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Phenotypic plasticity drives a depth gradient in male conspicuousness in threespine stickleback, *Gasterosteus aculeatus*

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Signal evolution is thought to depend on both a signal's detectability or conspicuousness (signal design) as well as any extractable information it may convey to a potential receiver (signal content). While theoretical and empirical work in sexual selection has largely focused on signal content, there has been a steady accrual of evidence that signal design is also important for trait evolution. Despite this, relatively little attention has been paid to spatial variation in the conspicuousness of a given signal, especially over small spatial scales (relative to an organism's dispersal distance). Here, we show that visual signals of male threespine stickleback vary in conspicuousness, depending on a male's nest depth within a given lake. Deeper nesting males were typically more chromatically conspicuous than shallow nesting males. This trend is partly because all male stickleback are more conspicuous in deep optical environments. However, deep males are even more conspicuous than environmentally driven null expectations, while shallow males tend to be disproportionally cryptic. Experimental manipulation of male nesting depth induced plastic changes in nuptial color that replicated the natural gradients in conspicuousness. We discuss a number of potential mechanisms that could produce depth gradients in conspicuousness in male stickleback, including concomitant depth gradients in diet, predation pressure, male/female density, female preference, and opportunity for sexual selection.

KEY WORDS: **Local adaptation, microclines, nuptial color, phenotypic plasticity, sensory drive.**

In many species of animals, one or both sexes employ secondary sexual characteristics during courtship (Darwin 1871; Andersson 1994). These traits may provide information about the sender's reproductive status (Andersson 1994), general condition and health (Hill 1990; Milinski and Bakker 1990; Boughman 2007), parental ability (Frischknecht 1993; Candolin 2000) and various other attributes (reviewed in Andersson and Simmons 2006). Many theoretical models have been developed to understand how courtship traits evolve (Fisher 1930; Lande 1981; Grafen 1990a,b; Kirkpatrick and Ryan 1991; Kirkpatrick 1996; Kirkpatrick and Barton 1997; Kokko et al. 2002, 2006; Chunco et al. 2007) and empirical studies have confirmed a number of these models do explain courtship trait evolution in natural populations (Andersson 1982, 1994; Berglund et al. 1986; Houde 1987; Kirkpatrick 1987; Hill 1990; Milinski and Bakker 1990; Bakker and Mundwiler 1994; Wilkinson and Reillo 1994; Jones and Ratterman 2009).

It is common in studies investigating sexual selection to assume a trait is equally detectable or conspicuous to all conspecifics, and thus any sexual selection on the trait is due to mate choice, intrasexual competition, or some combination of the two (reviewed in Andersson 1994; but see Endler and Théry 1996; Heindl and Winkler 2003; Gray et al. 2008; Hurtado-Gonzales et al. 2014). However, beginning with the work of Ryan et al. (1990; Ryan 1990) and Endler (1990, 1992, 1993) researchers increasingly emphasized that a trait's detectability can vary substantially across different environments and/or receivers and this,

in turn, can dramatically influence trait evolution (Endler and Basolo 1998; Ryan 1998; Boughman 2001; Fuller 2002; Fuller and Noa 2010; Fuller et al. 2005; Chunco et al. 2007; Cummings 2007; Gray et al. 2008; Ryan and Cummings 2013). For instance, environmental factors such as tree density and urban noise have been shown to influence the degradation and detectability of acoustic signals in birds. Richards and Wiley (1980) found that the effect of tree density on signal degradation favored songs that avoided repetition periods between elements at similar frequencies in forest-dwelling birds (see also Wiley 1991). Slabbekorn and Peet (2003) found that great tit (*Parus major*) populations from noisier urban environments sing higher pitched songs, presumably to increase signal contrast with the background din, thus increasing the song's conspicuousness to potential receivers (reviewed in Brumm and Slabbekorn 2005; Patricelli and Blickley 2006). Perceptual influences on acoustic signal perception and evolution have also been demonstrated. Ryan et al. (1990) showed that tuning in a specific auditory region (basilar papilla) of two species of *Physalaemus* frogs biased perception toward the lower frequency "chuck" elements of the calls of male *P. pustulosus*, even though calling males of the second species (*P. coloradorum*) do not produce this call element.

Visual courtship traits may also differ across distinct signaling environments (Reimchen 1989; Boughman 2001; Fuller 2002; Cummings 2007), possibly due to selection to maintain trait conspicuousness. For example, Cummings (2007) found that divergence in visual pigments of five species of embiotocid fishes was associated with differences among species' local optical environments. This, in turn, biased perception toward certain visual signals in each species and resulted in predictable divergence in male courtship signals (Cummings and Partridge 2001; Cummings 2007; reviewed in Ryan and Cummings 2013). Threespine stickleback from postglacial lakes in British Columbia also show strong divergence in male nuptial color across distinct optical environments (melanic vs. red-throated phenotypes in tea-stained versus clear lakes: Reimchen 1989; Boughman 2001; Scott 2001; Brock et al. in prep.). This across-population correlation between color and the local light environment has mostly been attributed to selection to maintain signal detectability (Reimchen 1989; Scott and Foster 2000; Boughman 2001; Scott 2001).

These phenotype-environment correlations have been most commonly documented over relatively broad spatial scales (e.g., different lakes) or in strongly contrasting environments (e.g., tea-stained vs. clear water, forest vs. rangeland). At these large geographic scales, the homogenizing effect of gene flow is weak and therefore less likely to impede adaptive divergence (Felsenstein 1976; Garcia-Ramos and Kirkpatrick 1997; Lenormand 2002; Richardson et al. 2014). However, signaling environments can vary across much finer spatial scales. Examples of signaling microhabitats can include sunflecks in terrestrial forests (Mollon 1989; Endler 1993) or marine kelp (Cummings 2004, 2007) forests, and depth gradients in background irradiance in aquatic environments (Seehausen et al. 2008; Sabbah et al. 2011; Brock et al. in review). This microspatial variation could potentially induce corresponding microspatial variation in phenotypes, despite gene flow, if individuals (1) choose habitats nonrandomly, or (2) adjust their phenotype to their local environment. As a result, it is possible to observe phenotype-environment correlations over very fine spatial scales relative to the scale of gene flow (Richardson et al. 2014; Bolnick et al. 2015a,b). Despite this potential for microspatial signal variation, the vast majority of studies of signal phenotype (i.e., nuptial color, call pitch, etc.) focus on trait variation across widely separated environments. Far fewer have investigated spatial variation in signal functionality (detectability or conspicuousness) across environments (but see Gray et al. 2008), and/or variation over fine spatial scales (but see Seehausen et al. 2008; Marques et al. 2017).

Here, we provide evidence for microspatial variation in the conspicuousness of male nuptial signals in threespine stickleback from postglacial lakes on Vancouver Island, British Columbia. We have documented repeatable depth gradients in stickleback male nuptial color across 15 lakes (Fig. 1; Brock et al. in review). However, the impact of these color gradients on male signal efficacy remained unknown. Here, we employ a visual model for stickleback to gain insight into the likely signaling effects of these previously documented male color gradients. This change in male color with nest depth could in principle allow males to maintain a constant level of conspicuousness across all depths, compensating for a changing background light environment. Alternatively, the optimal conspicuousness might itself change with depth. To distinguish between these alternatives, in this article we employ a stickleback visual model to show that these previously demonstrated depth-color correlations give rise to microspatial clines in male chromatic conspicuousness within each of these 15 lakes. By manipulating a male's nesting depth in a single lake (Gosling), we were able to experimentally recapitulate the depth gradient in male conspicuousness found in wild-caught Gosling male stickleback. Our results implicate phenotypic plasticity in nuptial color as the primary mechanism involved in producing this gradient in conspicuousness over microspatial scales. Additionally, the results presented here suggest that the optimal conspicuousness itself changes with depth, as in 12 of 15 lakes deep and shallow males are more, and less conspicuous, respectively, than null expectations. Finally, we find strong evidence that gradients in the ambient light environment are consistently correlated with male conspicuousness, with males from more red-shifted habitats being consistently more conspicuous than a randomly sampled male stickleback would be in that same environment. We discuss a number of potential biotic and abiotic factors that may vary over fine spatial scales, leading to microspatial variation in selection,

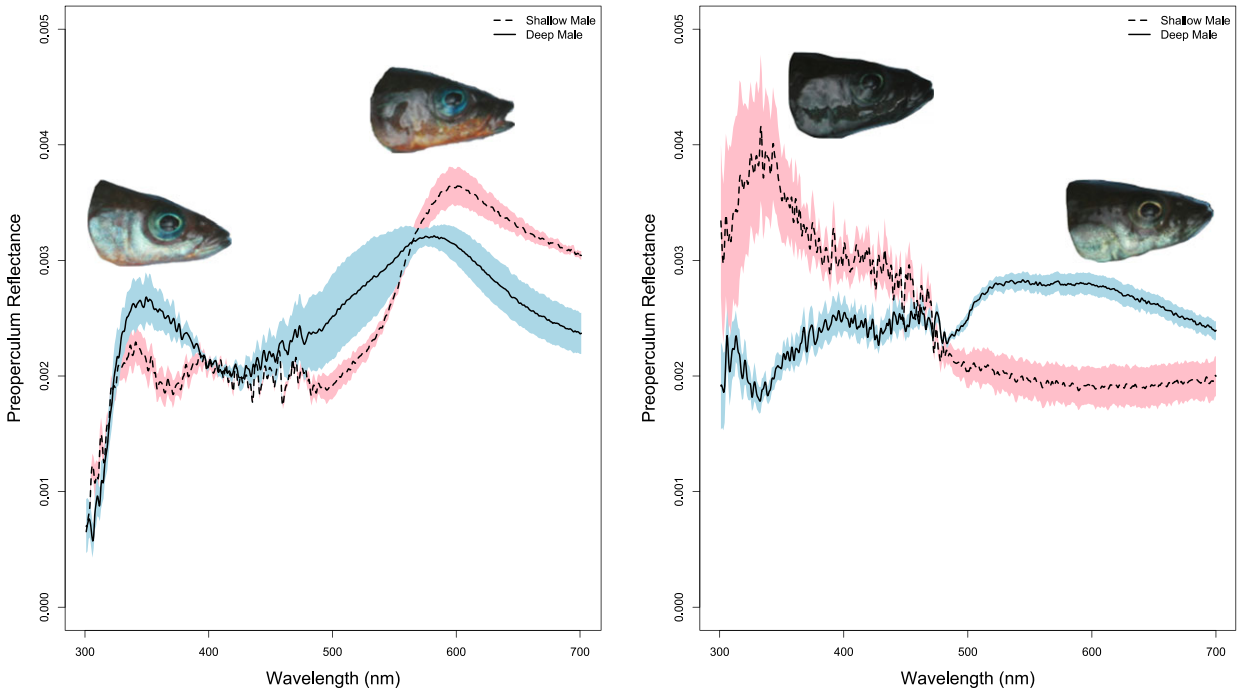

Figure 1. Male reflectance spectra for the preoperculum for shallow- and deep-nesting males from two lakes. (A) Median reflectance spectra for the preoperculum for a shallow- (light pink, hatched line) and deep-nesting (light blue, solid line) male from Gosling Lake. Photos were taken immediately before reflectance data was collected. (B) Median reflectance spectra for the preoperculum for a shallow- (light pink, hatched line) and deep-nesting (light blue, solid line) male from Farewell Lake. Note the opposite pattern in the shift in reflectance spectra between shallow and deep males across these two lakes.

which may then facilitate the maintenance of spatial gradients in signal detectability.

Methods

SAMPLING WILD-CAUGHT MALES FOR COLOR AND VISUAL CONTRAST MEASUREMENTS

We sampled actively nesting male stickleback from 15 lakes on Vancouver Island, British Columbia (Extended Data: Table S1). Each lake was sampled in one or a few consecutive days, to minimize temporal variance among same-population samples. Males were collected directly from their nests by a snorkeler (CDB) using a dipnet, to ensure they were actively breeding. We believe this approach is an improvement upon the common practice in which trapped stickleback males are retained or released depending on the researcher's evaluation of whether their color indicates breeding status.

For each nesting male collected, we recorded its nest depth, the distance to the nearest protective cover, and categorical descriptions of the substrate and nearby vegetation. Prior to capture, each male was observed for five minutes to note whether it displayed (1) aggression toward other stickleback, (2) courting behavior toward females, (3) nest-fanning behavior (a form of parental care). A darkened cooler with fresh lake water was used to transport males to shore for immediate collection of reflectance data (within 1–5 min after capture). This cooler was rinsed and refilled with lake water between males. Males were euthanized using MS-222 prior to the collection of reflectance data. As male color can change rapidly after death, the time of capture, euthanization, and reflectance measurements were also recorded for use as potential covariates in downstream analyses.

SPECTRAL REFLECTANCE MEASUREMENTS OF MALE NUPTIAL COLOR

For each male, spectral reflectance measurements were taken using a EPP200C UV-VIS spectrometer, SL-4 Xenon lamp, and a R400-7 reflectance probe for two body regions: (1) preoperculum (2) abdomen. All measurements were taken while holding the probe perpendicular to the surface of the fish at a fixed 3 mm distance. Three replicate measurements were collected for each body region, moving the probe slightly between replicates. Spectralon white standard measurements were taken between each fish to account for lamp drift.

AMBIENT LIGHT

In each lake, we measured both sidewelling and downwelling irradiance along a depth gradient (0–2 m) at 25–50 cm intervals. Irradiance data were collected with a EPP200C UV-VIS spectrometer and a UV-NIR cosine receptor. Sidewelling irradiance was collected with the probe oriented horizontally away from the

shore and represents the visual background against which a male stickleback is often viewed by females (Fig. S3). Downwelling irradiance was measured with the probe directed vertically toward the water surface and represents the primary source of light for target reflection (Fig. S4). All irradiance data was collected in areas where males were found nesting that year. Five replicate measurements were taken at each depth and these measurements were dispersed throughout the nesting environment. We used the median value at each wavelength of these five replicates as our estimate of the ambient light at a given depth. As both the time of day and the time of year can impact irradiance measurements, all irradiance data were collected between 9 and 10:30 AM and within a window of 20 days (June 13–July 2; this timeframe is in the middle of the breeding season of threespine stickleback in our study area). To control for ambient light conditions, we collected measurements above the water surface for each replicate to allow for the normalization of irradiance (Normalized $\text{Irradiance}_{\text{Depth}} = \text{Irradiance}_{\text{Depth}}/\text{Irradiance}_{\text{Surface}}$). Additionally, we documented the ambient conditions at the time of each irradiance measurement (e.g., partly cloudy, sunny, etc.). Preliminary analyses indicated there was no significant effect of ambient condition on our irradiance measurements, and our study results were qualitatively similar regardless of whether we employed normalized or raw irradiance data. As such, we focus here on the results using only the latter.

We calculated the ratio of the respective areas under the sidewelling irradiance curve for the wavelength intervals of 500–600 nm (green-orange) and 300–400 nm (UV-blue) at each depth. Within each lake, we regressed this ratio versus depth to estimate the ambient light depth gradient. Qualitatively similar light gradients were found using 550–650 and 350–450 nm. We focus on sidewelling rather than downwelling irradiance when assessing ambient light gradients because sidewelling light is the best predictor, by far, of among-population divergence in male color (Reimchen 1989; Boughman 2001; Scott 2001; Brock et al. in prep.). However, downwelling irradiance is incorporated in our calculations of both chromatic and luminance constrasts (see below).

STICKLEBACK VISUAL MODEL TO CALCULATE CHROMATIC AND LUMINANCE CONTRASTS

To assess whether the relationship between nuptial color and nest depth impacted male conspicuousness we calculated a male's signal contrast in a given environment. As previous research suggests that chromatic and luminance channels are processed independently (Cummings 2004; Endler and Mielke 2005; Osorio and Vorobyev 2005; Lind and Kelber 2011; but see Pignatelli et al. 2010) we calculated two measures of contrast: chromatic contrast, or ΔS, and luminance contrast, ΔL, using a stickleback-specific visual model. We developed a photoreceptor noise-limited color discrimination model for stickleback fish (Vorobyev and Osorio 1998) using MSP-estimated peak cone sensitivities (Rowe et al. 2004) and employed the parameters outlined in Govardovskii et al. (2000) to calculate spectral sensitivity functions for the four stickleback cone receptors (Fig. S6, S7). The absolute quantum catch, Q, for each class of photoreceptor is:

$$Q_c = \int_{\lambda=300}^{700} A_c(\lambda) S(\lambda) I(\lambda) d\lambda \ ,$$

where λ is the wavelength, A_c is the photoreceptor absorptance of cone class c, $S(\lambda)$ is the target reflectance, and $I(\lambda)$ is the environmental incident irradiance (= downwelling irradiance). Quantum catch was adjusted for the adapting light environment using von Kries transformations, where $q_c = k_c Q_c$, and

$$k_c = \frac{1}{\int_{\lambda=300}^{700} A_c(\lambda) I_b(\lambda) d\lambda} \ ,$$

where $I_b(\lambda)$ is the adapting background (= sidewelling irradiance). Quantum catch was calculated for each male's target reflectance and the local sidewelling background light. The signal for each photoreceptor when viewing a target in a given background is proportional to the logarithm of their adjusted quantum catches such that contrast between the two is

$$\Delta f_c = ln \frac{q_c(target)}{q_c(background)} \ ,$$

Threespine stickleback are tetrachromats, and thus have four cone classes: UV-sensitive (SWS1, abbreviated below as U), Short-wavelength sensitive (SWS2 = S), Medium-wavelength sensitive (MWS = M), and Long-wavelength sensitive (LWS = L) cone receptors (Rowe et al. 2004). As such, the perceptual distance in terms of chromatic contrast, ΔS, between the target and background was calculated for a tetrachromatic visual system as (Vorobyev et al. 1998; Siddiqi et al. 2004):

$$\Delta S = \sqrt{\frac{\begin{bmatrix} e_U e_S^2 (\Delta f_L - \Delta f_M)^2 + e_U e_M^2 (\Delta f_L - \Delta f_S)^2 + e_U e_L^2 (\Delta f_M - \Delta f_S)^2 \\ + e_S e_M^2 (\Delta f_L - \Delta f_U)^2 + e_S e_L^2 (\Delta f_M - \Delta f_U)^2 + e_M e_L^2 (\Delta f_S - \Delta f_U)^2 \end{bmatrix}}{\left[(e_U e_S e_M)^2 + (e_U e_S e_L)^2 + (e_U e_M e_L)^2 + (e_S e_M e_L)^2 \right]}}$$

where e_c is the signaling noise for a photoreceptor of class c, and is given by the following:

$$e_c = \sqrt{\frac{\omega}{\eta_c}} \ ,$$

where ω is the Weber fraction and η_c is relative density of photoreceptors of class c in the retina. A Weber fraction value of 0.05 was chosen as a conservative estimate (Vorobyev et al. 1998; Rick et al. 2012). We used hypothetical cone ratios of 1:1:2:2, though results were qualitatively similar using cone ratios of 1:1:1:1 (Rick et al. 2012).

Double cones are believed to be the primary receptor type involved in detecting contrast in the luminance channel in birds and fish (Smith et al. 1985; Hart et al. 2000; Osorio and Vorobyev 2005; but see Cummings 2004; Pignatelli et al. 2010). As such, luminance contrast was calculated as

$$\Delta L = \left|\Delta f_{double} \middle/ \omega_{double}\right|$$

Finally, it is ideal to account for the transmission of the lens, ocular media, and intracone oil droplets (if present) when calculating contrasts using visual models, as these can strongly influence spectral sensitivity in vertebrates, including fish (e.g., Bowmaker et al. 1997; Siebeck and Marshall 2001; Loew et al. 2002; Hofmann et al. 2010; Cheney et al. 2013; Lind et al. 2014; Stavenga and Wilts 2014). However, to our knowledge there are no transmission spectra data available for the lens or ocular media of threespine stickleback (Rick et al. 2012 measure lens transmission, but their data is not publicly available; See also Flamarique et al. 2013). Additionally, it is unknown whether oil droplets are present within the cones of threespine stickleback. As such, we were not able to include any of these components in our visual model calculations. While we recognize this is a limitation of our study, previous evidence from both optomotor and behavioral studies indicate that stickleback are sensitive to a variety of colors, including those of both long (e.g., red) and short (e.g., ultraviolet) wavelengths(e.g., Milinski and Bakker 1990; Boughman 2001; Rick and Bakker 2008a,b,c; Bolnick et al. 2016). Consequently, these results suggest the stickleback eye is at least partially transmissive to wavelengths between ~300 and 700 nm. Additionally, the lens of a close relative, the fifteen-spine stickleback (*Spinachia spinachia*), is highly transmissive across the visible spectrum, including UV(Thorpe et al. 1993), which is consistent with the presence of similar lens properties in the threespine stickleback. Given this previous research, we feel the conclusions of our visual model analyses should be qualitatively robust to the exclusion of these components from our calculations of the photon catch.

DEPTH GRADIENTS IN CONTRAST SCORES

Chromatic and luminance contrast scores were calculated using the normalized reflectance spectra from the preoperculum, as this is the body region that is most consistently related to nest depth, in multiple lakes and multiple years (Brock et al. in review). We used linear-mixed models to assess whether ΔS and ΔL for the preoperculum varied predictably across nesting depth for males from each of our 15 lakes. Preliminary analyses found no effect of behavior, nesting habitat (except for depth), or the time between collection, euthanization, and reflectance measurement on male color (Brock et al. in review) or contrast, so we do not consider these further. We fit a series of mixed models with preoperculum contrast (ΔS or ΔL) as the response and nest depth as the sole fixed predictor variable. Lake was also included as a random effect and the following nested models were compared using Akaike Information Criterion (AIC): (1) Random Intercept Only model (2) Nest Depth + Random Intercept model (3) Nest Depth + Random Slope + Random Intercept (Uncorrelated) (4) Nest Depth + Random Slope + Random Intercept (Correlated). Model (1) included no fixed effect of depth, and instead only allows for a different mean contrast for each lake. Model (2) includes nest depth as a fixed predictor, while also allowing for differences in mean contrast between lakes. Model (3) includes all terms from Model (2) as well as a random slope term that allows the relationship between contrast and depth to vary between lakes. Finally, Model (4) includes all terms from Model (3) with the addition of a correlation between the random intercept and random slope terms, which accounts for the possibility that lakes characterized by more contrasting males have predictably different depth gradients.

Changing contrast with depth could arise because of the change in male color with depth, or because the light environment changes, or both. If the trend is exclusively driven by male color, then the contrast-depth correlation should disappear if we permute males across depths and recalculate their contrast score at randomly assigned depths (e.g., an in silico transplant experiment). Alternatively, if the trend is exclusively driven by the ambient light (e.g., all stickleback are more visible in deep water), then we should observe a contrast-depth correlation even in permuted males. If both effects contribute, then the observed contrast-depth correlation should be more (or less) pronounced than the correlation obtained from depth-permuted males' contrast scores.

We therefore assessed whether a trend in contrast(s) across depth existed when males were randomly permuted between depths and contrasts were recalculated. Specifically, we calculated ΔS and ΔL for every male at all nesting depths and regressed the average contrast at each depth on their respective depth. We compared the slopes of the empirical versus permuted contrast-depth correlations using a t-test. Additionally, we calculated the deviation of a male's contrast score from the null expectation obtained from other males assigned to the focal male's depth ($\Delta\Delta S = \Delta S_{\text{observed}} - \Delta S_{\text{expected}}$) and regressed the results on nest depth. As our mixed model results (see Results) indicate that only ΔS showed significant depth gradients across multiples lakes we limited our analyses to the chromatic channel. We estimated depth gradients in male $\Delta\Delta S$ for each lake using linear regression. To

compare ambient light (irradiance) – and $\Delta\Delta S$-depth gradients we first used a sign test to assess whether gradient slopes were consistently associated across lakes. To investigate this further, we used a weighted regression (with the reciprocal of the standard errors of the slope estimates as weights) of $\text{Slope}_{\Delta\Delta S \text{ v. depth}} \sim \text{Slope}_{\text{irradiance v. depth}}$ to test for an association between the two. We applied both analyses to three datasets: (1) using all lakes ($n = 15$ lakes), (2) using all lakes with significant $\Delta\Delta S$-depth gradients, ($n = 8$ lakes), and 3) using only those lakes whose $\Delta\Delta S$-depth gradients survive multiple-test correction (FDR-corrected $P < 0.05$; $n = 6$ lakes).

PHENOTYPIC PLASTICITY AND SIGNAL CONTRAST

We previously demonstrated that male color changes in a predictable manner when breeding males from Gosling Lake are constrained to nest at one of two predetermined depth treatments (shallow treatment~0.5 m; deep treatment~1.85 m; Brock et al. in review). Here, we reanalyze the data to evaluate whether this color change led to adjusted chromatic and luminance contrast as well (see below). For the experiment, we constructed 40 open-bottomed cylindrical enclosures (~1 m in diameter) out of 0.5 cm square hardware cloth, and installed these in Gosling Lake. We placed 20 enclosures in 0.45–0.55 m deep water, and the other 20 in 1.8–1.9 m deep water, with a minimum of 1 m of open space between each cage.

We placed one male and one female into each cage at the start of the experiment. Females were collected by trapping and dip-netting. Males were hand-collected from nests as outlined above. Prior to being introduced into the enclosure, reflectance data were collected from males suspended in an aquarium constructed from UV-transmissive material by CDB. As males and females were collected along a depth gradient we feel it would be problematic to assign them to "shallow" and "deep" categories by setting an arbitrary cut-off to distinguish the two classes. As such, this prevented us from being able to reciprocally transplant individuals across depth treatments. Instead, we randomly distributed males and females among the 40 experimental cages. In addition to randomizing individuals among depth treatments, our study design also allows us to assess whether greater changes in nest depth were consistently associated with greater changes in contrast. We placed a fixed amount of natural nesting material into each cage, which was replenished weekly. The experiment was run for 28 days to ensure the males had enough time to acclimate to these enclosures and, if possible, modify their color. Males were removed from cages in the same order in which they were added and their final color was measured in aquaria as before. The total amount of time each male spent in an experimental enclosure was measured for use as a covariate.

Sidewelling and downwelling irradiance data were collected from each enclosure prior to our experiment. Comparison of the ambient light gradient of our enclosures to that of the lake as a whole ($t = 1.12$, $P = 0.137$; Fig. S3) was not significant, indicating that our experiment produced a reasonable replication of the natural variance in light conditions across depths.

For experimental males, contrast scores were calculated in the wild (their original nesting environment), as well as in their assigned enclosure using both their initial (preexperimental) reflectance and final (postexperimental) reflectance spectra. To assess whether color shifts in our plasticity experiment lead to significant changes in ΔS and/or ΔL across treatments we first compared the postexperimental mean contrast scores using a Student's t-test. We then fit a general linear-mixed model with the ΔS (or ΔL) as the response and treatment (deep vs. shallow) and time (wild vs. pre- vs. postexperiment), as well as their interaction, as fixed predictors. Male stickleback identity was included as a random effect. Order of introduction and the total time in the enclosure were not significant terms in the model and were excluded from the final analyses. Parametric bootstrapping (replicates = 10,000) was employed to calculate P-values for model terms. We also tested whether the change in male contrast (Δcontrast) depended on the magnitude and direction of change in nest depth (Δnest depth) using linear regression. Finally, to assess whether a male's contrast within its home cage differed from that expected by chance, we created a null contrast for each cage by taking the average contrast (for both ΔS and ΔL) of all possible males, digitally "transplanted" into the focal cage. We then compared a male's deviation from this null, or expected, contrast ($\Delta\Delta S$ *and* $\Delta\Delta L$) between treatments using a t-test. To assess whether there was significant deviation from the null, we ran one-sample t-tests on the contrast deviations separately for each treatment.

Results

DEPTH GRADIENTS IN ΔS AND ΔL

We begin by presenting results from one lake (Gosling Lake, for which we have two years of data) to introduce the basic pattern. We then expand our discussion to include the results across all 15 lakes. For both 2010 and 2012 in Gosling Lake, chromatic contrast (ΔS) increased in males nesting at greater depths (2010: $P = 0.0002$, $r^2 = 0.7385$; 2012: $P << 0.0001$, $r^2 = 0.606$). The null expectation, generated by in silico permutations of males across nest depths, also displays positive relationship between ΔS and nest depth. However, the empirical gradient exceeds the null expectation for both years, and these two gradients differ significantly in 2012 ($t = 2.67$, $P = 0.009$; Fig. 2), though not in 2010 ($t = 1.733$. $P = 0.09$). We therefore infer that in Gosling Lake changing optical environments with depth influence chromatic contrast on males, but that the changing nuptial color with depth further amplifies this gradient.

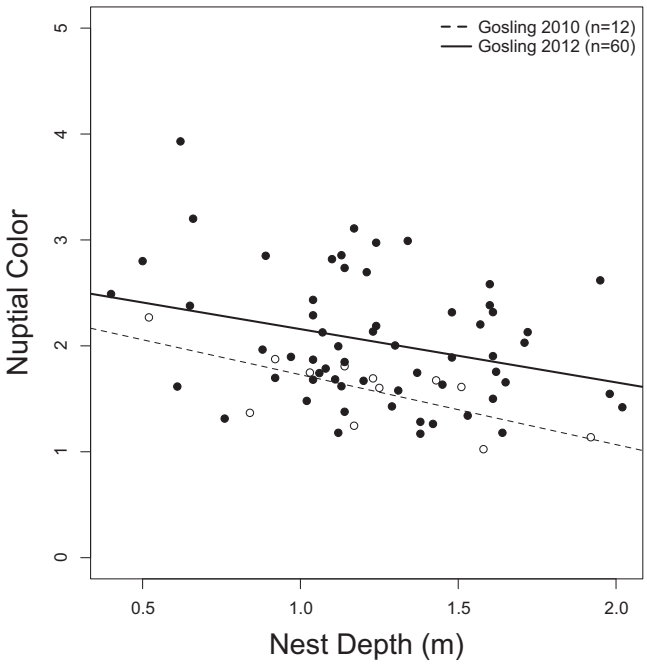

Figure 2. **Microspatial gradients in male nuptial color in threespine stickleback. Nuptial color is measured as the ratio of the respective areas under the reflectance curve for the wavelength intervals of 500–600 nm (green-orange) and 300–400 nm (UV-blue) for the preoperculum. We focused on these two intervals because previous research has shown that both long wavelengths (e.g., orange and red) and short wavelengths (e.g., ultraviolet) strongly influence both mate preference and male–male aggressive encounters (see text for details). Qualitatively similar results were found using the wavelength intervals of 550–650 nm and 350–450 nm. Stickleback from Gosling Lake show a significant association between male nuptial color and nesting depth over spatial scales of <2 meters (Brock et al. in review). Males collected in both 2010 ($n = 12$, $r = –0.706$, $P = 0.01$; dotted line) and 2012 ($n = 60$, $r = –0.3$, $P = 0.019$; solid line) display significant microspatial gradients in nuptial color.**

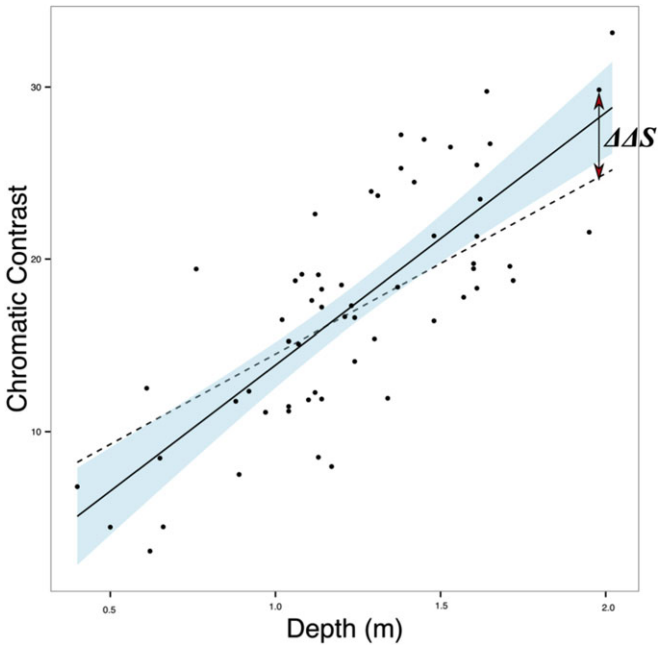

Figure 3. **Depth gradients in nuptial color lead to concomitant gradients in chromatic contrast (ΔS). Deeper nesting males are more conspicuous in the chromatic channel than shallow nesting males in Gosling Lake. Plotted is the data from Gosling Lake (2012) and the line of best fit from a linear regression (solid line; shaded region = 95% CI). The dotted line indicates the null expectation for ΔS (see main text) and the double-headed arrow indicates graphically the deviation from this null ($\Delta\Delta S$) for a single male.**

Closer inspection of the empirical and null trends in Gosling Lake reveal an interesting result. Males' deviations from their null ΔS ($\Delta\Delta S$) differs across nesting depths (2012: $P = 0.008$, $r^2 = 0.114$; 2010: $P = 0.029$, $r^2 = 0.366$). Deeper nesting males contrast more than expected in the chromatic channel ($\Delta\Delta S > 0$), implying they are more conspicuous than a randomly assigned male would be. In contrast, shallow males contrast less ($\Delta\Delta S < 0$, implying they tend to be more cryptic; Fig. 2).

In the luminance channel, we found nest depth was a significant predictor of ΔL for Gosling males collected in 2012, but not those from 2010 (2012: $P = 0.012$, $r^2 = 0.105$; 2010: $P = 0.75$, $r^2 = 0.009$). As with ΔS, the null gradient was positive for both years, though the empirical gradient was significantly steeper than the null in 2012 (2012: $t = 4.34$, $P = 0.00004$), but not 2010 (2010: $t = 0.687$, $P = 0.49$). The deviation from the null expectation ($\Delta\Delta L$) also differed significantly across nest depths in 2012 ($P = 0.008$, $r^2 = 0.1142$), though not 2010 ($P = 0.377$, $r^2 = 0.072$). Deeper nesting males contrast more than expected in the luminance channel, while shallow males contrast less, though again this result was only significant in Gosling males collected in 2012.

Our multilake (= 15 lakes) mixed model analyses indicate that depth gradients in male contrast are common in many lakes. However, the direction of these gradients varies significantly between lakes for ΔS, but not ΔL (Extended Data Tables 1 and 2). Furthermore, these results indicate that gradient slope is correlated with mean ΔS. Specifically, lakes with less chromatically contrasting males showed negative depth gradients of ΔS (deeper males are cryptic), while more contrasting lakes displayed the opposite pattern (deeper males are conspicuous).

For our multilake analysis of $\Delta\Delta S$, eight of the 16 lakes/years surveyed showed a significant correlation between $\Delta\Delta S$ and nest depth, of which six remain significant after correcting for multiple tests (FDR-corrected P-values <0.05; Table S4). All but one of these eight lakes (Comida Lake) showed a concordant orientation between the $\Delta\Delta S$ and ambient light depth gradients: males in more red-shifted environments were more conspicuous than

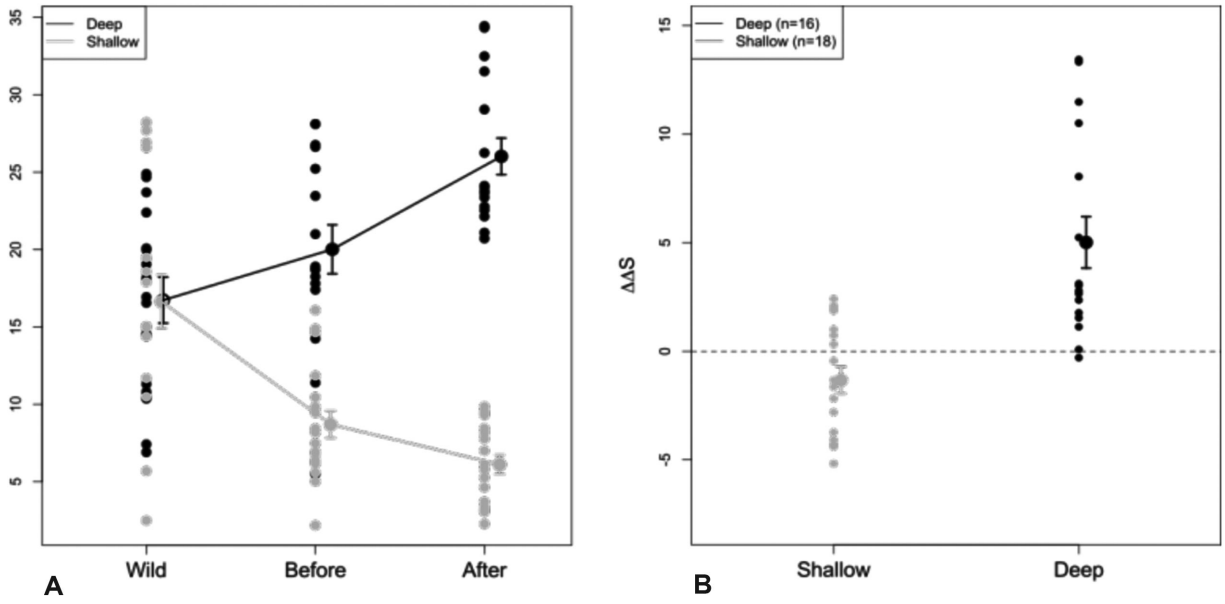

Figure 4. **Plasticity leads to dramatic shifts in male conspicuousness. (A) Males restricted to deep nests (~1.85 m) show a significant increase in chromatic contrast (ΔS) while males from the shallow nest treatment (~0.5 m) decrease their ΔS. Shown are ΔS for each male in: (1) the wild (Wild: initial color in original nesting environment), (2) the nesting enclosure at the start of the experiment (Before: initial color in males' enclosure), and (3) the nesting enclosure at the end of the experiment (After: final color in males' enclosure). ΔS changes significantly in both treatments across all timepoints, indicating that both optical environment and trait plasticity influence male conpicuousness. (B) Males from the deep nest treatment contrasted more than expected ($\Delta\Delta S$) in the chromatic channel while shallow males contrasted less. In both treatments $\Delta\Delta S$ differed significantly from 0 (dotted line). Plotted are the mean and standard error of $\Delta\Delta S$ for each treatment.**

null expectations (from randomly permuted males placed in the same visual setting). Using sign tests, we found evidence for an association between the slopes of depth gradients in optical environment and $\Delta\Delta S$ for the full 15 lake dataset ($P = 0.018$) and the 8-lake dataset ($P = 0.035$), but not for the 6-lake dataset ($P = 0.10$). However, further investigation of this relationship using weighted linear regression demonstrated that the two gradients slopes were positively correlated in all three datasets, though the result was only marginally significant in the 8-lake subset (15 lakes: $r^2 = 0.36$, $P = 0.014$; 8 lakes: $r^2 = 0.491$, $P = 0.053$; 6 lakes: $r^2 = 0.713$, $P = 0.034$; Fig. 5). These results indicate that across lakes more strongly red-shifted environments, regardless of depth, showed concomitantly larger shifts toward greater $\Delta\Delta S$ (disproportionate conspicuousness) in nesting males.

PHENOTYPIC PLASTICITY AND ΔS AND ΔL

ΔS differed significantly between the deep and shallow treatments at the end of the experiment ($t = 3.808$, $P = 0.0003$), with chromatic contrast, on average, being greater in the deep treatment ($\mu_{deep} = 26.02$, $\mu_{shallow} = 6.11$). There was a significant interaction between time and treatment in our linear-mixed model, indicating there was a significant change in ΔS across the duration of the experiment, though the direction of this change differed between treatments (GLMM Time × Treatment $P = 0.0002$). In general, ΔS increased in the deep treatment (i.e., males became more conspicuous), while it decreased in the shallow treatment (i.e., males became more cryptic; Fig. 3). Males subjected to the greatest change in nest depth exhibited the largest change in color contrast, though not luminance contrast (ΔS: $r^2 = 0.782$, $P < 0.001$; ΔL: $r^2 = 0.043$, $P = 0.234$). Deviation of ΔS from the null expectation ($\Delta\Delta S$) also differed significantly between depth treatments ($t_{32} = 4.74$, $P < 0.0001$), with the deep treatment contrasting more than expected (one-sample $t_{17} = 4.23$, $P = 0.0007$) and the shallow treatment contrasting less ($t_{15} = 2.15$, $P = 0.046$). This experimentally induced gradient in chromatic contrast recapitulates the naturally occurring depth gradient ($t_{90} = 0.293$, $P = 0.39$; Extended data: Fig. S1). Furthermore, this short-term change in male contrast clearly indicates that phenotypic plasticity allows males to dynamically adjust their contrast to be more or less pronounced in response to distinct signaling environments.

Postexperiment ΔL did not differ significantly between treatments ($t = 0.13$, $P = 0.9$). Similarly, there was no significant difference between ΔL across the different time points of our experiment, and the interaction between time and treatment was not significant (GLMM Time × Treatment $P = 0.250$). Deviation of ΔL from the null expectation did not differ significantly between depth treatments ($t = 0.486$, $P = 0.631$), and neither treatment's

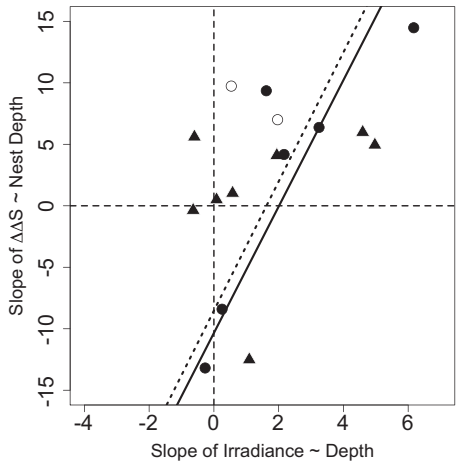

Figure 5. Depth gradient in the optical environment predicts microspatial variation in $\Delta\Delta S$. The magnitude and orientation of the $\Delta\Delta S$ depth gradient ($Slope_{\Delta\Delta S}$) and background light depth gradient ($Slope_{irradiance}$) are correlated, and weighted regression analyses indicate this result is significant for the 15 lake and six lake (solid line, filled circles) datasets, and marginally significant for the eight lake dataset (hatched line, open, and filled circles). Triangles indicate lakes where the $\Delta\Delta S$ depth gradient ($Slope_{\Delta\Delta S}$) was not significantly different from 0. See the main text for the details of these analyses.

mean deviation from the null differed significantly from 0 (deep: $t = 0.524$, $P = 0.607$; shallow: $t = 1.074$, $P = 0.298$).

Discussion

We found that in 12 of 15 lakes that we sampled on Vancouver Island, deeper nesting male stickleback had more conspicuous nuptial signals than shallow nesting males. Focusing on the eight lakes with statistically significant gradients in conspicuousness, in seven of these the deep-nesting males were more conspicuous. These trends were also temporally repeatable between years in Gosling Lake. This pattern was evident primarily in the chromatic signaling channel, though was also observed in the luminance channel (ΔL) in fish from Gosling Lake in 2012. These results are remarkable, especially considering the narrow range of nest depths stickleback typically occupy (spanning ~0.5–2 m). Additionally, we demonstrated that microspatial variation in chromatic contrast exceeded that expected from shifts in optical environment alone. Repeatedly across most lakes, deeper nesting males contrasted more than expected (for a randomly assigned male), while shallow nesting males contrasted less, indicating that increasing contrast may not always be in a males' best interest. While these results suggest a general pattern of deeper nesting males contrasting more than shallow nesting males, our results also find that depth gradients in the optical environment were consistently associated with concordant gradients in $\Delta\Delta S$ across a number of lakes. These results provide evidence for a significant role of ambient light gradients in producing microspatial variation in $\Delta\Delta S$. Specifically, regardless of depth, more red-shifted optical environments had males that were more chromatically conspicuous than null expectations, while more blue-shifted optical environments had males that were more cryptic. The varying direction of the ambient light depth gradients among these 15 lakes provides an unprecedented opportunity to assess the effect of the local signaling environment on male nuptial color and contrast. These results suggest that while nesting depth has a significant influence on signal contrast, this effect is at least partially mediated by the optical environment.

Our experimental results are consistent with results from Gosling males in the wild and indicate that phenotypic plasticity in male nuptial color is the predominant mode through which these microspatial depth gradients in conspicuousness are contrived.

Table 1. Model descriptions and fit statistics for nested model comparisons for deviance in ΔS across 15 lakes from Vancouver Island.

Model description	AIC	ΔAIC	AIC_{weight}	Conditional pseudo-r^2
(I) Random intercept (Lake)	1680.56	214.97	0	0.727
(II) Nest depth + random Intercept (lake)	1590.74	125.15	0	0.821
III) Nest depth + random slope and intercept (Uncorrelated)	1474.94	9.35	0.009	0.922
IV) Nest depth + random slope and Intercept (Correlated)	**1465.59**	**0**	**0.991**	**0.946**

Table 2. Model descriptions and fit statistics for nested model comparisons for ΔL across 15 lakes from Vancouver Island.

Model description	AIC	ΔAIC	AIC_{weight}	Conditional pseudo-r^2
(I) Random intercept (Lake)	**1272.08**	**0**	**0.605**	**0.504**
(II) Nest depth + random intercept (lake)	1273.89	1.81	0.247	0.499
(III) Nest depth + random slope and intercept (Uncorrelated)	1285.96	13.88	0.006	0.539
(IV) Nest depth + random slope and intercept (Correlated)	1274.91	2.83	0.148	0.548

Males constrained to nest in deeper environments increased their chromatic contrast, while shallow nesting males did the opposite, recapitulating the natural depth gradients in ΔS for Gosling Lake. Furthermore, these shifts in contrast were not due solely to differences in the incident and background light between depth treatments. As in the wild, Gosling males in the deep and shallow depth treatments contrasted more ($\Delta\Delta S>0$) and less ($\Delta\Delta S<0$) than expected by chance, respectively. Again, these results match those from wild stickleback, and, remarkably, the slope of our experimental depth gradient in $\Delta\Delta S$ ($\text{Slope}_{\text{experiment}} = 4.7$) does not differ appreciably from that of the natural depth gradient ($\text{Slope}_{\text{wild}} = 4.2$) in Gosling Lake (Fig. S1).

While our results strongly suggest that optical environment influences male signal contrast in threespine stickleback, the exact nature of this influence remains unclear. As male color changes predictably in the opposite direction ("countergradient") to ambient light spectral shifts (Brock et al. in review), a reasonable expectation would be that male color tracks the local signaling environment to remain conspicuous across nest depths. However, our visual model results suggest that instead of maximizing chromatic contrast across nest depths, as predicted by signaling theory for intraspecific communication (Endler 1992; but see Chunco et al. 2007; Gray et al. 2008), shallow nesting males were typically more cryptic than expected, while deep nesting male were more conspicuousness. This pattern suggests that microspatial variation in male nuptial coloration may be a product of divergent forces across depth clines. A number of factors have previously been shown to influence male color displays, including diet (Grether et al. 1999; Hill et al. 2002), female preference (Endler and Houde 1995; Hill 1994; Boughman 2001), and predation (McPhail 1969; Moodie 1972; Endler 1980; Stuart-Fox et al. 2003; Stuart-Fox et al. 2004).

The red coloration of breeding male stickleback is, at least in part, carotenoid-based, being composed primarily of astaxanthin, lutein, and tunaxanthin fatty acyl esters (Brush and Reisman 1965; Matsuno and Katsuyama 1976; Wedekind et al. 1998; Barber et al. 2001; McClennan 2007; Pike et al. 2011). As vertebrates are unable to synthesize these compounds (Simpson and Chichester 1981), stickleback rely on carotenoids extracted from their diet to produce the red throat patch (Barber et al. 2001; Pike et al. 2007; Pike et al. 2010). Thus, diet-mediated microspatial variation in carotenoid availability could lead to variability in signal intensity and conspicuousness. Stickleback diet may vary spatially within lakes, including across nest depth gradients (Snowberg and Bolnick 2012). Furthermore, diet has been posited as a potential factor driving the divergence in the extent of red coloration between benthic and limnetic stickleback species (Boughman 2007). While data exist on the distribution of carotenoid compounds among common prey items of threespine stickleback (McClennan 2007), they are not quantitative in nature, making it difficult to estimate carotenoid intake based on diet alone. However, an initial step would be to assess whether diet, and specifically, the proportion of carotenoid-containing prey items, shows similar gradients across nesting depths and whether this helps to predict gradients in male conspicuousness across lakes.

A female's preferred signal may also vary across depth. For instance, females that prefer particular microhabitats may be exposed to a limited range of optical environments. This may result in concomitant modifications of their visual systems, via either selection or plasticity to optimize foraging, increase predator detection, etc. (Boughman 2001; Fuller et al. 2005; Cummings 2004; Rick et al. 2012; Ryan and Cummings 2013; Veen et al. in press). Alternatively, initial differences in perception could drive habitat preference via selection for different optical environments. Regardless of the mechanism, this covariation between perception and habitat could lead to microspatial variation in signal preference, creating depth gradients in male conspicuousness. Optical environment has been shown to influence both sensitivity (McDonald and Hawryshyn 1995; Rowe et al. 2004) and preference for red throats in threespine stickleback (Boughman 2001). Furthermore, opsin expression in stickleback is plastic (Flamarique et al. 2013; but see Rennison et al. 2016), and data from females from Gosling Lake show evidence for depth gradients in the expression of particular opsin pigments (Veen et al. in press). Our stickleback visual model assumes a constant relative proportion of cone pigments, and does not account for potential differences in the perceptual systems of female observers across depths. Future work employing more idiosyncratic visual models could better assess the degree to which male conspicuousness varies across nest depths as well as the role sensory drive may play in driving microspatial variation in male conspicuousness (Boughman 2002; Maan et al. 2006; Seehausen et al. 2008).

Predation pressure may also vary across nesting depth. Specifically, in the 12 lakes where male conspicuousness increased at greater nest depths, predation may be more intense in shallow-water environments favoring males that are more cryptic. Previous work has demonstrated elevated levels of predation in shallow versus deep water in a number of freshwater fish species (loricarriid catfishes: Power 1984; haplochromine cichlids: Maan et al. 2008; but see Rypel et al. 2007 and Bossu and Near 2015). In threespine stickleback, Reimchen (1980) provided evidence for spatial heterogeneity in predation mode, though not intensity, across limnetic versus littoral environments, with the former being dominated by avian predators, while benthic invertebrates were implicated as the primary predator in the latter. How this spatial variability translates across water depth, however, is unclear. Doucette et al. (2004) made qualitative assessments of predation pressure in Icelandic threespine stickleback from five sites across three lakes, finding that predation pressure was generally higher in deeper environs. Contrary to this, Brown and

Moyle (1997) found the presence of the piscivorous Sacramento squawfish (*Ptychocheilus grandis*) lead to shifts in habitat preference toward shallow water (where predation was less intense) in a number of freshwater fish, including threespine stickleback. These results suggest that while predation intensity may vary with depth, the nature of this relationship most likely varies across environments and/or predators. Preliminary data from a series of GoPro cameras placed along a depth gradient in Gosling Lake (see Bolnick et al. 2016) suggest that trout and bellastomatid density are somewhat higher in deep and shallow environments, respectively. However, these results are not significant (Fig. S2). Furthermore, as three of our 15 lakes show the opposite trend (i.e., deep males are more cryptic), higher predation in shallow environments cannot explain depth gradients in male conspicuousness across all of the lakes investigated. While we know very little about predation intensity in these 15 lakes, it is interesting to note that ΔL shows little microspatial variation across depth. This suggests that some mechanism may be in place to maintain luminance contrast within some optimal range (but see Cole and Endler 2015). Consistent with a role for predation in mediating ΔL, luminance contrast is often invoked as the primary channel employed in motion, shape, and texture perception, and thus of particular importance in prey detection by predator species (Osorio et al. 1999; Osorio and Vorobyev 2005; Stuart-Fox et al. 2006; Prudic et al. 2007; Pignatelli et al. 2010). Future work could not only more assiduously assess predation intensity at different depths, but also employ predator-specific visual models to properly assess male conspicuousness in a receiver-dependent manner (Crothers and Cummings 2013).

Finally, other potential factors could influence male conspicuousness across nest depths. Microspatial variation in immune function (Bolnick et al. 2015a) could potentially influence the expression of male color, as the two are interrelated in stickleback (Kurtz et al. 2007; Bolnick et al. 2015a). Spatial variation in stickleback density could mediate the nature and/or potential strength of sexual selection (Candolin and Salesto 2009), and the latter has been shown to vary across depth in Gosling Lake (Bolnick et al. 2015b). Finally, a more conspicuous signal is not necessarily more preferred (Gong and Gibson 1996). In addition to a signal's design, its content is also potentially important. Nuptial color in male stickleback is correlated with health (Milinski and Bakker 1990), condition (Frischknecht 1993; Boughman 2007), parental ability (Frischknecht 1993; Candolin 2000), immune function (Kurtz et al. 2007) and functional fertility (Pike et al. 2010). Assuming some minimal detection threshold is met, females may choose to mate with redder males, regardless of the overall conspicuousness of the signal, if they attain some direct (or indirect) benefit by doing so. Shallow males across seven of the nine lakes in which depth gradients in nuptial color are present reflect more orange/red than deeper nesting males, who reflect more UV/blue (Brock et al. in review). This could indicate that females prefer redder males when the local optical environment does not prevent its detection. However, as red coloration is increasingly obscured in males nesting in red-shifted optical environments, the latter may have no recourse aside from increasing the overall conspicuousness of their signal through more UV/blue reflectance. Our results suggest that optical background is a strong predictor of male conspicuousness and provide further support for this argument, as across lakes males from red-shifted environments were typically more conspicuous than males from blue-shifted environments. In situ mate choice trials, perhaps using model stickleback (e.g., Bolnick et al. 2016), could be employed to assess whether female detection and/or preference differs across depth. Additionally, nuptial color is also involved in intrasexual interactions in stickleback (Bakker and Sevenster 1983; McLennan and McPhail 1989; Baube 1997; Rick and Bakker 2008; but see Mckinnon and McPhail 1996), and thus a similar argument could be made that color-mediated, male–male agonistic interactions drive depth gradients in male conspicuousness. Intriguingly, previous evidence suggests that color not only mediates intrasexual aggression in threespine stickleback, but that this affect varies across optical environments and depths (Bolnick et al. 2016).

In conclusion, our results demonstrate that variation in male conspicuousness occurs over small spatial scales ($<$ 2 meters) in threespine stickleback, and provide another example of microgeographic trait variation in natural populations (Gray et al. 2008; Snowberg and Bolnick 2012; Richardson et al. 2014; Langin et al. 2015; Bolnick et al. 2015a; Brock et al. in review). Furthermore, the repeatable nature of these gradients, in both space and time, strongly imply they are adaptive and at least partially mediated by gradients in the local optical environment. These results also highlight the importance of phenotypic plasticity in producing this microspatial variation over abbreviated time scales, thus allowing males to adjust their conspicuousness in accordance with their nesting habitat. Finally, they highlight the importance of employing species-specific visual models when investigating signal evolution as these may provide interesting and surprising insights into the functional consequences of predictable associations between color and signaling environment.

AUTHOR CONTRIBUTIONS

C.D.B., D.I.B., and M.J.C. designed the study, C.D.B. conducted the study and collected the data. C.D.B., D.I.B., and M.J.C. conducted the analyses and wrote the article.

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CONFLICT OF INTERESTS

The authors declare no competing interests.

DATA ARCHIVING

The doi for our data is https://doi.org/10.5061/dryad.ct240.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Sample size (n), year sampled, and latitude and longitude for each study population of stickleback fish.
Table S2. Slopes and standard errors for $\Delta\Delta S \sim$ nest depth ($\text{Slope}_{\Delta\Delta S}$) and irradiance $\sim$ depth (Slopeirradiance).
Figure S1. Depth gradient in $\Delta\Delta S$ from wild-caught (solid black line) and experimental fish (dotted grey line).
Figure S2. The number of trout (a) and bellastomatid (b) visits to nests at a given depth for a two-hour period in Gosling Lake.
Figure S3. Sidewelling irradiance spectra for shallow (hatched, red) and deep (solid, light blue) treatment cages from the plasticity experiment compared with equivalent measurements from the wild.
Figure S4. A depth series of the median downwelling irradiance from Gosling Lake.
Figure S5. Depth gradients in sidewelling ambient light for Blackwater (hatched line, blue points) and Gosling Lake (solid line, red points).
Figure S6. Threespine stickleback are tetrachromats.
Figure S7. Spectral sensitivity curve for threespine stickleback.