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*Proc. R. Soc. B* 2012 **279**, 1684-1690 first published online 23 November 2011  
doi: 10.1098/rspb.2011.2132

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- Supplementary data** ["Data Supplement"](http://rsob.royalsocietypublishing.org/content/suppl/2011/11/17/rspb.2011.2132.DC1.html)  
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# Female mate preference explains countergradient variation in the sexual coloration of guppies (*Poecilia reticulata*)

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We tested the hypothesis that mate choice is responsible for countergradient variation in the sexual coloration of Trinidadian guppies (*Poecilia reticulata*). The nature of the countergradient pattern is that geographical variation in the carotenoid content of the orange spots of males is counterbalanced by genetic variation in drosoplerin production, resulting in a relatively uniform pigment ratio. A female hue preference could produce this pattern, because hue is the axis of colour variation most directly affected by the pigment ratio. To test this hypothesis, we crossed two populations differing in drosoplerin production and produced an F<sub>2</sub> generation with variable drosoplerin levels. When the carotenoid content of the orange spots was held constant, female guppies preferred males with intermediate drosoplerin levels. This shows that females do not simply prefer males with greater orange spot pigment content; instead, the ratio of the pigments also affects male attractiveness. To our knowledge, this is the first direct evidence for a hypothesized agent of countergradient sexual selection.

**Keywords:** mate choice; carotenoid; drosoplerin; sexual selection; plasticity; genetic compensation

## 1. INTRODUCTION

Countergradient variation is a geographical pattern in which environmental variation in a trait is masked by genetic variation, resulting in reduced phenotypic variation across environments (electronic supplementary material, figures S1 and S2) [1,2]. The most commonly described cases involve diminished variation in body size or development time across altitudinal or latitudinal gradients [3]. Countergradient variation patterns have been documented in numerous species (reviewed in Conover *et al.* [3]), but relatively few studies have confirmed the hypothesized agents of selection [4–6]. Countergradient variation is usually hypothesized to arise from selection opposing the influence of the environmental gradient on the development of the trait (i.e. countergradient selection).

In this paper, we report the results of an experiment designed to investigate a hypothesized mechanism of countergradient selection on the sexual coloration of male guppies (*Poecilia reticulata*). Male guppies have complex and highly polymorphic colour patterns that typically include orange spots [7,8]. The orange colour is produced by a combination of yellow carotenoids (tunaxanthins; absorbance maxima ( $\lambda_{\max}$ ) near 440 nm) and red pteridines (drosoplerins;  $\lambda_{\max}$  near 480 nm) [9,10]. Drosoplerins are synthesized de novo from carbohydrates and amino acids, whereas tunaxanthins are obtained by metabolic conversion of ingested carotenoids [10]. The primary source of

carotenoids for guppies is unicellular algae [11]. In the upper reaches of Trinidad's watersheds, algae production is light-limited [11]. Algae availability generally increases in the downstream direction, as streams widen, creating larger gaps in the forest canopy [11,12]. Population comparisons along this gradient showed that the mean carotenoid content of the orange spots increases asymptotically with algal carotenoid intake [13]. Nevertheless, the ratio of carotenoid and drosoplerin pigments remains relatively constant across sites owing to correlated variation in drosoplerin production [14]. A common garden experiment showed that interpopulation variation in drosoplerin production is largely genetic [14]. This is a countergradient pattern in which genetic variation in drosoplerin production counteracts the effects of the carotenoid availability gradient on the pigment composition of the orange spots [14].

Two alternative explanations for the countergradient pattern that do not require countergradient selection have been ruled out [14]. First, production costs might constrain guppies to produce less drosoplerins at sites with lower food (algae) availability. This hypothesis was ruled out by showing that food limitation in the laboratory does not affect drosoplerin levels in the orange spots. Second, if female guppies prefer males with brighter colours in darker environments, then this might select for reduced pigment deposition at darker sites (see Marchetti [15]). This hypothesis was rejected by showing that drosoplerin production correlates better with carotenoid intake than with ambient light levels, and that orange spot brightness is only weakly correlated with ambient light.

The basis of our countergradient selection hypothesis is as follows. Tunaxanthins and drosoplerins have

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Electronic supplementary material is available at <http://dx.doi.org/10.1098/rsob.2011.2132> or via <http://rsob.royalsocietypublishing.org>.

different absorbance spectra; hence, the shape of the orange spot reflectance spectrum depends on the ratio of the two pigments [9]. To human eyes, the orange spots appear yellower (less red) as the carotenoid:drosopterin ratio increases. While variation in total pigment content affects the chroma (colour saturation) and, to a lesser extent, the brightness (total reflectivity) of the orange spots, the axis of colour variation that is most directly affected by the pigment ratio is the hue [14]. Researchers often treat hue as a categorical variable (yellow, orange, red, etc.), but in this case, the variation in hue occurs within the category that we call 'orange'. How guppies perceive hue is not known. Nevertheless, it is reasonable to hypothesize that a female mate preference based on hue is responsible for the observed countergradient variation pattern [14,16]. That is, the relatively constant pigment ratio observed across populations in nature might have resulted from females choosing males based, in part, on the hue of their orange spots. For simplicity, we call this the hue preference hypothesis, although as will become clear shortly, this name should not be taken too literally.

Our basic approach to testing the hue preference hypothesis was to allow female guppies to choose among males with different carotenoid:drosopterin ratios. To obtain a robust test of this prediction, it was important to control the pigment ratio experimentally. The simplest way to control the pigment ratio would be to raise males on different dietary carotenoid levels. However, previous research using this method showed that females prefer males raised on the highest carotenoid level, whether the males come from high-drosopterin populations or low-drosopterin populations [17,18]. These results ruled out the hypothesis that females invariably prefer the same hue, but they did not rule out the hypothesis that females take chroma and hue into account simultaneously. In principle, females could prefer males with greater total pigment content (hence, higher chroma) so long as the carotenoid:drosopterin ratio (hue) remains above some level.

In human vision terms, females might prefer orange spots that are more saturated and yellower to orange spots that are less saturated and redder. Increases in carotenoid content would always be favoured by such a preference, but for every average carotenoid level, there would be a different optimal drosopterin level. To test for this sort of hue preference, we varied drosopterin content of the orange spots over a wider range than occurs within-wild populations, while holding the carotenoid content at an intermediate level (i.e. between the extremes that occur in nature). We accomplished this by performing a reciprocal cross between parental (wild-type) low- and high-drosopterin populations with full-sibling crosses in the  $F_1$  generation, thereby producing  $F_2$  fish with variable drosopterin levels. Mate choice trials were carried out using virgin females from the parental population and  $F_2$  males that had been raised on a standardized carotenoid diet. The hue preference hypothesis predicts that, under these circumstances, females should prefer males with intermediate drosopterin levels [14]. In addition to presenting the results of this experiment, we show how the predicted pigment ratios compare with the average pigment ratios in wild-caught fish from 18 field sites in Trinidad.

## 2. METHODS

### (a) *Study populations and cross design*

To create populations of male guppies that were variable in drosopterin production, we performed a reciprocal full-sibling cross hybridization experiment using two outbred populations from the Northern Mountains of Trinidad: Small Crayfish River (SC) and Marianne River (MR) [9,11,18] (electronic supplementary material, table S1, figures S3 and S4). These populations were chosen to represent the available extremes in orange spot drosopterin content (with SC on the low end and MR on the high end). The difference in colour (chroma) is so distinct that males from these populations are easily distinguished from one another (electronic supplementary material, figure S3).

First or second generation laboratory-reared parental stock population males and virgin females ( $F_0$ ) were mated in separate tanks to produce multiple  $F_1$  families. Full siblings from the  $F_1$  population were then crossed to produce the  $F_2$  generation. This was performed for each side of the cross (cross 1: SC female  $\times$  MR male and cross 2: MR female  $\times$  SC male). In total, there were 43  $F_0$  families, 18 from cross 1 and 25 from cross 2.

Siblings were reared together in 21 plastic tanks with a maximum of six fish per tank and the sexes were separated prior to male sexual maturity (3–6 months). Families were not mixed. Virgin females from the parental populations (MR or SC) to be used in the mate choice tests were housed in 38 l tanks at 1–20 fish per tank and were visually isolated from males prior to mate choice test.

$F_2$  fish were fed ad libitum a special flake food containing a known quantity of carotenoids. Based on skin carotenoid content assays, the carotenoid content of this diet, which is referred to in previous publications as the 'low-carotenoid diet', is well within the range of average carotenoid levels that guppies experience in Trinidadian streams (for full details of this diet, see Kolluru *et al.* [19]).  $F_0$  and  $F_1$  fish were fed ad libitum a high-growth diet (Tetra-Min high-growth flakes: liver past and live *Artemia*). Pigment analyses (see §2c) were carried out on mature males from each generation.

### (b) *Mate choice tests*

We used an open-aquarium design in which three virgin parental population females (SC or MR) were allowed to interact freely with three  $F_2$  males from one cross population (cross 1 or cross 2). Each group of males was tested, in separate sessions, with virgin females from each parental population. Male guppies perform discrete courtship displays, sigmoid displays, and females give stereotyped responses that correlate with their probability of mating [20]. We scored female responses to male sigmoid displays as follows: 0, no response but probably did not see the display; 1, no response and probably saw the display; 2, orient towards the male (look); 3, approach the male (follow); 4, receptivity display (glide); and 5, copulation (spiral). At least three 5 min focal observations were carried out on each male. The order of observations of males with a trio was chosen at random. If a male did not perform at least one courtship display while being observed, he was observed for up to four additional 5 min periods. Counts of courtship displays ( $n_{\text{attempts}}$ ) and successes ( $n_{\text{success}}$ ) were recorded for each trial. Courtship displays were considered successful if the female gave a receptivity display or copulated with the male, as in several previous studies of mate choice in guppies [17,18,21].

While the open-aquarium design has previously been shown to yield reliable mate preference estimates [17,20,21],

it is possible for male–male interactions to influence the results. In the present context, the chief concern is whether orange spot pigment levels are correlated with the outcome of male–male interactions. To test for such effects, during the mate choice trials we collected detailed data on male–male interactions using the same protocol and behavioural categories as Kolluru & Grether [22]. The two primary categories were: (i) competitive interactions that take place when one of the interacting males is actively following a female, and (ii) dominance interactions that take place when none of the interacting males is actively following a female. Success in competitive interactions was defined as the focal male keeping or stealing the female (versus losing or failing to steal her). Success in dominance interactions was defined as the focal male supplanting, biting or chasing his opponent (versus being supplanted, bitten or chased). Interactions without a clear winner were excluded from the analysis.

In total, we carried out 47 mate choice trials using cross 1 males and 50 trials using cross 2 males (97 trials in total). In two of the 291 observations (0.7%), the male performed no courtship displays, and another 24 observations were discarded because of data handling and pigment measurement errors, leaving 265 valid observations for the statistical analysis.

### (c) Pigment analysis

After the mate choice trials, males were euthanized with an overdose of MS-222, frozen instantly in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until pigment extractions were performed. Fish were thawed just prior to pigment extractions. The skin on the body of the fish was peeled off with surgical instruments and separated into orange spot and non-orange spot fractions. First, carotenoids were extracted with acetone from both fractions. The acetone extract was dried in a stream of nitrogen and then re-suspended in hexane. Drospterins were extracted from the orange spot skin using 30 per cent acidified (with HCl to pH 2) ethanol. Absorbance spectra of the pigment extracts were measured with an Ocean Optics USB-2000 spectrometer equipped with a cuvette holder and a deuterium–tungsten light source (Ocean Optics DT-1000). Five replicate measurements of each extract were averaged. A dark and reference standard of pure solvent (hexane for carotenoids and ethanol for drospterins) were stored prior to any measurements and between samples during measurements. For both carotenoids and drospterins, absorbance was measured at the wavelength of peak absorbance. Prior to statistical analysis, pigment absorbance ( $A_{ij}$ ) was transformed into pigment transmittance ( $T_{ij} = \exp(-A_{ij})$ ), which corresponds more directly to the effect of a pigment on the light reflected from a colour patch and hence the perceived colour [14]. It should be noted that transmittance ( $T_{ij}$ ) is inversely related to absorbance ( $A_{ij}$ ); thus, an increase in transmittance corresponds to a decrease in pigment content, and vice versa.

### (d) Data analysis

As explained in §1, the goal of this experiment was to determine whether female guppies would prefer males with intermediate drospterin levels if orange spot carotenoid levels were held at an intermediate level. Therefore, we fit regression models of the form  $f(x) = \beta_0 + \beta_1 T_d + \beta_2 T_d^2 + \beta_3 T_c$ , where  $f(x)$  represents male courtship success and  $T_d$  and  $T_c$  represent drospterin and carotenoid transmittance, respectively. The squared term for  $T_d$  was included to test the prediction that, holding carotenoid levels constant, there is a nonlinear

relationship in which females prefer males with intermediate drospterin levels to those with lower or higher drospterin levels. Specifically,  $\beta_1$  was predicted to be positive and  $\beta_2$  was predicted to be negative. To evaluate whether this model adequately describes the relationship between male courtship success and pigment levels, we also fit more complex models including terms for  $T_c^2$  and  $T_d T_c$ . We analysed the data using a generalized-linear mixed effects model (GLMM) with the logit link function (R v. 2.10.0 lme4 package), with counts of  $n_{\text{success}}$  and  $n_{\text{attempts}}$  entered as a binomial dependent variable (using `cbind`) [23–26]. To account for the experimental design, males were nested within male group and male group was modelled as a random effect. These random effects terms were correlated ( $r > 0.7$ ), which can cause numerical instability in some cases; however, in our case, these effects were part of the experimental design and estimates of the fixed effects were stable. Therefore, we retained both random effects terms in the analysis [23]. In the full model, with both female populations and both male crosses, female population and male cross were included as fixed effects. We tested for interactions between female population, male cross and pigment levels, but none of these effects were significant (all  $p > 0.1$ ) and the fit of the model was better (lower Akaike information criterion (AIC)) with the interaction terms removed (not shown). To evaluate whether male–male interactions influenced the results, we tested the following effects by including the covariates in models: (i) male body mass, (ii) success in competitive interactions, and (iii) success in dominance interactions. All terms with  $p < 0.1$  for inclusion were retained in the final model.

To estimate the preferred drospterin level, i.e. the drospterin level at which male courtship success peaks ( $T_d^*$ ), we used the equation  $T_d^* = -\beta_1/2\beta_2$ , which was obtained by taking the partial derivative of the regression equation above with respect to  $T_d$ , setting the partial derivative equal to 0, and solving for  $T_d^*$ . We obtained independent estimates of  $T_d^*$  for each female population (i.e. MR and SC) by analysing the data for the two female populations separately. To bracket the preferred pigment ratio, we calculated  $T_c/T_d^*$  using the upper 75th and lower 25th quartile of the observed range of  $T_c$  values in the  $F_2$  populations.

We carried out additional analyses to evaluate possible alternative explanations for the results. To evaluate whether male body size or male–male interactions correlated with orange spot pigment levels in such a way as to confound the mate preference results, we ran the GLMM (described earlier) with: (i) male body mass, (ii) success in competitive interactions, and (iii) success in dominance interactions as the dependent variables. Likewise, to determine whether male courtship rate correlated with orange spot pigment levels in such a way as to confound the mate preference results, we ran the GLMM with the number of courtship displays ( $n_{\text{attempts}}$ ) as the dependent variable.

### (e) Comparison with field populations

Data on skin pigment contents of wild-caught fish from 18 field sites in Trinidad (electronic supplementary material, table S1) were used for comparison with the preferred pigment ratios ( $T_c/T_d^*$ ). All of these are classified as ‘low-predation’ sites, where the only piscine predator of guppies is the killifish *Rivulus hartii* [8]. These sites were chosen to represent the available extremes in forest canopy cover among low-predation sites in their respective drainages, excluding logged forest. ‘High light’ sites with less forest

Table 1. Mate preference generalized-linear mixed model. (The dependent variable in this analysis is a measure of male courtship success (or attractiveness) based on the responses of females to male courtship displays (see §2). Significant *p*-values are in bold. AIC, 474.3.)

groups	name	variance	s.d.	correlation
<i>random effects</i>				
fish	(intercept)	0.4141	0.6435	
	male group	0.0002	0.0136	−0.9530
<i>n</i> <sub>observations</sub> : 263				
<i>n</i> <sub>males</sub> : 132				
variable	estimate	s.e.	<i>z</i> -value	<i>p</i> -value
<i>fixed effects</i>				
intercept	−5.0384	1.1428	−4.409	<b>1.04 × 10<sup>−5</sup></b>
<i>T</i> <sub>d</sub>	8.5774	3.0806	2.784	<b>0.0054</b>
<i>T</i> <sub>d</sub> <sup>2</sup>	−6.2442	2.1817	−2.862	<b>0.0042</b>
<i>T</i> <sub>c</sub>	1.0316	0.3456	2.985	<b>0.0028</b>
male cross	−0.1821	0.0934	−1.951	0.0511
female population	0.4660	0.0626	7.444	<b>9.73 × 10<sup>−14</sup></b>
male competition	0.0398	0.0068	5.816	<b>6.03 × 10<sup>−9</sup></b>
male mass	0.0044	0.0025	1.76	0.0784

canopy cover tend to have greater carotenoid availability for guppies than ‘low-light’ sites [13]. Pools and fish within pools were selected at random.

### 3. RESULTS

#### (a) *Mate preference functions*

As predicted by the hue preference hypothesis, the linear *T*<sub>d</sub> coefficient was statistically significantly positive and the quadratic *T*<sub>d</sub> coefficient was statistically significantly negative, both in the full model (table 1), and also in separate models for the two female populations (electronic supplementary material, table S4). MR females responded more strongly to male courtship displays than did SC females (table 1 and figure 1), but the shape of the preference functions with respect to male drosoplerin levels was very similar for the two female populations (figure 1). Separate regression models for the two female populations retained exactly the same predictor variables (electronic supplementary material, table S4). The optimal drosoplerin level, where the quadratic function peaked (*T*<sub>d</sub><sup>\*</sup>), was nearly identical for the two female populations (0.66 for SC females and 0.69 for MR females).

Neither the quadratic *T*<sub>c</sub><sup>2</sup> coefficient nor the correlational *T*<sub>d</sub>*T*<sub>c</sub> coefficient was a significant predictor of male courtship success (electronic supplementary material, table S2). The linear *T*<sub>c</sub> coefficient was significantly different from zero in the final model (table 1) and in the SC model, but non-significant in the MR model (electronic supplementary material, table S4). Both populations of females were more receptive to males of cross 1 than to males of cross 2 (table 1 and figure 1).

#### (b) *Male body size and male–male interactions*

Controlling for male–male competition only strengthened support for the hue preference hypothesis. Male body mass was unrelated to *T*<sub>d</sub> but negatively correlated with *T*<sub>c</sub> (electronic supplementary material, table S5), which means that larger males had more carotenoids in their orange spots. Success in competition and dominance interactions was unrelated to male pigment levels (electronic supplementary material, table S5). Based on these

results alone, it does not seem possible for the female preference results to have been driven by effects of male colour on male–male interactions. To check this inference, we included male body mass, competition success and dominance success as covariates in the mate preference model (electronic supplementary material, table S3). Neither male body mass nor dominance success was a significant predictor of male courtship success, but competition success was a highly significant positive predictor. Compared with the model without these covariates (electronic supplementary material, table S2c), the coefficients for *T*<sub>d</sub>, *T*<sub>d</sub><sup>2</sup> and *T*<sub>c</sub> were all larger in magnitude, and the corresponding *p*-values were smaller (table 1; electronic supplementary material, table S3), which suggests that controlling for male–male competition improved the resolution of the female mate preference function. Qualitatively, the results were unchanged, except that *T*<sub>c</sub> emerged as a significant predictor of male courtship success (table 1).

#### (c) *Male courtship rate*

SC females were courted at significantly higher rates than MR females, and males from cross 1 displayed at significantly higher rates than males from cross 2, but courtship rate was unrelated to male pigment levels (electronic supplementary material, table S6). Thus, the mate preference results could not be confounded by a correlation between male pigment levels and courtship rate.

#### (d) *Comparison with field populations*

All of the field populations sampled had lower *T*<sub>c</sub>/*T*<sub>d</sub> ratios than predicted based on the laboratory mate choice tests (electronic supplementary material, figure S5). This means that the ratio of drosopterins to carotenoids in wild-caught fish is lower than expected. Possible reasons for this departure are discussed in §4.

### 4. DISCUSSION

Our results support the hypothesis that a female mate preference is responsible for countergradient variation in the sexual coloration of male guppies. The key result was

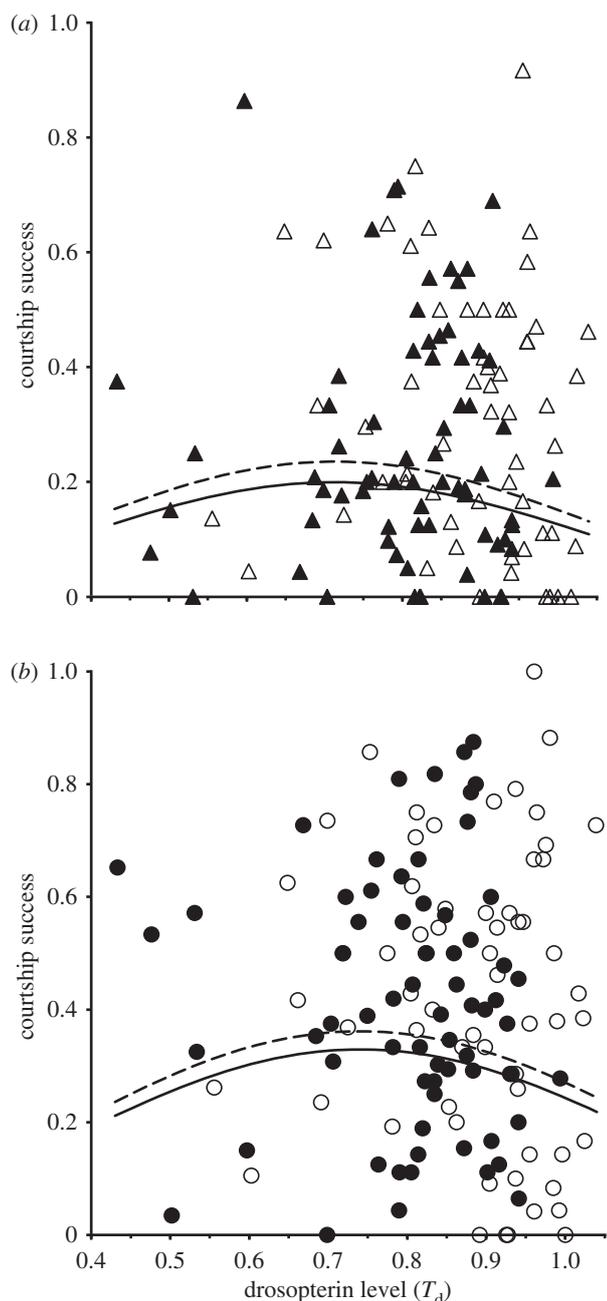


Figure 1. Male courtship success (attractiveness) in relation to orange spot drosopterin level  $T_d$ . (a) Small Crayfish River (SC) females. (b) Marianne River (MR) females. Open triangles, cross 1 males with SC females; filled triangles, cross 2 males with SC females; open circles, cross 1 males with MR females; filled circles, cross 2 males with MR females. Predicted values were obtained from the separate female population regression models in the electronic supplementary material, table S4. Dashed line, predicted courtship success of cross 1 males; solid line, predicted courtship success of cross 2 males.

that, when male carotenoid levels were controlled experimentally and statistically, female guppies preferred males with intermediate drosopterin levels (figure 1). This shows that females do not simply prefer males with greater orange spot pigment content (chroma); instead, the ratio of the pigments (hue) must also affect male attractiveness. Our experiment was designed to give females a choice between males with pigment ratios above and below the average pigment ratios observed in nature, hence it was

robust to predict that females would prefer males with intermediate drosopterin levels. In the wild, we would not expect females to always prefer males with intermediate drosopterin levels, and especially not if carotenoid availability has recently changed.

In recent years, there has been a surge of interest in the role of phenotypic plasticity in evolution [27–32]. Much attention has been focused on the hypothesis that environmentally induced changes in development enable populations to reach new adaptive peaks. As with genetic mutations, however, most environmental perturbations of development are likely to reduce fitness. While deleterious genetic mutations can usually be purged by selection, deleterious environmental effects cannot be eliminated so readily. Unlike mutations, changes in the environment can simultaneously affect the entire population. If a deleterious environmental effect persists for multiple generations, then selection will favour alleles that counteract it. Genetic compensation (*sensu* [32–34]) is a general term for this process that can be applied to environmental change over both time and space. Genetic compensation is similar to genetic assimilation [35] in that both processes involve the selective accumulation of genetic changes that alter the norm of reaction to an environmental factor, but they are opposites in terms of their effect on the fate of novel, environmentally induced phenotypes [32]. Conover *et al.* [3] suggested that genetic compensation is ‘essentially synonymous’ with the evolution of countergradient variation, but countergradient variation patterns could arise from other processes (see §1), and genetic compensation does not necessarily generate a geographical pattern. We think both terms are useful, one for describing a geographical pattern and the other for describing an evolutionary process.

In the case considered here, the environmental perturbation is the change in carotenoid availability that occurs when guppies colonize a site that differs in forest canopy cover from the site to which they had previously adapted (or when canopy cover changes *in situ*). For example, if a waterfall is breached and a new upstream site is colonized, then the founding population will probably experience lower carotenoid availability than the source population [11]. If males in the source population produced orange spots of the hue that females prefer, then males in the founding population would initially produce orange spots that have lower-than-optimal carotenoid : drosopterin ratios. The response to selection caused by the female preference could take the form of an increase in carotenoid assimilation efficiency or a decrease in drosopterin production (both of which are heritable in guppies; [36]). This simple evolutionary process could account for the observed countergradient variation pattern. Here, we have provided direct evidence that such a hue preference exists.

Many studies have provided evidence that female guppies prefer males with larger and more chromatic orange spots [17,18,37]. This has usually been interpreted as a preference for high-carotenoid males, and indeed experimental manipulations of dietary carotenoid levels have consistently shown that females prefer males with higher orange spot carotenoid concentrations [17,18,37]. By contrast, we found that the attractiveness of  $F_2$  males tended to decrease as orange spot carotenoid levels increased. As our experiment was designed to test for an effect of drosopterin on mate choice while carotenoids

were held constant, the carotenoid level in the diet of the F<sub>2</sub> fish was held at a low and constant level. Consequently, the power of this experiment to detect effects of carotenoids on male attractiveness should have been considerably lower than in previous studies where carotenoid levels were either not controlled or manipulated experimentally. Thus, it would not have been surprising if our experiment had yielded non-significant results with respect to male attractiveness and carotenoid content. The significant negative correlation for one of the two female populations is surprising, however, and merits further investigation. We do not think it undermines our conclusion that, holding carotenoid levels constant, female guppies prefer males with intermediate drosopeterin levels.

To evaluate the generalizability of our experimental results, we compared the predicted preferred carotenoid : drosopeterin transmittance ratio with observed transmittance ratios in field populations. The median transmittance ratio of all 18 field populations sampled lies below the preferred value predicted from our mate choice experiment (electronic supplementary material, figure S5). Because pigment transmittance is inversely proportional to pigment content, these results indicate that drosopeterin levels in wild-caught fish are lower than predicted by the mate choice trials. What prevents males from attaining the preferred drosopeterin level? Perhaps there is a trade-off between the effects of drosopeterins on attractiveness to females versus conspicuousness to predators. This explanation seems unlikely, however, because only low-predation sites, where survival selection on coloration is relaxed, were included in this analysis. Another possible explanation is that genetic constraints prevent males from achieving the optimal drosopeterin level. Drosopeterin production is heritable [14,36], however, so this explanation also seems unlikely.

Finally, the disparity between the predicted peak drosopeterin value and the field values might be an artefact of the laboratory environment. Females used in our mate choice experiments were first or second generation laboratory-reared fish, and while females raised in laboratories may exhibit a preference, it is not necessarily the same preference that they would exhibit in the wild [38]. Exposure to males has been shown to influence mate preference development in female guppies [38–42]. We kept the females in our experiment visually isolated from males prior to the mate choice tests, specifically to avoid biases that might have resulted from exposing them to males with abnormal colour phenotypes. It would be informative to repeat our experiment using females that were exposed to males with pigment ratios characteristic of wild populations. It is also possible that diet affects the development of colour-based mate preferences. Rodd *et al.* [43] showed that guppies are preferentially attracted to orange objects while foraging and suggested that this might be an adaptation for finding orange fruits that occasionally fall into their native streams. Perhaps a female's innate sensory bias for orange is refined after exposure to the stimulus. This hypothesis could be tested by exposing juvenile guppies to food items of different colours and examining the effects on attraction to coloured objects in a foraging context.

In conclusion, our results support the hypothesis that female mate choice—specifically, an orange hue preference—is the agent of selection responsible for countergradient variation in the sexual coloration of guppies.

To our knowledge, this study provides the first direct evidence for a hypothesized mechanism of countergradient sexual selection. Further research will be required to determine whether this mechanism fully accounts for the observed geographical pattern.

All procedures employed in this study were approved by the UCLA Institutional Animal Care and Use Committee.

We thank R. P. A. Macheemer, R. K. Wayne, N. Losin, P. Green, C. Anderson, A. Gonzalez-Karlsson, J. Drury, C. Storch and three anonymous reviewers for many helpful comments on the manuscript. Marc Suchard and UCLA ATS Statistical Consulting Group provided statistical advice; G. R. Kolluru provided advice on fish husbandry and experimental design; Vivian Kuei, Linda Petch, Amanda Tran and 100 additional University of California undergraduates assisted with the laboratory work. Ocean Star International, Inc., produced and donated the experimental diets; Roche Vitamins, Inc., donated carotenoids; and D. F. Millie and L. Boiherm analysed the carotenoid content of the diets. This investigation was supported by the United States Public Health Service grants (GM53275, MH59490, T32-HG02536, T32-GM008185), and by the National Science Foundation (IBN-0130893).

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