journal, I would expect more discussion of the

broad implications to the field.

Minor comment: L 160-165: Some additional details of when this occurred and how often turbidity was measured would be helpful.

>The measurement was taken the month before the phenotyping was done – a statement indicating this was added to Line 240

L 172-180: I find this description to be vague (or possibly just awkwardly worded). For example, you state that 'Significance testing of pigmentation treatment effects was done using one-sample t-tests'. Would it be possible to state what you are testing for biologically in the stats description? E.g. 'To determine if trout predation influenced pigmentation, we used t-tests in which we determined if the control and predation ponds differed in striping.' (or something similar).

>This has been revised as suggested.

L 174-175, 'Treatment effects were estimated within each of the four F1 families (i.e. within the control and predation ponds that were paired)': Does this mean that individual fish are being treated as replicates? Or are ponds your level of replication?

>Ponds are the replicate as indicated by the 3 degrees of freedom for our test statistics. This has been clarified in the methods section – Line 258

Also, does this mean that a separate analysis was run for each family (which seems to be implied in L 194 but not elsewhere)? If so, it seems like a single analysis for each response variable would be more appropriate. For example, why not run a single ANOVA (or ANCOVA) for each response variable in which family and predation treatment are factors and stripe contrast? I would think that turbidity and diet could also be included as covariates in such a model. Perhaps I am missing or misunderstanding something, though.

>No, a single test was run for each trait. This has been clarified in the methods section – Line 256.

L 231-232: Good point.

>Thank you.

Discussion in general: The discussion focuses largely on sticklebacks. Do your findings relate to work in other systems? Are there any bigger-picture implications to our understanding of the expression of variation in general?

>The discussion has now been thoroughly re-written in an attempt to broaden the implications of the findings.

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_2 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, 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 divergence in body pigmentation between benthic and limnetic ecotypes.

Key words: natural selection, pigmentation, patterning, adaptation, species interactions

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 inter-specific 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 non-reproductive 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 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 (*Onchorhynchus 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 and 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 non-reproductive 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 (Cuthill *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_2 benthic - limnetic stickleback in a selection experiment conducted under semi-natural conditions in artificial ponds. Four experimental ponds were exposed to cutthroat trout predation and four ponds were kept as trout-free controls. After ten 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 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 of light penetrance 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 & Lily, 1985; Seehausen *et al.,*1997; Engström-Öst & Candolin, 2007).

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.

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 one year. In spring 2012, these F_1 fish were introduced into 8 semi-natural 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 Supplementary Figure 1 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 - 31 individuals were added per pond). The experimental ponds were 15 x 25m in size with a maximal depth of 6m (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 (*Onchorhynchus 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 two-year-old F_1 fish and the approximately one-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-sibling 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 nine to ten months of natural selection) adult (~1 year old) reproductively mature F_2 stickleback were caught using a combination of un-baited minnow traps, open water seining, and dip netting. One hundred F_2 individuals were randomly sub-sampled 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_2 individuals were photographed using a Nikon D300 camera with a 60mm macro lens (Nikon, Melville, NY). The photos were illuminated with ambient light, the camera flash and an external ring flash. The camera settings were ISO 200, automatic white balance, 2.5 second 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 done 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 20x20 pixels in size, with one square placed on a dark patch, 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 – 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 20x150 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 photos, the sex and reproductive state of some individuals could unequivocally be determined, 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) 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 clusters to classify the individuals of unknown or ambiguous sex/reproductive state; there were 163 individuals classified as reproductive males and 639 as females or non-reproductive 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 ~10cm below surface. The data was then converted into ug1^{-1}phytoplankton by applying a lab 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 micrometer (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 done in R (R Development Core Team 2017) and R Studio version 3.2.3 (R Studio Team 2015). To determine if trout predation influenced pigmentation, we used a paired t-test (two-sided with a null of zero); this allowed us to determine if 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_1 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 (Figure 1A; mean = 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 (Figure 1B; mean = 6.85, $t_3 = 1.90$, $P = 0.15$, 95% CI: -4.62 – 18.32), although there was a significant effect in two of the four pairs (Figure 1B). There was a non-significant trend of a reduced blue eye colouration in reproductive males, with an effect seen in two predation treatment ponds (Figure 1C; mean = -0.14, $t_3 = -2.59$, $P = 0.08$, 95% CI: -0.31 – 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 (Figure 2A; r = -0.764, $t_6 = -2.90$, $P = 0.027$, 95% CI: -0.96 – -0.13). The extent of barring was also negatively correlated with water turbidity (Figure 2B; r = -0.903, $t_6 = -5.16$, $P = 0.0025$, 95% CI: -0.98 – -0.55). Thus, fish with a greater degree of barring were found in ponds with lower turbidity and were less likely to consume zooplankton; *i.e*. a more benthic habitat usage.

There was a significant negative correlation between the proportion of zooplankton in the diet and dorsal greenness (Figure 3A; r = -0.803, $t_6 = -3.30$, $P = 0.016$, 95% CI: -0.96 – -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. (Figure 3B; r = -0.56, $t_6 = -1.66$, $P = 0.15$, 95% CI: -0.907 – 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_2 hybrids.

The environmental factors of diet and turbidity did not explain patterns of divergence in eye colour; there was 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 – 0.85; diet r = -0.19, $t_6 = -0.42$, $P = 0.69$, 95% CI: -0.82 – 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 (Supplementary Figure 2; 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 non-reproductive pigmentation traits, but not in the blue eye pigmentation. It seems plausible that these non-reproductive 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 remains 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 or 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 or 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 and Cooper, 1985). Object mimicry is not likely to be the mechanism that stickleback would be utilizing 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.* Schafer & Stobbe, 2006; Stevens & Cuthill, 2006), 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 a*l., 2004; Sherratt *et a*l., 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 & Kolloru, 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*., 2005; Giery *et al.*, 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 divergence 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); given that the experiment was conducted within a generation, there may not have been sufficient time for adaptation of this trait.

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, 2007). 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, 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 suggest 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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Data sharing statement

All raw data will be archived in dryad doi: to be determined

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Figure Captions:

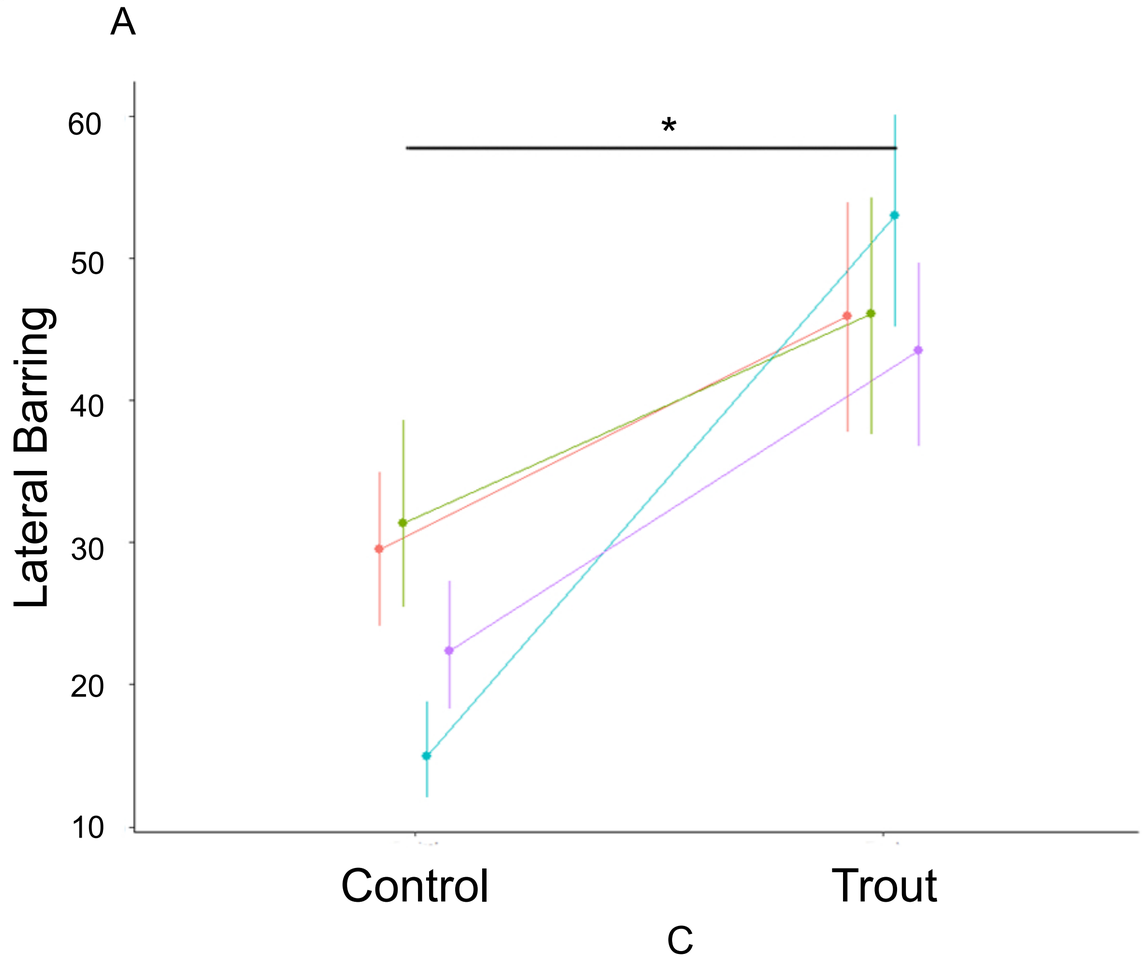
Figure 1. Effect of trout predation on four pigmentation traits. (A) Barring on the lateral flank (mode-difference). (B) Dorsal greenness (mean green pixels). (C) Male eye blueness (ratio of blue to red pixels). Colour is consistent across panels and indicates ponds derived from the same F_1 family (paired ponds). * indicates a significant treatment effect.

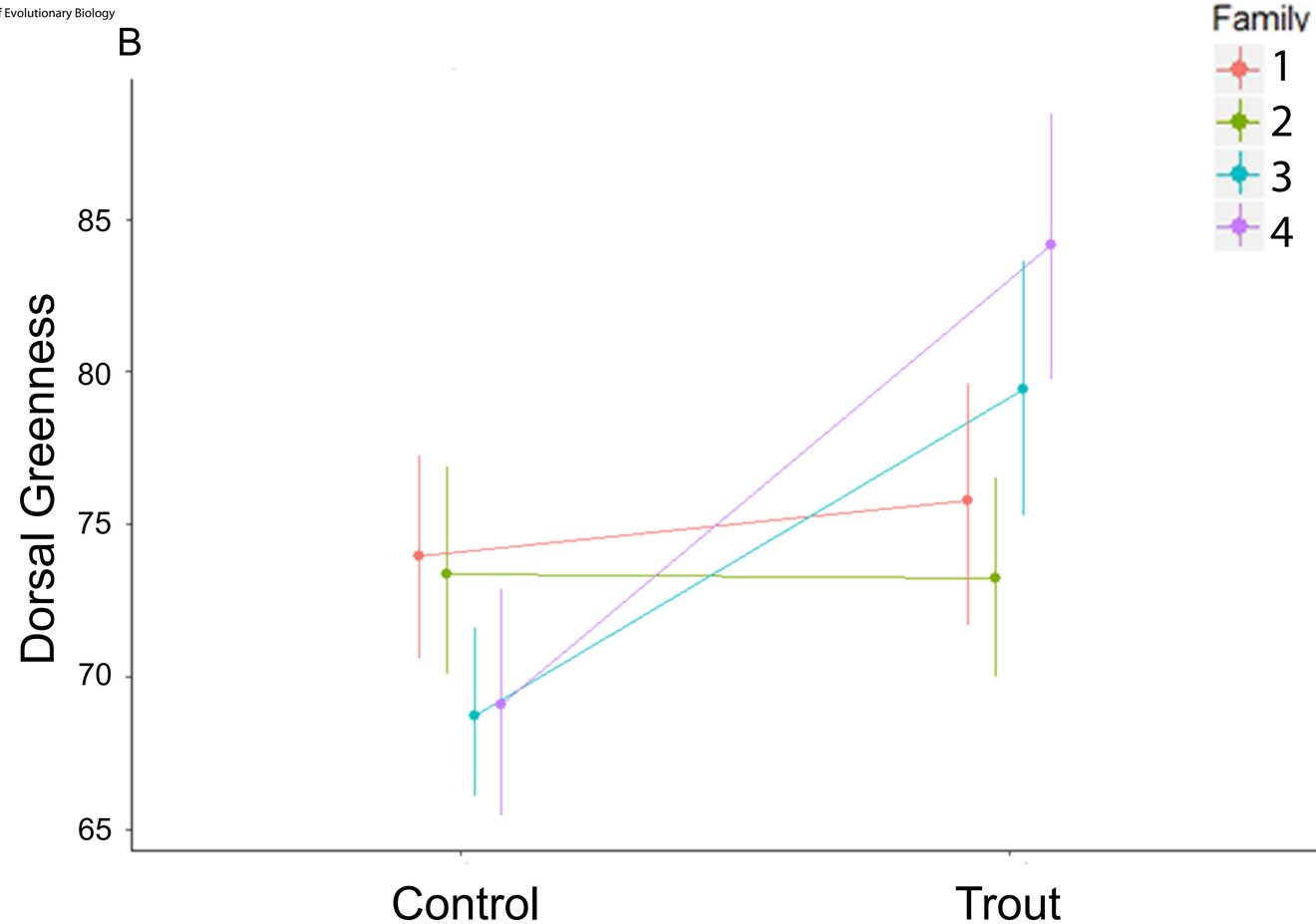
Figure 2. Relationship between barring on the lateral flank (mode-difference) and (A) proportion of zooplankton in the diet and (B) water turbidity (ug1^{-1}phytoplankton). In both panels triangles indicate trout treatment ponds and circles indicate control ponds.

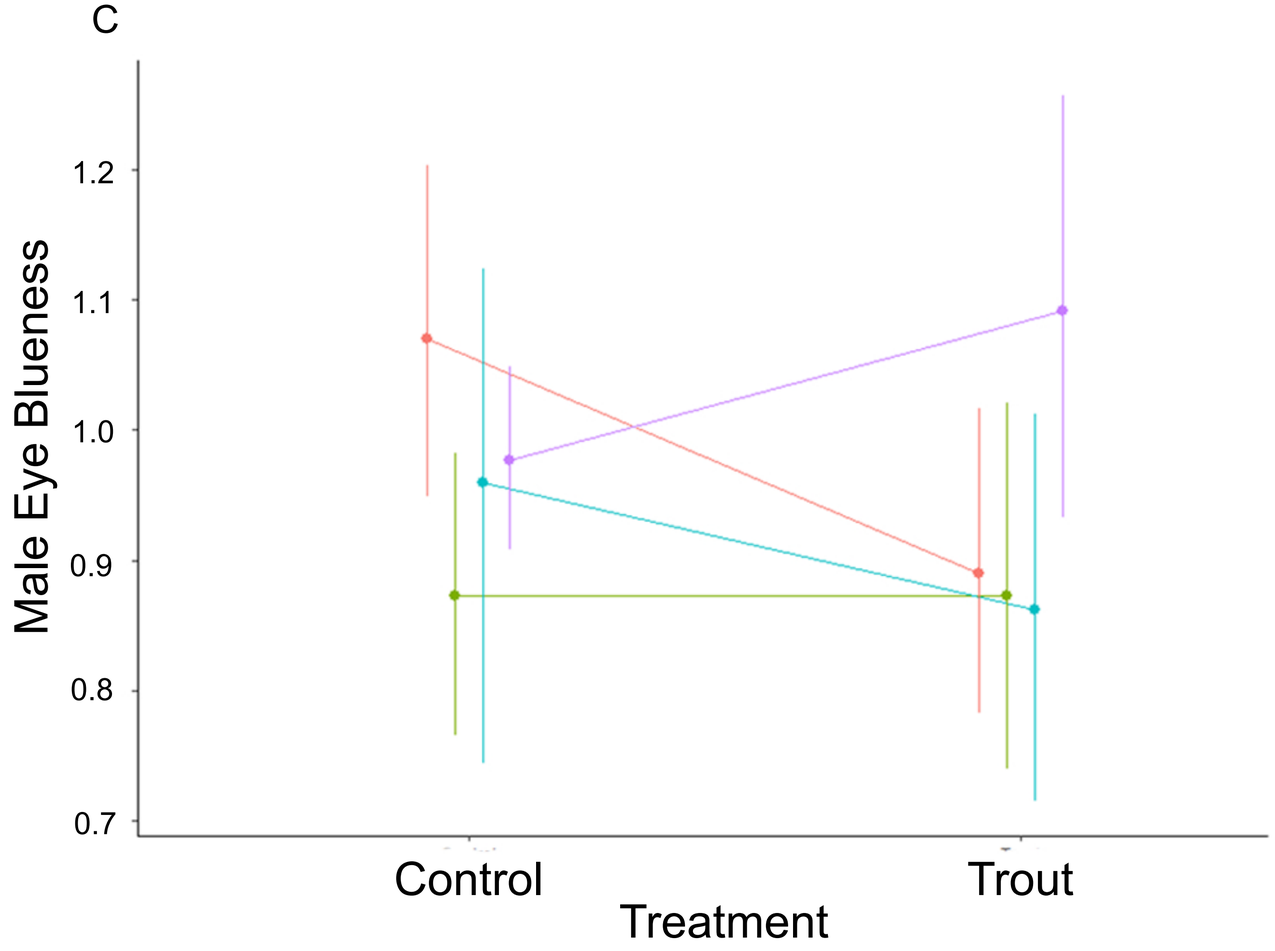
Figure 3. Relationship between dorsal greenness (mean green pixels) and (A) the proportion of zooplankton in the diet and (B) water turbidity (ug1^{-1}phytoplankton). In both panels triangles indicate trout treatment ponds and circles indicate control ponds.

Supplementary Figure 1. 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.

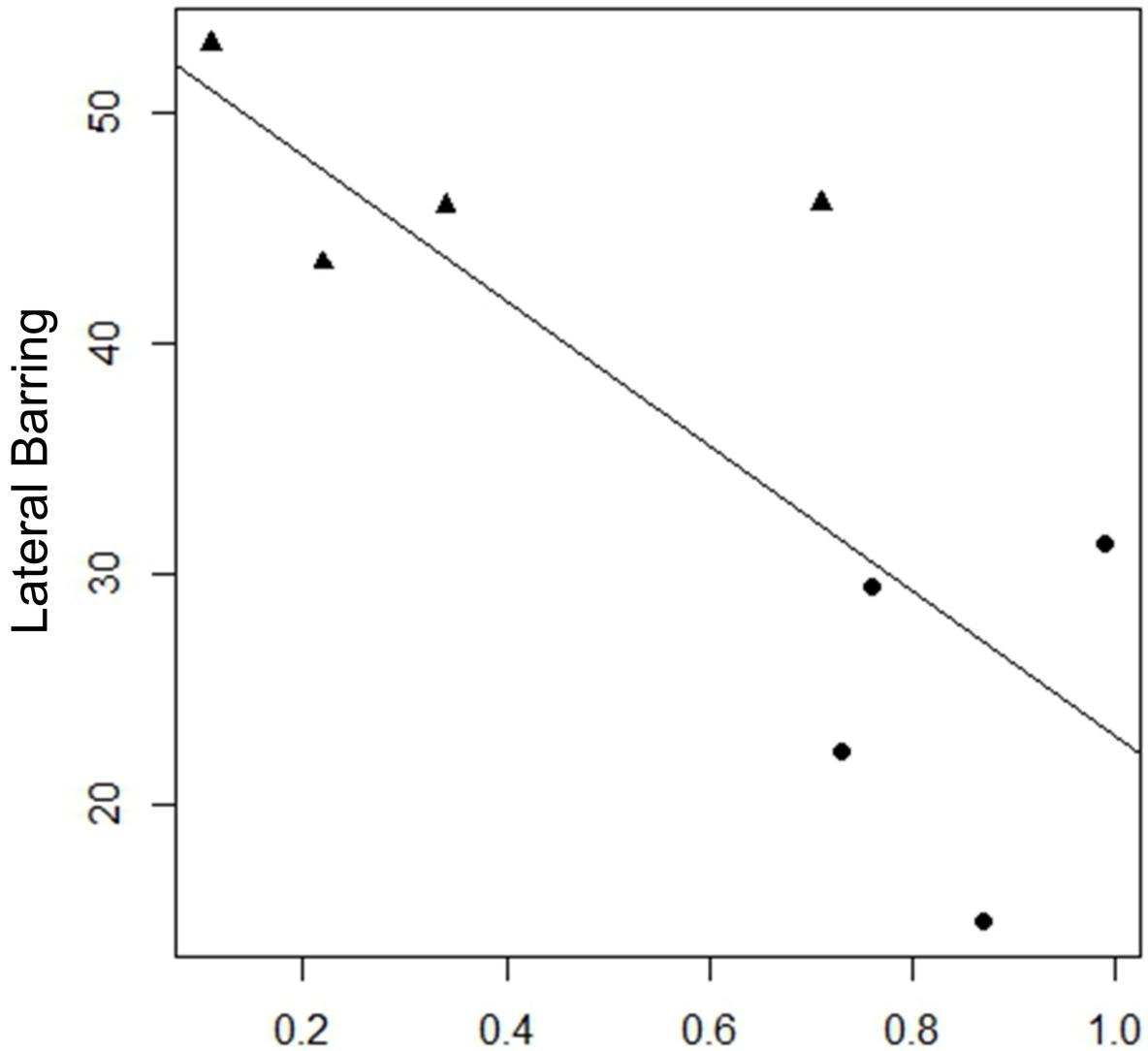
Supplementary Figure 2. Relationship between dorsal greenness (mean green pixels) and male eye blueness (ratio of blue to red pixels).


A
Lateral Barring
60
50
40
30
20
10
*
Control
Trout

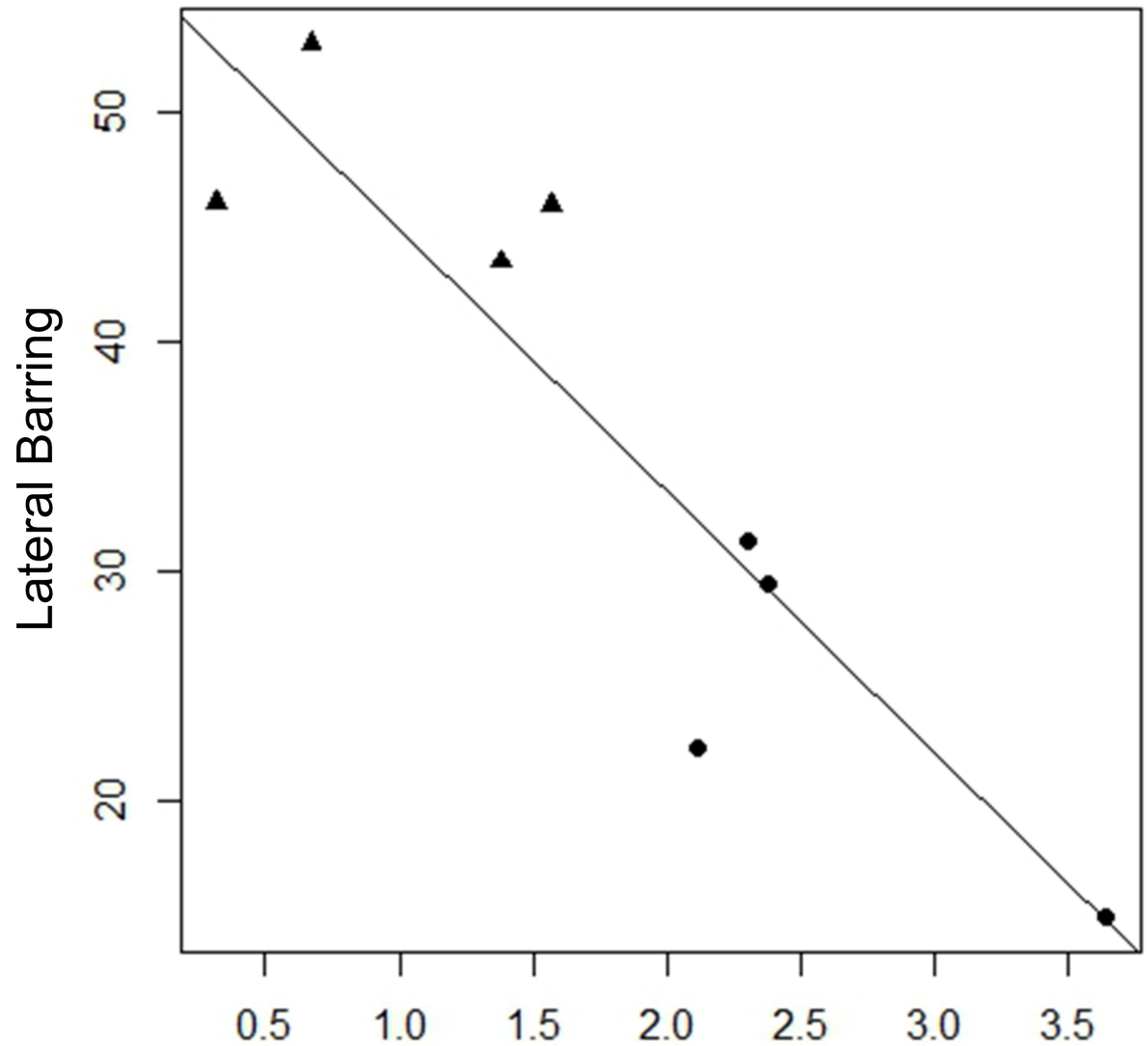

B
Dorsal Greenness
85
80
75
70
65
Control
Trout
Family
1
2
3
4


C
Male Eye Blueness
1.2
1.1
1.0
0.9
0.8
0.7
Control
Trout
Treatment

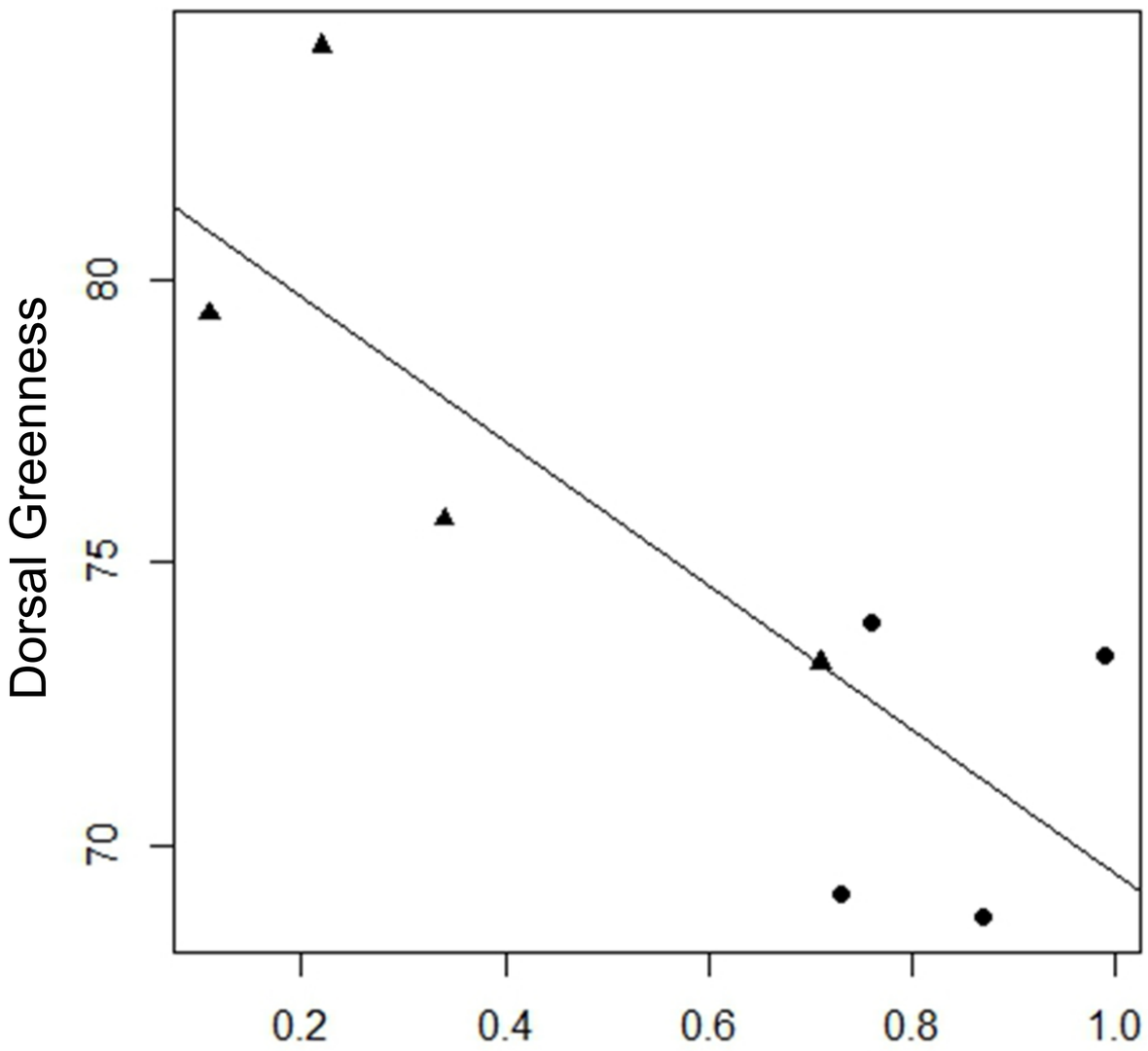
A


Lateral Barring
50
40
30
20
0.2
0.4
0.6
0.8
1.0
Proportion of Zooplankton
in the Diet

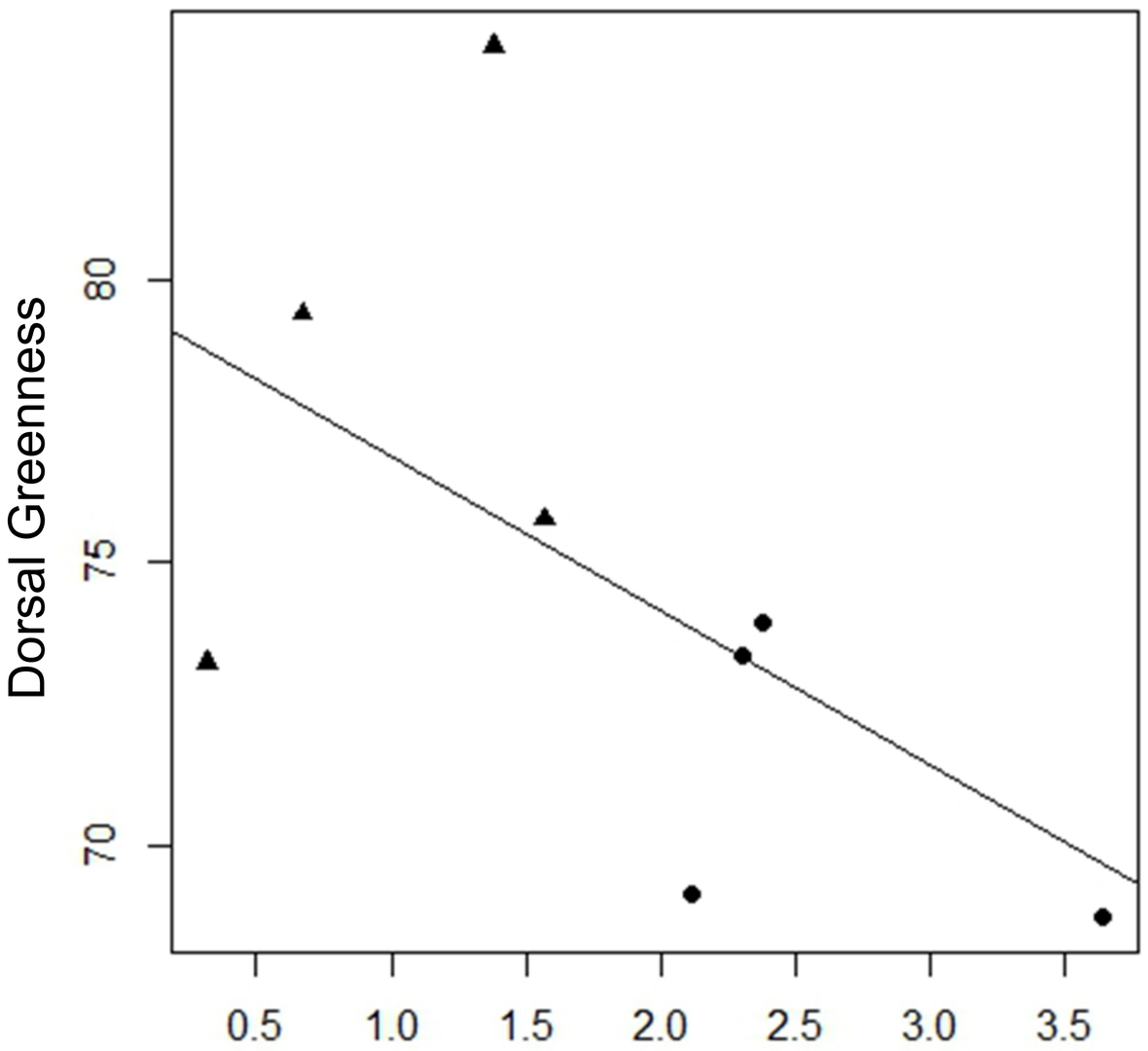
B

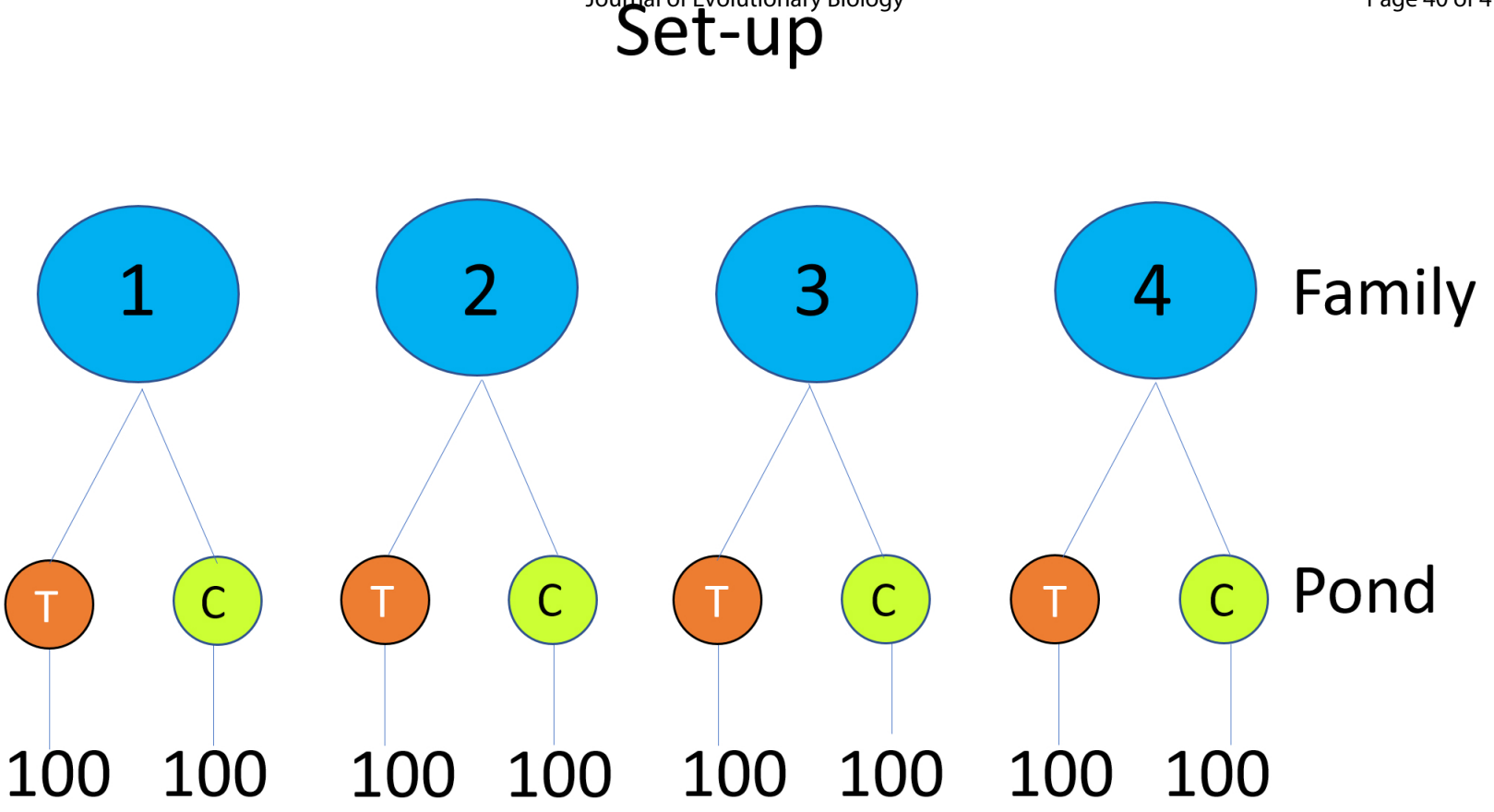

Lateral Barring
50
40
30
20
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Turbidity

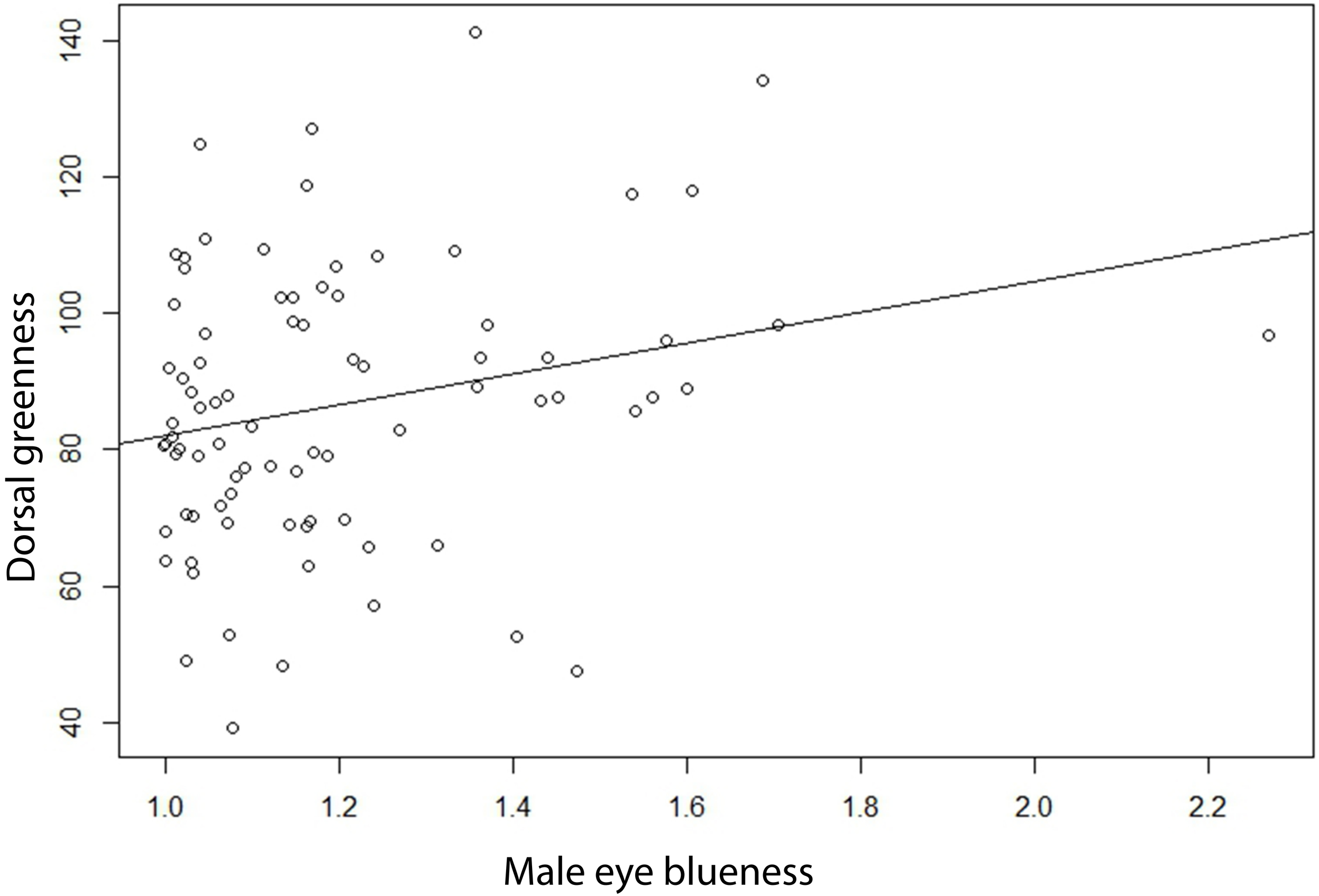
A


Dorsal Greenness
80
75
70
0.2
0.4
0.6
0.8
1.0
Proportion of Zooplankton
in the Diet

B


Dorsal Greenness
80
75
70
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Turbidity


Set-up
1
2
3
4
Family
T
C
T
C
T
C
T
C
Pond
100
100
100
100
100
100
100
100


Dorsal greenness
140
120
100
80
60
40
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Male eye blueness