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# Intruder colour and light environment jointly determine how nesting male stickleback respond to simulated territorial intrusions

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Variation in male nuptial colour signals might be maintained by negative frequency-dependent selection. This can occur if males are more aggressive towards rivals with locally common colour phenotypes. To test this hypothesis, we introduced red or melanic three-dimensional printed-model males into the territories of nesting male stickleback from two optically distinct lakes with different coloured residents. Red-throated models were attacked more in the population with red males, while melanic models were attacked more in the melanic male lake. Aggression against red versus melanic models also varied across a depth gradient within each lake, implying that the local light environment also modulated the strength of negative frequency dependence acting on male nuptial colour.

## 1. Introduction

Many dramatic male nuptial signals such as song, behavioural displays and colouration have evolved to attract females' attention [1]. However, males' nuptial signals can also attract the attention of other viewers, including predators or rival males [2], who may negatively impact the signaller's fitness. It is well established that the local signalling environment (e.g. wavelength availability and background light) affects how visible a males' nuptial signals are to viewers [3–5]. However, signal visibility might also depend on the relative abundance of other males' phenotypes. If males acquire search images facilitating recognition of locally common signals, then males with locally common signal phenotypes may suffer disproportionately strong intrasexual conflict [6]. Such negative frequency-dependent antagonism can facilitate the persistence of rare signal types (e.g. immigrants or rare morphs), thereby maintaining signal polymorphism [7,8].

We tested whether aggressive interactions among male fish (three-spined stickleback, *Gasterosteus aculeatus*) are modulated by an interaction between the visual environment and local male nuptial colours. Breeding male stickleback exhibit dramatic red throats and blue eyes in clear water habitats [9]. In more tannic habitats, males are typically blue–black ('melanic'), more visible against the redder side-welling light [9]. However, both the light environment and stickleback male colour also vary with depth within lakes (C. D. Brock, M. Cummings & D. I. Bolnick, in preparation). We hypothesized that male–male antagonism disproportionately affects locally common male colour morphs, but this is modulated by fine-scale gradients in light environment. Specifically, we predicted that locally atypical males (e.g. red in tannic lakes, melanic in clear lakes) would experience less male–male
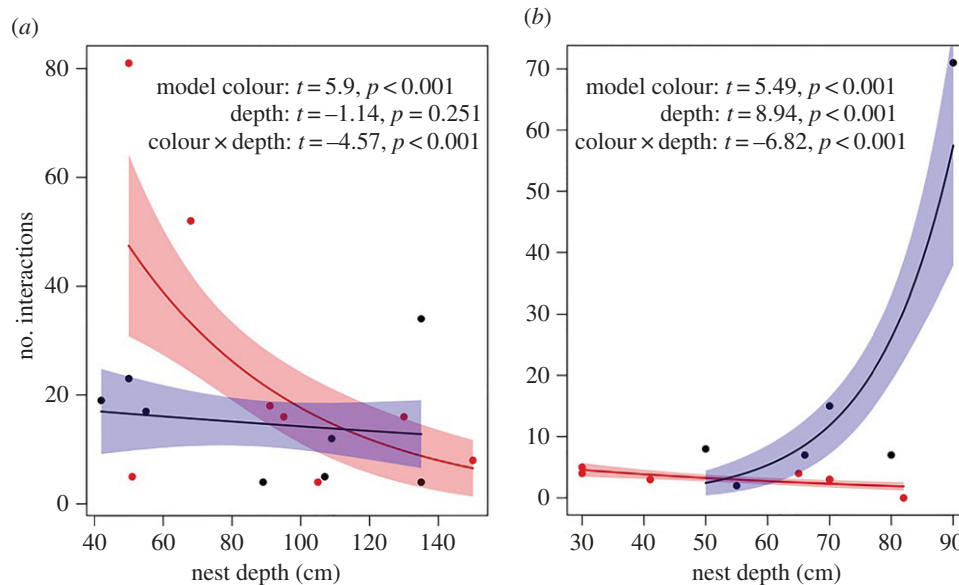
**Figure 1.** (*a*) In Gosling Lake (red males), red models were inspected or bitten more often than melanic models, especially at shallow depths. (*b*) In Blackwater Lake (melanic males), melanic models were inspected or bitten more often than red models, especially at deeper nests. Red and blue models are, respectively, denoted by red and blue points and curves, with 1 s.e. confidence intervals (shading).

antagonism, but within each lake this bias changes with the nest depth (light environment) of the male aggressor.

## 2. Methods

During the June 2014 stickleback breeding season, we used three-dimensional printed-model stickleback to simulate intrusion into wild males' territories in two lakes. Gosling Lake (50.0680° N, −125.5059° E) and Blackwater Lake (50.1804° N, −125.5874° E) are medium-sized lakes (62.5 and 37.5 ha, respectively) on northern Vancouver Island that contain predominantly red-throated and melanic males, respectively [10]. However, male colour does vary within each population as well (e.g. intensity and area of red throats and blue abdomens in Gosling Lake; and intensity of black or iridescent blue colouration in Blackwater) [10]. Within each lake, the spectral composition of the ambient side-welling light changes with depth: in Gosling Lake, shallower water is blue-shifted and deeper sites are red-shifted, while the reverse is true in Blackwater [10]. Males' nuptial colour (red/blue ratio) also varies across depths within each lake (C. D. Brock, M. Cummings & D. I. Bolnick, in preparation).

We used an HDI Advance white light scanner to obtain a three-dimensional digital rendering of a formalin-preserved adult male stickleback from Gosling Lake (electronic supplementary material S1). We used an Objet Eden 500 three-dimensional printer to create 50 plastic models of this male, which we hand-painted using Testors enamel modelling paint (half melanic, half red-throated). Painting was done to provide superficial rather than quantitative resemblance (electronic supplementary material S2), because we found no paints with the necessary UV reflectance. A pilot study confirmed that painted models elicited aggressive responses from nesting males. Males did not respond to unpainted models or cylinders.

In each lake, snorkellers searched for actively nesting males (fully constructed nest with eggs or fry). A GoPro camera was placed underwater 1 m from the nest, 30 cm above the substrate, and set to record video for 2 h. Three-dimensional male models were suspended by thin fishing filament from an overhead poly-vinyl chloride (PVC) pipe arm (electronic supplementary material S3), 20 cm above the substrate and 30 cm horizontally away from the nest. We recorded 20 resident males per lake (10 with red and 10 with melanic models, randomly assigned), sampling across a range of nest depths. In a few cases, we did not obtain usable

video (e.g. faulty camera battery, waterproof case failure, nest abandonment or nest predation by conspecifics (a common natural phenomenon)). Ultimately, we obtained videos of seven red and six melanic intruders in Blackwater Lake; eight red and eight melanic in Gosling Lake.

Videos were relabelled to remove treatment information (source lake, depth) and scored by K.H. and her 9th–11th grade science students at Northwest High School in Texas, independently verified by Surabhi Tyagi. Observers recorded the frequency, duration and timing of physical attacks (bites) on the model, as well as inspections of the model (defined as swimming to within one body length while facing the model). For each lake separately, we used a Poisson general linear model to test whether the number of bites (or inspections) directed at the model depended on model colour, nest depth and a model colour × depth interaction. Data to recreate analyses presented here are available on Dryad [11]. Raw videos are available upon request.

## 3. Results

Most resident males inspected and attacked the model intruder. In Gosling Lake, all males inspected the model multiple times (mean of 12.1 times, s.d. = 12.1), and 10/16 males bit the model at least once (mean of 6.3 bites, s.d. = 9.1). In Blackwater Lake, all but one male inspected the model (a mean of 4.2 times, s.d. = 2.9), and all but four bit the model (mean of 6.0 bites, s.d. = 17.2). In one instance, a gravid female approached the model rather than the resident male. Bites and inspections were concentrated in the first half an hour of the experiment, after which males acclimated to the intruder.

Nest depth and intruder colour both affected the rate of resident male aggression against the model. In Gosling Lake, the mostly red native males interacted with red models (bites or inspections) 20% more often than with blue models (figure 1*b*, median of 3 versus 2.5 interactions; univariate Poisson general linear mixed (GLM) model colour effect $Z = 2.15$, $p = 0.032$; or $Z = 4.38$, $p < 0.0001$ with depth as a covariate). Conversely, in Blackwater Lake (melanic males), the melanic models received 50% more bites than red models (median of 1.5 versus 1, respectively; colour effect $Z = 3.6$, $p = 0.0057$ in a Poisson GLM with depth as a covariate; not significant without depth, $p = 0.235$).

The same trend held for all interactions ($Z = 2.8$, $p = 0.0215$). Thus, we observe a symmetric pattern of greater aggression against locally common male models (red in Gosling Lake; melanic in Blackwater Lake).

Within each lake, territorial males' reaction to intruder colour varied across nest depths. In Gosling Lake, males were more aggressive against red models in shallower territories (depth effect $Z = -7.70$, $p < 0.001$) where side-welling light is more blue-shifted [10] than in red-shifted deeper water. Aggression towards melanic models was rare and insensitive to depth (depth effect $Z = -0.37$, $p = 0.724$). The result, in a combined model with both colours, is significant effects of model colour and a colour × depth interaction (figure 1a, effect sizes and significance in the figure). In deeper water, interaction with red versus melanic models converged. Similar results were observed when considering only bites, excluding inspections (depth $p = 0.222$; colour $p < 0.001$; depth × colour interaction $p < 0.001$). We observed the inverse trend in Blackwater Lake, where the side-welling light is more red-shifted in shallow than deep water. There, resident males were more aggressive towards melanic models in deeper water (counting both attacks and inspections; depth effect for blue models only, $Z = 8.94$, $p < 0.001$; figure 1b), but there was a non-significant trend to be more aggressive towards red models in shallow water (depth effect for red models only, $Z = -2.37$, $p = 0.064$). In a combined model, these opposing trends generated significant main effects of depth, model colour and a depth × colour interaction (p-values and effect sizes in figure 1b). Although both lakes exhibited this colour × depth interaction, in Gosling it arose from more attacks on red models in shallow water, whereas in Blackwater it resulted from attacks on melanic models in deep water. Although the light gradient does change with water depth, over the 1.5 m vertical range studied here this change is not enough to obscure colour differences between our models (judging by snorkeller observations), nor colour differences between redder versus more melanic resident males within each lake (based on visual modelling of conspicuousness using down- and side-welling light and male reflectance measured by spectrophotometer (C. Brock, M. Cummings & D. I. Bolnick, in preparation)).

The median interval between bites at a model indicates the intensity of attack. This metric is not redundant with the total number of bites ($r = -0.304$, $p = 0.361$) because of variance in the acclimation rate. In Gosling, the attack interval was shorter for red than melanic models (figure 2; $t = -2.59$, $p = 0.036$). For red models, the attack interval tended to be shorter in shallower water (non-significant), whereas for blue models the interval was shorter in deep water ($t = -2.95$, $p = 0.0418$), resulting in a non-significant trend towards a depth × colour interaction ($t = 2.07$, $p = 0.077$). The equivalent analysis was not possible for Blackwater Lake, where most models received only one or two bites so intervals cannot be calculated.

## 4. Discussion

We found that within a population of predominantly red-throated male stickleback, territorial males were more aggressive towards red-throated model intruders, whereas melanic models were attacked more in a melanic population. Thus, one colour was not inherently more vulnerable to attack (unlike results reported previously [12]); rather, the more-targeted phenotype depends on the local populations' typical morphology.
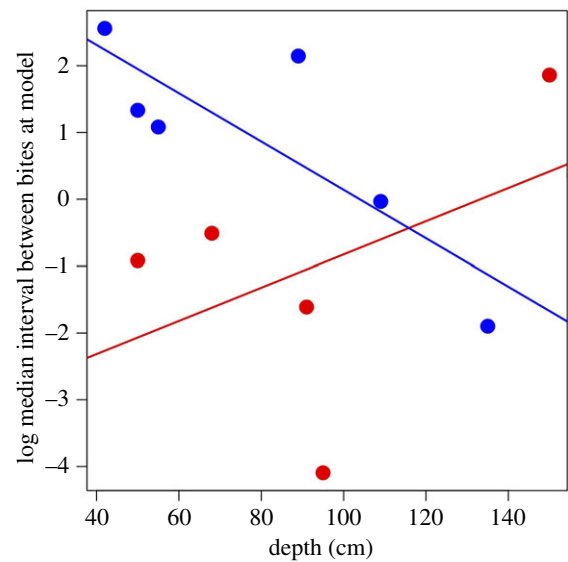


**Figure 2.** The intensity of attacks as a function of nest depth and intruder colour. Intensity is inversely related to the log of the median time interval (minutes) between sequential bites. Melanic models (blue points) were attacked more intensively (shorter intervals) at deep nests in Gosling Lake, whereas red models (red points) were attacked marginally more intensely at shallow nests. The interval can only be calculated when there were at least two attacks, so the sample size is lower than in figure 1a and too low to analyse for Blackwater Lake.

This result is consistent with previous evidence, from East African cichlids, of frequency-dependent aggression [6].

If male–male interactions are costly, this frequency-dependent targeting of locally common male colour types may confer a fitness advantage on rare types. Intrasexual selection could then contribute to sustaining within-population phenotypic variation in male nuptial signals [8], such as the colour variation within stickleback populations [10]. Of course, signal variation might also be due to variation in female visual systems or preferences, or gradients in local light environments.

Within each lake, stickleback nests range from 0.5 to 2.0 m deep, and the wavelengths of side-welling light change appreciably across this remarkably small gradient [10]. For as-yet unknown reasons, this light gradient is in opposite directions in Gosling and Blackwater Lakes. This light environment gradient is associated with micro-spatial variation in male–male aggression. In both lakes, the red models tended to be inspected or attacked more in shallow sites (significant in Gosling, nearly so in Blackwater), whereas melanic models tended to be inspected or attacked more in deeper water (significant in Blackwater, not in Gosling). Thus, the effect of depth is more pronounced for red models in the red-native Gosling Lake and melanic models in the melanic-native Blackwater Lake. We infer that metre-scale variation in light environments modulates males' tendency to attack red versus melanic intruder males. Given the proximity of the model to the nest, we doubt this is because the resident males were more or less able to notice the intruder. Rather, we expect that depth effects on aggression arise from variation in stimulus intensity or the degree of familiarity with the intruder phenotype. Within both focal populations, we have found that quantitative measures of resident male colour covary with nest depth (C. D. Brock, M. Cummings & D. I. Bolnick, in preparation). Consequently, the locally common male colour type does change with depth, which may explain the depth gradient

in aggression observed here. However, owing to plasticity in male stickleback colour, we cannot experimentally tease apart the direct effects of depth (light environment) on aggression towards our models versus indirect effects of depth via changing local male colour.

Our results show that male–male antagonism in stickleback is both frequency dependent among lakes [6] and modulated by depth within lakes. These trends may contribute to the maintenance of male signal polymorphism within natural populations [8]. To the extent that dispersal occurs between disparate light environments, negative frequency dependence may also favour immigrants with atypical colour phenotypes who will evade intrasexual antagonism (but might anyway fail to attract mates). More generally, our result highlights the strong potential for negative frequency-dependent interactions among conspecifics to promote phenotypic diversity within populations [13–15].

# References

1. Darwin C. 1871 *The descent of man and selection in relation to sex.* London, UK: John Murray.

2. Crothers L, Gering E, Cummings M. 2011 Aposematic signal variation predicts male–male interactions in a polymorphic poison frog. *Evolution* **65**, 599–605. (doi:10.1111/j.1558-5646.2010.01154.x)

3. Cummings M. 2007 Sensory trade-offs predict signal divergence in surfperch. *Evolution* **61**, 530–545. (doi:10.1111/j.1558-5646.2007.00047.x)

4. Seehausen O *et al.* 2008 Speciation through sensory drive in cichlid fish. *Nature* **455**, 620–626. (doi:10.1038/nature07285)

5. Millar NP, Reznick DN, Kinnison MT, Hendry AP. 2006 Disentangling the selective factors that act on male coloration in wild guppies. *Oikos* **113**, 1–12. (doi:10.1111/j.0030-1299.2006.14038.x)

6. Dijkstra PD, Seehausen O, Gricar BLA, Maan ME, Groothuis TGG. 2006 Can male-male competition stabilize speciation? A test in Lake Victoria haplochromine cichlid fish. *Behav. Ecol. Sociobiol.* **59**, 704–713. (doi:10.1007/s00265-005-0100-1)

7. Dijkstra PD, Seehausen O, Pierotti MER, Groothuis TGG. 2006 Male–male competition and speciation: aggression bias towards differently coloured rivals varies between stages of speciation in a Lake Victoria cichlid species complex. *J. Evol. Biol.* **20**, 496–502. (doi:10.1111/j.1420-9101.2006.01266.x)

8. Seehausen O, Schluter D. 2004 Male–male competition and nuptial-colour displacement as a diversifying force in Lake Victoria cichlid fishes. *Proc. R. Soc. Lond. B* **271**, 1345–1353. (doi:10.1098/rspb.2004.2737)

9. Reimchen TE. 1989 Loss of nupital color in threespine sticklebacks (*Gasterosteus aculeatus*). *Evolution* **43**, 450–460. (doi:10.2307/2409219)

10. Bolnick DI, Brock CD, Schmerer M. 2015 Population-specific covariation between immune function, sexual signals, and microhabitat in male threespine stickleback. *PLoS ONE* **10**, e0126000. (doi:10.1371/journal.pone.0126000)

11. Bolnick DI, Hendrix KA, Jordan LA, Veen T, Brock CD. 2016 Data from: Intruder colour and light environment jointly determine how nesting male stickleback respond to simulated territorial intrusions. Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.92q55.

12. Tinghitella RM, Lehto WR, Minter R. 2015 The evolutionary loss of a badge of status alters male competition in three-spine stickleback. *Behav. Ecol.* **26**, 609–616. (doi:10.1093/beheco/aru242)

13. Rueffler C, Van Dooren TJM, Leimar O, Abrams PA. 2006 Disruptive selection and then what? *Trends Ecol. Evol.* **21**, 238–245. (doi:10.1016/j.tree.2006.03.003)

14. Bolnick DI. 2004 Can intraspecific competition drive disruptive selection? An experimental test in natural populations of sticklebacks. *Evolution* **87**, 608–618. (doi:10.1111/j.0014-3820.2004.tb01683.x)

15. Kopp M, Hermisson J. 2006 The evolution of genetic architecture under frequency-dependent disruptive selection. *Evolution* **60**, 1537–1550. (doi:10.1111/j.0014-3820.2006.tb00499.x)