OXFORD

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Color and behavior differently predict competitive outcomes for divergent stickleback color morphs

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Abstract

Our knowledge of how male competition contributes to speciation is dominated by investigations of competition between within-species morphs or closely related species that differ in conspicuous traits expressed during the breeding season (e.g. color, song). In such studies, it is important to consider the manner in which putatively sexually selected traits influence the outcome of competitive interactions within and between types because these traits can communicate information about competitor quality and may not be utilized by homotypic and heterotypic receivers in the same way. We studied the roles of breeding color and aggressive behaviors in competition within and between two divergent three-spine stickleback *Gasterosteus aculeatus* color types. Our previous work in this system showed that the switch from red to black breeding coloration is associated with changes in male competition biases. Here, we find that red and black males also use different currencies in competition. Winners of both color types performed more aggressive behaviors than losers, regardless of whether the competitor was of the same or opposite color type. But breeding color differently predicted competitive outcomes for red and black males. Males who were redder at the start of competition were more likely to win when paired with homotypic competitors and less likely to win when paired with heterotypic competitors. In contrast, black color, though expressed in the breeding season and condition dependent, was unrelated to competitive outcomes. Placing questions about the role of male competition in speciation in a sexual signal evolution framework may provide insight into the "why and how" of aggression biases and asymmetries in competitive ability between closely related morphs and species.

Key words: male competition, speciation, sexual signal, color, threespine stickleback, *Gasterosteus aculeatus*

Darwin (1859, 1871) reasoned that intrasexual selection would lead to differential fitness outcomes when males varied in traits that enhance success in competition for mates or mating resources (Darwin 1871; Simmons 2001). Indeed, male competition is an important determinant of reproductive success. This is particularly true in systems in which males compete for access to pools of mating resources such as harems of females or territories (e.g., LeBouf 1974; Alatalo et al. 1986), and when male competition and female choice jointly determine mating outcomes (as when the two act simultaneously or sequentially, and when mate choice is based on male dominance; Wong and Candolin 2005; Hunt et al. 2009). Despite this, there has been a strong and persistent bias in the sexual selection literature toward investigating the role of female choice, rather than male competition, in driving diversification of sexually selected traits (McCullough et al. 2016) and generating reproductive isolation (Panhuis et al. 2001; Seehausen and Schluter 2004; Qvarnström et al. 2012; Tinghitella et al. in press). This is likely because early sexual selection work focused on mate choice (the more controversial of Darwin's mechanisms of sexual selection; Anderson 1994), male ornaments are commonly correlated with female preferences and female sensory perception, implicating mate choice in ornament evolution (e.g. Boughman 2001; Mendelson and Shaw 2002; Rodriguez et al. 2006), and because there is a direct relationship between accepting or rejecting a mate and reproductive isolation (Qvarnström et al. 2012).

Evidence is accumulating in support of a role for male competition in speciation, however. For instance, recent work sheds light on how competitive phenotypes and competitor recognition coevolve (e.g., Martin and Mendelson 2016; Scordato 2017). Male competitive strategies (anything that leads to success in competition in a particular environment) can come to reflect variation in the ecological and social environments (predators and parasites, competitor densities, mating resources, habitat patchiness, and transmission properties of the environment, mate availability) with the result that well-adapted males can be socially dominant and gain access to mates. When divergent types come into contact, male competition researchers often find evidence of aggression biases (e.g., Dijkstra et al. 2006, 2007; Tyers and Turner 2013; Lackey and Boughman 2013; Lehtonen 2014; Tinghitella et al. 2015) and asymmetries in competitive ability (e.g., Owen-Ashley and Butler 2004; Dijkstra et al. 2005; Pryke 2009; Winkelmann et al. 2014; Martin et al. 2017). Each could contribute to reproductive isolation, though the consequences for hybridization and speciation are not well characterized. For instance, aggression biases may 1) facilitate co-existence of multiple morphs/species in sympatry through negative frequency dependent selection if aggression is directed toward homotypic males (Seehausen and Schluter 2004) or 2) lead to competitive exclusion (Hardin 1960) and habitat isolation if aggression is biased toward heterotypics (e.g., Peiman and Robinson 2007). Asymmetries in dominance can also facilitate habitat and reproductive isolation if one competitor is able to displace the other, compelling the latter to use less ideal habitat (Winkelmann et al. 2014) or drive local extinction of the lesser competitor. Previous work has nicely identified these patterns and their implications for speciation, but less attention has been paid to why those patterns occur from a sexual signal evolution (signalers/receivers) perspective. Here, we illuminate the "how and why" of aggression biases and asymmetries.

Many of the study systems in which the role of male competition in speciation has been addressed are systems in which alternate color morphs are maintained within species (e.g. Kissenda Island cichlids (Dijkstra et al. 2005; Dijkstra et al. 2009), sticklebacks (Tinghitella et al. 2015; Bolnick et al. 2016), European wall-lizards (While et al. 2015), or in which color is a primary phenotype that differs between closely related species [e.g., lake Victoria cichlids (Seehausen and Schluter 2004); Nicaraguan cichlids (Lehtonen et al. 2015); darters (Martin and Mendelson 2016; Roberts and Mendelson 2017); rubyspot damselflies (Anderson and Grether 2010a, 2010b), limnetic-benthic stickleback species pairs (Lackey and Boughman 2013; Keagy et al. 2016)]. In a handful of these systems, variation in the sensory environment is implicated in color shifts (Reimchen 1989; Boughman 2001; Scott 2001; Maan et al. 2006; Seehausen et al. 2008). Environment-associated differences in color can influence male aggressive responses to heterotypic competitors (e.g., Macedonia et al. 1994; Macedonia and Stamps 2010; Macedonia et al. 2013, 2015; Tinghitella et al. 2015; Bolnick et al. 2016), as identifying appropriate competitors is a first step in competitive interactions. If agonistic color signal evolution is hypothesized to contribute to reproductive isolation, it is important to understand how divergent colors function in male competition (the extent to which and which components of putative signals dictate competitive outcomes) and whether the relationship between signals and competitive outcomes depends on the competitor (receiver) type.

We tested whether two threespine stickleback *Gasterosteus aculeatus* color types have diverged in the manner in which male-limited breeding color is associated with male competition

outcomes. The study system is one in which male competition interacts with female choice to influence reproductive success and male color functions in both male competition and female choice contexts (e.g., Candolin 1999). During the breeding season, most male threespine sticklebacks *Gasterosteus aculeatus*, including the marine ancestors of the freshwater fish studied here, express a carotenoid-based red throat that can extend from the mouth to the pelvic spines. The red throat is strongly preferred by females in mate choice because it is correlated with health, condition, and parental care ability (e.g., Milinksi and Bakker 1990; Frischknecht 1993; Candolin 2000; Boughman 2007; Kurtz et al. 2007) and is also used in male competition (reviewed in Bakker and Sevenster 1983; McLennan and McPhail 1989; Rowland 1994; Rowland et al. 1995; Bolyard and Rowland 1996; McKinnon 1996; Baube 1997; Rick and Bakker 2008; but see McKinnon and McPhail 1996). Male threespine sticklebacks compete with one another for breeding territories and males must have a territory and a nest built *before* they can court potential mates (Tinbergen and van Iersel 1947; van Iersel 1953), so male competition is an important determinant of reproductive success. Females then search and choose amongst nesting males, and males subsequently provide all parental care. In some studies, brighter red males are more aggressive (Rowland 1984; McLennan and McPhail 1989) and more likely to win in dominance tests (Bakker and Sevenster 1983; McKinnon 1996; Baube 1997). The dominance status of a male may also reflect his parental ability (McKinnon 1996; Candolin 2000). Males frequently destroy one another's nests, and conspecifics eat and steal eggs regularly (Wootton 1976), so dominance likely influences success in parenting.

In several locations across the west coast of North America, male sticklebacks no longer display red nuptial coloration and instead have full-body black coloration (McPhail 1969; Moodie 1972; Reimchen 1989; von Hippel 1999; Boughman 2001; Catchen et al. 2013; Bolnick et al. 2016). Black color is expressed during the breeding season and is condition-dependent (Scott 2011), suggesting a sexual selection function. The best-supported explanation for the switch from red to black coloration is sensory drive, which posits that signals and sensory systems coevolve to maximize signal detectability in a given habitat (Endler 1992; Boughman 2002; Maan and Seehausen 2011). Black populations are typically found in locations with red-shifted water color, whereas red populations are found in clear water (Reimchen 1989; Boughman 2001; Scott 2001; Tinghitella, unpublished data). Habitats with red-shifted and full-spectrum light within each drainage are not separated by physical barriers and there are documented polymorphic regions where red and black fish interact (Hagen and Moodie 1979). The difference in coloration between red and black males affects male competitive behavior in both regions where it has been investigated (Tinghitella et al. 2015; Bolnick et al. 2016). In simulated mixed color assemblages in the lab, black males from Washington state (WA) rivers biased their aggression toward males with red nuptial coloration, whereas red males show no bias in aggressive behavior (Tinghitella et al. 2015). This pattern of aggression (bias toward heterotypic males) suggests that red males are the recipients of more aggression overall which could allow black males to exclude red males from preferred breeding sites (e.g., Adams 2004; Peiman and Robinson 2007; Vallin and Qvarnström 2011; Winkelmann et al. 2014; Lehtonen et al. 2015), enhancing habitat use differences. However, our understanding of how male competition might contribute to genetic divergence (through male competition outcomes) in this

system has been limited by not knowing whether black nuptial coloration itself is related to male competition outcomes.

We conducted dyadic within and between color morph male competition trials to determine whether and how red and black breeding color contribute to competition outcomes. Previous work also supports a possible relationship between measurements of male aggressiveness (performing more aggressive behaviors) and territory establishment in threespine stickleback males with red coloration (Bakker and Sevenster 1983), so we also tested for a role of aggressive behaviors. We predicted that red throat coloration would predict competitive outcomes, with males who have a larger proportion body coverage of red winning more territories (Bakker and Sevenster 1983; Baube 1997). Given that black body coloration likely evolved as a consequence of sensory drive, is conspicuous to the stickleback visual system (Boughman 2001), and is condition-dependent (Scott 2011), we also predicted that melanin-based black body coloration would predict competitive outcomes, with more melanic males winning territories. There is reason to believe this may be the case. Melanin-based coloration is correlated with increased aggression and sexual activity in a wide variety of vertebrates through pleiotropic effects of the melanocortin system (reviewed in Ducrest et al. 2008). However, if signals and competitor recognition have co-evolved within color types, we predict that body color (red or black) and/or the number of aggressive competitive behaviors will be differentially associated with competition outcomes in homotypic versus heterotypic interactions and that more homotypic than heterotypic trials will end with territory establishment (assuming homotypic competitors have mutually "understood" signals).

Materials and Methods

Competition trials

We collected sexually mature sticklebacks in regions of WA where red and black fish are allopatric and transferred them to the University of Denver by air in summers of 2014–2017. Fish with red nuptial coloration were collected from the Chehalis River (46°56′22″N, 123°18′46″W) and Campbell Slough (47°2′40″N, 124°3′33″ W), and fish with black nuptial coloration were collected from Vance Creek (46°59′48″N, 123°24′43″W), Scatter Creek (46°49′20″N, 123°3′11″W), Conner Creek (47°4′10″N, 124°9′58″W), and Black River (46°49′45″N, 123°8′1″W). We housed fish in visually isolated 110-L tanks (77 cm × 32 cm × 48 cm) separated by collection location and sex at densities of no more than 30 fish per tank. Tanks were housed in a temperature and photoperiod controlled room set to 17 °C and a 15: 9-h light: dark at the beginning of the season. Temperature and photoperiod conditions tracked those in the field for the remainder of the season. We fed the fish a mixture of frozen bloodworms (*Chironomus* spp.) and brine shrimp (*Artemia* spp.) daily *ad libitum*.

We conducted dyadic competition trials between two fish with the same nuptial coloration and between two fish with different nuptial coloration. In the field, the red populations we worked with are typically found in full-spectrum light and the black populations are found in red-shifted light environments. All trials were conducted under full spectrum lighting because our previous work with these fish revealed no differences in competitive or mate choice biases under red-shifted light in the lab (Tinghitella et al. 2015). Trials followed established methods (Bakker and Sevenster 1983; Rowland 1989) wherein two size-matched sexually mature males were introduced to a tank (77 cm × 32 cm × 48 cm) and allowed to compete for a single nesting space (in this case, a sand-filled

tupperware container 19 cm × 12 cm × 2 cm). Larger mass provides a competitive advantage for red sticklebacks (Rowland 1989), so we controlled for this by pairing males that differed in mass by no more than 10% at the start of the trial. Males were given two days from introduction into the tank to interact and to establish a territory and/or build a nest. This time period was chosen because male sticklebacks begin to build nests within two days of introduction to a new nesting area when alone, and must have an established territory in order to build a nest (Tinbergen and van Iersel 1947; van Iersel 1953). Twenty-four hours and 48 h after introduction to the competition tanks, we presented competing males with a gravid female in a clear glass jar for 10 min near the nesting site (following Lackey and Boughman 2013). This reinforced that sexually mature females were present and motivated males to compete and build a nest. We also recorded whether males had established a territory, and inspected the sand substrate for signs of nest-building. When a territory has been established, the losing male becomes restricted to a small area of the tank, and upon moving toward the territory edge is attacked by the dominant fish (Rowland 1989). Forty-eight hours after introduction to the competition tank and immediately after experience with the gravid female, we observed the competing males for 20 min and recorded all aggressive behaviors (approaches, bites, and chases) using the event recorder JWatcher (Blumstein et al. 2006). During approaches a male swims, sometimes rapidly, towards the other, often at territorial boundaries, coming within one body-length. These have variously been described as "charges" in the literature as well. Bites can range in intensity from nips to sustained biting. Chases occur when one male swims vigorously behind the other (within one body-length), and can involve movement across the length of the tank multiple times.

Color quantification

To measure nuptial color, we photographed fish immediately before placing them into competition tanks. For 187 of the 195 competition trials, we also photographed males immediately after the 20-min focal observation period. Red breeding color is quite plastic, including during competitive interactions between males (Candolin 1999, personal observation). Photographing males before and after competition trials allowed us to capture the starting conditions *and* changes in body color that might occur during the course of the trial. All photographs were taken with a digital camera (Canon PowerShot G15) under standardized lighting (four evenly spaced xenon 20 W bulbs) inside a photobox that held the camera and blocked ambient light. In each photo the fish was on its right side, unanesthetized, against a neutral background with a millimeter ruler in view for scale. The process was brief, minimizing the potential for color change and stress.

Using FIJI (Schindelin et al. 2012), we quantified red and black coloration as a proportion of total body area from each photo (Figure 1). To obtain red color area, we selected red coloration using the Threshold Color plugin (Y = 32–255, U = 0–143, V = 141–255; following Wong et al. 2007). To obtain black area, we selected black coloration using the Threshold Color plugin (Y = 0–20; U = 0–255; V = 0–255). We determined total body area using the SIOX: Simple Interactive Object Extraction and scaled all measured areas using the millimeter ruler in each photo.

Statistical analysis

We assessed the effects of proportion red body color and aggressive behaviors of the focal red male on competitive outcomes in dyadic
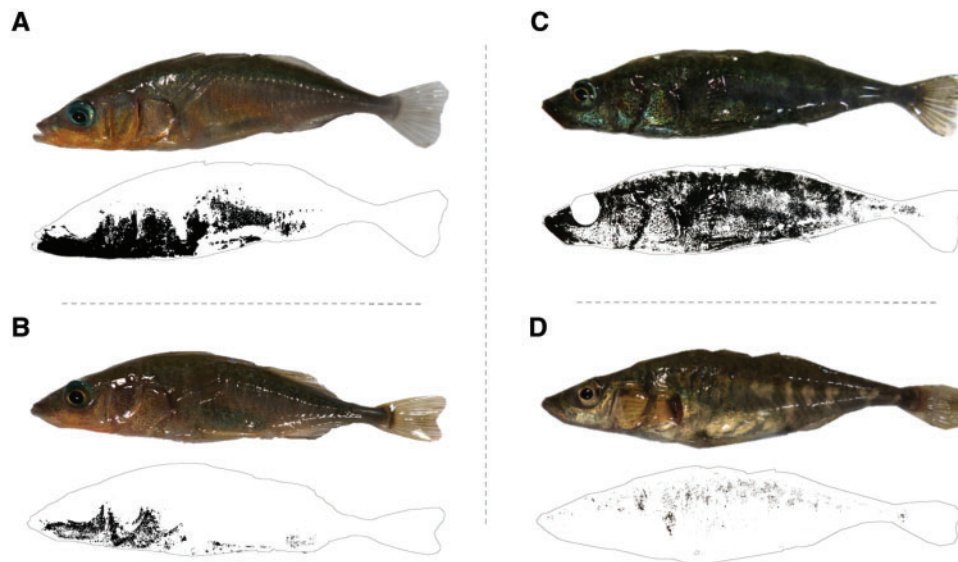
Figure 1. Quantification of red and black body color using ImageJ. Panels A and B show representative red males with a high (A) and low (B) proportion coverage of red coloration. Panels (C) and (D) show representative black males with a high (C) and low (D) proportion coverage of black coloration.

trials between two red competitors and between one red and one black male. A focal red male was chosen randomly within each red × red trial for analyses. To examine territory establishment by red focal males, we ran two sets of models: one using "before-competition" red area and the other using difference in red area, calculated from before and after competition photos, as measures of male color. "Before-competition" red area captures color at the start of competition trials and the difference in red area (after competition − before competition) captures color change. We also ran a model using "after" red area as a predictor variable, which we provide in the Appendix. To examine the effect of aggressive behaviors on competitive outcomes, we combined approaches, bites, and chases using PCA as a variable reduction technique. All factors loaded evenly onto PC1, which explained 70.2% of the variation. We tested the effects of red color and behavior on competition outcomes using logistic regression via a generalized linear mixed model (family = binomial) in the lme4 package (version 1.2−12) in R (version 3.3.1; RStudio version 1.0.143; Bates et al. 2017). The "before-competition" model included before red color area, aggressive behaviors (PC1-behaviors), trial type (homotypic or heterotypic), and the interactions between color and trial type and between aggressive behaviors (PC1-behaviors) and trial type as independent variables. Trial outcome [whether or not the focal (red) male established a territory] was the dependent variable. The model also included the difference in mass between the two males in a trial as a covariate and the source populations of the red and black fish as random effects. The difference in red area model had the same structure. "Before-competition" color measurements were square-root-transformed prior to analysis to meet assumptions of normality.

We assessed the effects of proportion black body color and aggressive behaviors of the focal black male on competitive outcomes in dyadic trials between two black competitors and between one red and one black male. Again, a focal black male was chosen at random within each black × black trial for analyses. We performed a parallel analysis for focal black males using "before-competition" black area and difference in black area (after competition − before competition) as our measures of male color in separate models. Again, we also ran a model using only "after" black color area

which we discuss in the Appendix. The measure of focal male aggressive behavior was again PC1-behaviors from the PCA described above and the model structure was as described above.

For all models, we followed a model comparison method in which the full models were compared to reduced models using χ^2 tests (performed using the anova function in lme4 in R) to assess whether the fit of the model decreased significantly when the effects of interest were systematically removed (Winter 2013). For interaction effects, we plotted logistic curves using geom_smooth (method = "glm", family = "binomial") in the ggplot 2 package (version 2.2.1) in R (Wickham 2017) and for fixed effects, we plotted logistic curves using sjp.glmer in the sjPlot package (version 2.3.3) in R (Lüdecke and Schwemmer 2017).

Results

When competing with homotypic competitors (other red males), males with greater "before" red area were more likely to establish a territory, but when competing with heterotypics (black males) red males with less "before" red body area were more successful in establishing a territory (A in Table 1 and Figure 2A). Red males were also more likely to establish a territory, regardless of trial type, when they performed more aggressive behaviors. We found no interaction between behaviors and trial type and no effect of trial type alone or "before" red area alone on whether red focal males established a territory (A in Table 1).

The difference in red area (after competition − before competition) was also associated with territory establishment (B in Table 1 and Figure 3A). Males who established a territory gained more red body area during competition, regardless of trial type. As above, red males that successfully established a territory performed more aggressive behaviors (Figure 3B). We found no interaction between difference in red and trial type, no interaction between behaviors and trial type, and no effect of trial type alone on whether red focal males established a territory (B in Table 1). Because the relationships between "before" red color and territory establishment and difference in red color and territory establishment were different (see A versus B in Table 1), we also investigated the relationship between
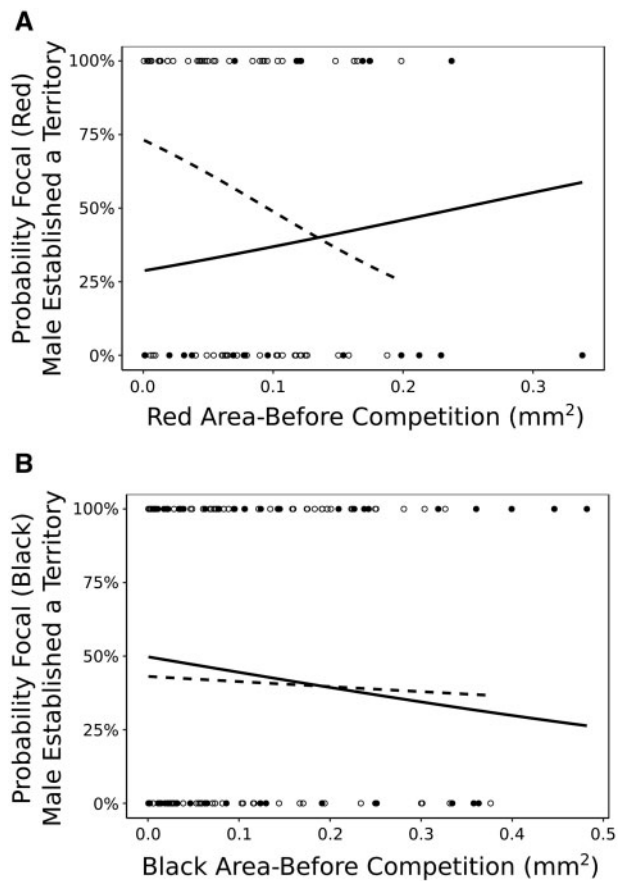
Figure 2. Dependence of territory establishment on the interaction between "before-competition" color area and competitor type. Panel A shows the outcomes of competition for red focal males and B shows the outcomes for black focal males. In both panels, closed circles and solid lines indicate outcomes in homotypic competition [other red males (A) or other black males (B)] and open circles and dashed lines indicate outcomes in heterotypic competition [black males (A) and red males (B)].

"before" red color and difference in red color. Males that entered a competition trial with less red body area (before) gained more red coloration than males who entered a competition trial with more red body area ($\chi^2_1 = 65.7$, $P < 0.0001$; mixed effects linear model with red focal male population = random).

In contrast to red body area, and contrary to our hypotheses, we found no effect of "before" black body area or the difference in black body area (after competition − before competition) on territory establishment by black males, regardless of competitor type (C and D in Table 1; Figure 3C). The only measured variable significantly related to the likelihood of a black male establishing a territory was aggressive behaviors; black males performed more aggressive behaviors when successfully establishing a territory (Figure 3D). The interactions between "before" black body area and trial type and difference in black area and trial type on territory establishment were non-significant. Trial type alone did not affect territory establishment in models using either black measurement (C and D in Table 1).

Of 195 male competition trials, 148 (∼76% overall) ended with a clear winner who had established a territory. A total of 20 of 23 red × red trials, 51 of 74 black × black trials, and 77 of 98 red × black trials ended with territory establishment. The proportion of trials ending with a territory did not differ among the three trial

Table 1. Color measures and aggressive behaviors associated with territory establishment by focal red or black males in heterotypic and homotypic competition trials

		χ^2	df	P
Red focal males				
A	Before red area * trial type	4.58	1	0.03
	Aggressive behaviors * trial type	1.41	1	0.24
	Before red area	1.28	1	0.26
	Aggressive behaviors	13.7	1	<0.001
	Trial type	0.12	1	0.73
B	Difference in red * trial type	0.57	1	0.45
	Aggressive behaviors * trial type	0.76	1	0.38
	Difference in red	3.97	1	0.046
	Aggressive behaviors	15.01	1	<0.001
	Trial type	0.11	1	0.74
Black focal males				
C	Before black area * trial type	0.29	1	0.59
	Aggressive behaviors * trial type	0.33	1	0.57
	Before black area	1.64	1	0.20
	Aggressive behaviors	49.95	1	<0.0001
	Trial type	0.01	1	0.94
D	Difference in black * trial type	1.28	1	0.26
	Aggressive behaviors * trial type	1.37	1	0.24
	Difference in black	0.85	1	0.36
	Aggressive behaviors	51.29	1	<0.0001
	Trial type	0.12	1	0.73

types ($\chi^2_1 = 3.89$, $P = 0.14$). There was a trend toward a greater proportion of red × red trials than black × black trials ending with territory establishment, but this was not significant ($\chi^2_1 = 2.91$, $P = 0.09$). Males performed 135% more aggressive behaviors (PC1-behaviors) in trials in which a territory was established (LS means ± SE: territory = 0.19 ± 0.20, no territory = −0.54 ± 0.30; $\chi^2_1 = 5.13$, $P = 0.02$), but the number of aggressive behaviors performed during competition did not differ across trial types (LS means ± SE; red × red = -0.33 ± 0.48, red × black = 0.25 ± 0.23, black × black = −0.09 ± 0.26; $\chi^2_2 = 2.18$, $P = 0.34$). Neither color type was more likely to win in heterotypic competition (red wins = 42, black wins = 35, $\chi^2_1 = 0.46$, $P = 0.50$), and both red and black focal males performed similar numbers of aggressive behaviors (LS means ± S.E.; red males = −0.33 ± 0.49, black males = 0.10 ± 0.21; $\chi^2_1 = 0.861$, $P = 0.35$).

Discussion

In this study, we first asked whether male nuptial coloration and aggressive behavior function differently in the competitive interactions of two diverging stickleback color types, one with red nuptial coloration (the ancestral type; McLennan 1996) and one with black nuptial coloration (the derived type). Overall, we found that different combinations of traits influence the outcome of dyadic competitive interactions involving red and black males. Males of both color types who performed more aggressive behaviors were more likely to establish a territory, regardless of whether the competitor was homotypic or heterotypic (Figure 3B, D; Table 1). But, whereas competition outcomes for red males varied with "before-competition" red body area and the degree to which red body area changed during competition (difference in red area), black body area was
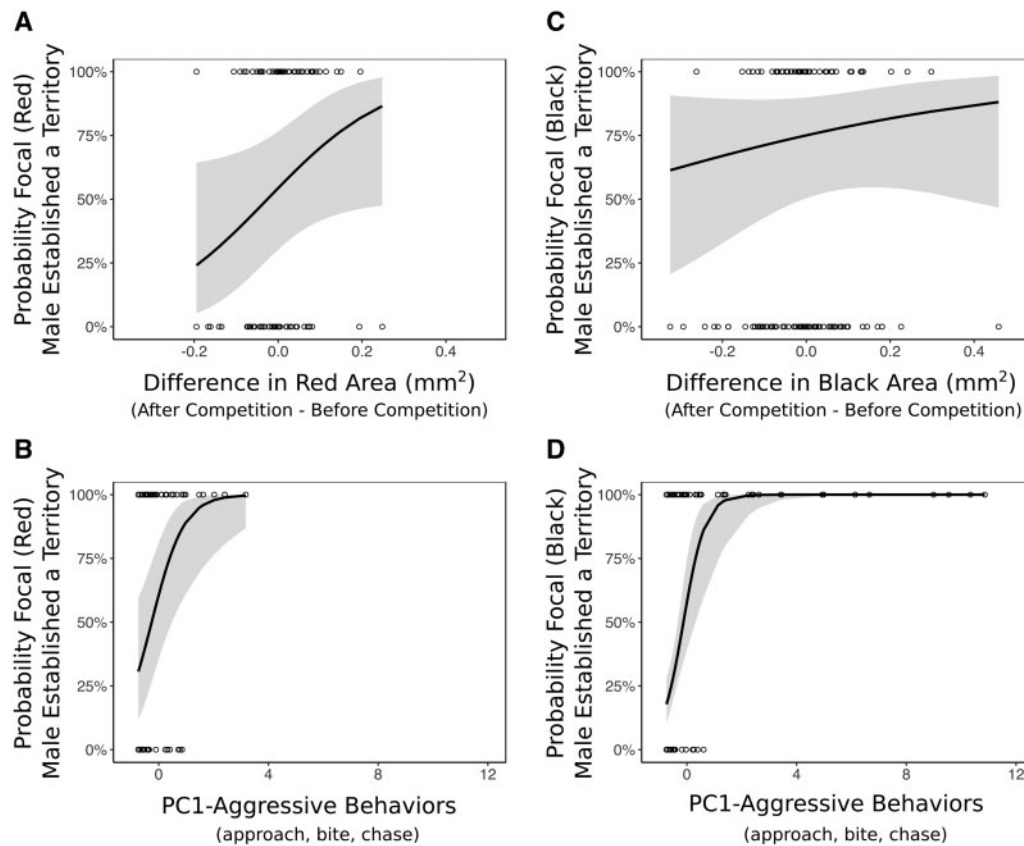
Figure 3. Difference in red area and aggressive behavior during competition correlate with territory establishment by red males, but only aggressive behaviors vary with territory establishment by black males. Pictured are the effects of A) difference in red area and B) PC1-behaviors of red focal males on probability of establishing a territory and the effects of C) difference in black area and D) PC1-behaviors of black focal males on the probability of establishing a territory (from the logistic regression). Increasing values of PC1-aggressive behaviors mean more behaviors were performed. Shading indicates the 95% confidence intervals, and circles represent individual focal male scores.

unrelated to competitive success (Table 1; Figure 3A, C). This is contrary to our original hypothesis that condition-dependent (Scott 2011) melanism expressed during the breeding season is sexually selected and that more melanic males would win territories, but supports the idea that competitive strategies (anything that leads to success in competition in a particular environment) of red and black males have diverged. Divergent competitive strategies may lead to aggression biases and asymmetries upon secondary contact.

What, then, is the function of black coloration, if any? Several authors have suggested that black coloration was favored through sensory drive and increases male conspicuousness in red-shifted light habitats (Boughman 2001; Scott 2001; Boughman 2002). Black coloration may function in female choice, as suggested in some work (Scott 2004; but see Tinghitella et al. 2015 and below). Other evidence suggests that black is maximally expressed during care for eggs and fry (Scott 2004) and thus may signal parental care ability to female conspecifics (Scott 2011) and/or act as a threat to egg predators or conspecific egg thieves (McPhail 1969; Hagen et al. 1980; but see Scott and Foster 2000). Alternatively, black coloration may not be a signal at all, but instead have been "pulled along" by selection on another trait (Ducrest et al. 2008). Experiments in which black body coloration is manipulated directly to assess effects on sexual selection or success in parental care would reveal whether black coloration is a signal and, if so, of what.

We also asked whether color or aggressive behavior varied with competitive outcomes differently when the competitor is homotypic versus heterotypic, and whether competition between homotypic males was more likely to be resolved than competition between heterotypic males. First, we found no evidence that homotypic trials were resolved more often that heterotypic trials, though there was a trend toward more red–red than black–black trials ending with a territory holder. In Pacific field crickets that have lost the ability to produce an aggressive song that mitigates the costs of fighting, fights involving one or more silent males are more physically aggressive than those involving two calling males (Logue et al. 2010). Fights between two threespine stickleback males who do not have the ancestral red threat signal did not involve more physically aggressive behaviors. However, red males with greater "before-competition" red color were more likely to establish a territory in homotypic red x red trials (consistent with previous work in threespine sticklebacks; Bakker and Sevenster 1983; Baube 1997), and the same trait (extensive red color) led to reduced success in competition with heterotypic (black) males (A in Table 1; Figure 2A). The relationship between black color (either measure) and competitive outcomes did not differ depending on whether a male faced a homotypic or heterotypic competitor (C and D in Table 1; Figure 2B). We interpret this to mean that the ancestral color, which does function in competition among red males, does not influence competitive outcomes with the derived color type in the same way—competitive strategies have diverged and the relationship between color and competitive outcomes differs depending on the reciever. This supports the hypothesis that signals and competitor recognition have co-evolved within color types.

In a previous study, we found that in mixed sex assemblages in the lab, black males biased their aggressive behaviors toward red males, while red males had no aggression bias (Tinghitella et al. 2015). The aggression bias we observed previously and difference in competition currencies (which traits are correlated with success in competition) uncovered here are likely related. For instance, less red (dull) males may be more successful in competition with black males because black males bias aggression toward red competitors. Said another way, if black males bias aggression toward red males with greater red body area, it would be reasonable to expect males with greater red body area to lose in one on one competitive interactions with black males, relative to dull red males (as we find here).

In the mixed sex assemblage experiment described above, we found no evidence that the preferences of females from red and black populations had diverged; all females, regardless of color type preferentially directed courtship interest behaviors toward red males (Tinghitella et al. 2015; but see Scott 2004). Ongoing no-choice courtship trials in the lab support this pattern (R.T., unpublished data). It is relatively straightforward to imagine how male competition and female choice might act in concert to drive speciation, for instance, if both favor the same alternative male trait combinations or females choose mates on the basis of competition outcomes. If females retain a preference for ancestral type males, however, there must be some other mechanism contributing to reproductive isolation.

To illuminate whether male competition contributes to reproductive isolation in this system, we suggest several lines of inquiry. It will be critical to know, for instance, whether the patterns we have uncovered in the lab also exist in the field. Are red males the recipients of more aggression *in the field*, and does this pattern lead red males to nest in less preferred habitat, reinforcing isolation? Are competitive outcomes and female preferences environment-dependent, varying, for instance, with light environment? If competitor types do differ in habitat use, natural selection can further differentiate competitive phenotypes and selection against migrants and hybrids might facilitate ecological specialization and speciation. Where the color types co-occur (in regions of polymorphism or upon secondary contact), black males biasing aggression toward red males should lead to a pattern whereby black males' nearest neighbors are other black males or "dull" red males, rather than red males with extensive red coloration. This could be easily observed and tested *in situ*. However, while black males that nest adjacent to homotypics may enjoy a fitness advantage if they lose fewer mating opportunities to red males who are preferred by female conspecifics, it is unclear whether nesting near "dull" red males would reinforce divergence between the color types. Further, comparing and contrasting competitor recognition and competitive outcomes between red and black sticklebacks from regions of their distribution where the two color types are allopatric versus sympatric would be particularly revealing. Where the two color types commonly co-occur, for instance, we might expect to find greater divergence in aggressive signals and competitive responses relative to allopatric populations, such that heterotypic competition is rare (e.g., Anderson and Grether 2010a, 2010b). Finally, future experiments similar to those conducted here, but with fish from regions where red and black males interact frequently would reveal whether competitors who commonly interact easily establish dominance through mutually "understood" signals.

Author contributions

RMT conceived of and designed the experiment. RMT and VFL conducted male competition trials. VFL quantified color from photos with undergraduate assistants, using methods developed by WRL. WRL analyzed the data and made the figures. All co-authors contributed to writing and editing the manuscript.

References

Adams DC, 2004. Character displacement via aggressive interference in Appalachian salamanders. *Ecology* 85:2664–2670.

Alatalo RV, Lundberg A, Glynn C, 1986. Female pied flycatchers choose territory quality and not male characteristics. *Nature* 323:152–153.

Anderson CN, Grether GF, 2010a. Character displacement in the fighting colours of *Hetaerina* damselflies. *Proc R Soc Lond B* doi: 10.1098/rspb.2010.0935

Anderson CN, Grether GF, 2010b. Interspecific aggression and character displacement of competitor recognition in Hetaerina damselflies. *Proc R Soc Lond B* 277:549–555.

Bakker TCM, Sevenster P, 1983. Determinants of dominance in male sticklebacks *Gasterosteus aculeatus* L. *Behavior* 86:55–71.

Bates D, Maechler M, Bolker B, Walker S, 2017. Package "lme4". Linear mixed-effects models using Eigen and S4. Available from: https://github.com/lme4/lme4/.

Baube CL, 1997. Manipulations of signaling environment affect male competitive success in three-spined sticklebacks. *Anim Behav* 53:819–833.

Blumstein DT, Evans CS, Daniel JC, 2006. Jwatcher v. 1.0 [Internet] [cited 2017 July 25]. Available from: www.jwatcher.ucla.edu.

Bolnick DL, Hendrix K, Jordan LA, Veen T, Brock CD, 2016. Intruder colour and light environment jointly determine how nesting male stickleback respond to simulated territorial intrusions. *Biol Lett* 8:20160467.

Bolyard KJ, Rowland WJ, 1996. Context-dependent response to red coloration in stickleback. *Anim Behav* 52:923–927.

Boughman JW, 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* 411:944–948.

Boughman JW, 2002. How sensory drive can promote speciation. *Trends Ecol Evol* 17:571–577.

Boughman JW, 2007. Condition dependent expression of red color differs between species. *J Evol Biol* 20:1577–1590.

Candolin U, 1999. Male-male competition facilitates female choice in sticklebacks. *Proc Biol Sci* 266:785–789.

Candolin U, 2000. Changes in expression and honesty of sexual signaling over the lifetime of sticklebacks. *Proc R Soc Lond B* 267:2425–2430.

Catchen J, Bassham S, Wilson T, Currey M et al. 2013. The population structure and recent colonization history of Oregon threespine stickleback determined using restriction-site associated DNA sequencing. *Mol Ecol* 22:2864–2883.

Darwin CR, 1859. *On The Origin of Species.* London: John Murray.

Darwin CR, 1871. *The Descent of Man, and Selection in Relation to Sex.* London: John Murray.

Dijkstra PD, Seehausen O, Groothuis TG, 2005. Direct male-male competition can facilitate invasion of new colour types in Lake Victoria cichlids. *Behav Ecol Sociobiol* **58**:136–143.

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.

Dijkstra PD, Seehausen O, Pierotti MER, Groothuis TGG, 2007. 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.

Dijkstra PD, Hemelrijk CK, Seehausen O, Groothuis TGG, 2009. Colour polymorphism intrasexual selection in assemblages of cichlid fish. *Behav Ecol* **20**:138–144.

Ducrest A, Keller L, Roulin A, 2008. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends Ecol Evol* **23**:502–510.

Endler JA, 1992. Signals, signal conditions, and the direction of evolution. *Am Nat* **139**:S125–S153.

Frischknecht M, 1993. The breeding colouration of male three-spined sticklebacks *Gasterosteus aculeatus* as an indicator of energy investment in vigour. *Evol Ecol* **7**:439–450.

Grether GF, Anderson CN, Drury JP, Kirschel ANG, Losin N et al., 2013. The evolutionary consequences of interspecific aggression. *Ann NY Acad Sci* **1289**:48–68.

Hagen DW, Moodie GEE, 1979. Polymorphism for breeding colors in *Gasterosteus aculeatus* I. Their genetics and geographic distribution. *Evolution* **33**:641–648.

Hagen DW, Moodie GEE, Moodie PF, 1980. Polymorphism for breeding colors in *Gasterosteus aculeatus* II. Reproductive success as a result of convergence for threat display. *Evolution* **34**:1050–1059.

Hardin G, 1960. The competitive exclusion principle. *Science* **131**: 1292–1297.

Heathcote RJP, While GM, Macgreagor HEA, Sciberras J, Leroy C et al. 2016. Male behavior drives assortative reproduction during the initial stage of secondary contact. *J Evol Biol* **29**:1003–1015.

Hunt J, Breuker CJ, Sadowski JA, Moore AJ, 2009. Male-male competition, female mate choice and their interaction: determining total sexual selection. *J Evol Biol* **22**:13–26.

Keagy J, Lettieri L, Boughman JW, 2016. Male competition fitness landscapes predict both forward and reverse speciation. *Ecol Lett* **19**:71–80.

Kurtz J, Kalbe M, Langefors A, Mayer I, Milinski M et al. 2007. An experimental test of the immunocompetence handicap hypothesis in teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. *Am Nat* **170**:509–519.

Lackey ACR, Boughman JW, 2013. Divergent sexual selection via male competition: ecology is key. *J Evol Biol* **26**:1611–1624.

LeBouf BJ, 1974. Male-male competition and reproductive success in elephant seals. *Amer Zool* **14**:163–176.

Lehtonen TK, 2014. Colour biases in territorial aggression in a Neotropical cichlid fish. *Oecologia* **175**:85–93.

Lehtonen TK, Sowersby W, Wong BB, 2015. Heterospecific aggression bias towards a rarer colour morph. *Proc R Soc B* **282**: 20151551.

Logue DM, Abiola IO, Rains D, Bailey NW et al. 2010. Does signalling mitigate the cost of agonistic interactions? A test in a cricket that has lost its song. *Proc R Soc Lond B* **277**: 2571–2575.

Lüdecke D, Schwemmer C, 2017. Package "sjPlot". Data Visualization for Statistics in Social Science. Available from: https://github.com/strengejacke/sjPlot/.

Maan ME, Hofker KD, van Alphen JJ, Seehausen O, 2006. Sensory drive in cichlid speciation. *Am Nat* **167**:947–954.

Maan ME, Seehausen O, 2011. Ecology, sexual selection and speciation. *Ecol Lett* **14**:591–602.

Macedonia JM, Evans CS, Losos JB, 1994. Male anolis lizards discriminate video–recorded conspecific and heterospecific displays. *Anim Behav* **47**: 1220–1223.

Macedonia JM, Stamps JA, 2010. Species recognition in *Anolis grahami* (Sauria, Iguanidae): evidence from responses to video playbacks of conspecific and heterospecific displays. *Ethology* **98**:246–264.

Macedonia JM, Clark DL, Riley RG, Kemp DJ, 2013. Species recognition of color and motion signals in *Anolis grahami*: evidence from responses to lizard robots. *Behav Ecol* **24**:846–852.

Macedonia JM, Clark DL, Brown ZN, Gensterblum S, McNabb L et al., 2015. Responses of *Anolis grahami* males to manipulations of species identity and components of displays in lizard robots. *Herpetologica* **71**: 110–116.

Martin MD, Mendelson TC, 2016. Male behavior predicts trait divergence and the evolution of reproductive isolation in darters (Percidae: *Etheostoma*). *Anim Beh* **112**:179–186.

Martin PR, Freshwater C, Ghalambor CK, 2017. The outcomes of most aggressive interactions among closely related bird species are asymmetric. *PeerJ* **5**:e2847.

Mendelson TC, Shaw KL, 2002. Genetic and behavioral components of the cryptic species boundary between *Laupaula cerasina* and *L. kohalensis* (Orthoptera: gryllidae). *Genetica* **116**:301–310.

McCullough EL, Miller CW, Emlen DJ, 2016. Why sexually selected weapons are not ornaments. *Trends Ecol Evol* **31**:742–751.

McKinnon JS, 1996. Red coloration and male parental behaviour in the threespine stickleback. *J Fish Biol* **49**:1030–1033.

McKinnon JS, McPhail JD, 1996. Male aggression and color in divergent populations of threespine stickleback: experiments with animations. *Can J Zool* **74**:1727–1733.

McLennan DA, 1996. Integrating phylogenetic and experimental analyses: the evolution of male and female nuptial coloration in the stickleback fishes (Gasterosteidae). *Syst Biol* **45**:261–277.

McLennan DA, McPhail JD, 1989. Experimental investigations of the evolutionary significance of sexually dimorphic nuptial coloration in the *Gasterosteus aculeatus* L.: the relationship between male color and male behavior. *Can J Zool* **67**:1778–1782.

McPhail JD, 1969. Predation and the evolution of a stickleback (*Gasterosteus*). *J Fish Res Board Can* **26**:3183–3208.

Milinski M, Bakker TCM, 1990. Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature* **344**:330–333.

Moodie GEE, 1972. Morphology, life history, and ecology of an unusual stickleback *Gasterosteus aculeatus* in the Queen Charlotte Islands, Canada. *Can J Zool* **50**:721–732.

Owen-Ashley NT, Butler LK, 2004. Androgens, interspecific competition and species replacement in hybridizing warblers. *Proc R Soc B Biol Sci* **271**: S498–S500.

Panhuis TM, Butlin R, Zuk M, Tregenza T, 2001. Sexual selection and speciation. *Trends Ecol Evol* **16**:364–371.

Peiman KS, Robinson BW, 2007. Heterospecific aggression and adaptive divergence in brook stickleback *Culaea inconstans*. *Evolution* **61**: 1327–1338.

Pryke SR, 2009. Is red an innate or learned signal of aggression and intimidation?. *Anim Beh* **78**:393–398.

Qvarnström A, Vallin N, Rudh A, 2012. The role of male contest competition over mates in speciation. *Curr Zool* **58**:493–509.

Reimchen TE, 1989. Loss of nuptial color in threespine sticklebacks *Gasterosteus aculeatus*. *Evolution* **43**:450–460.

Rick IP, Bakker TCM, 2008. Males do not only see red: UV wavelengths and male territorial aggression in the threespined stickleback *Gasterosteus aculeatus*. *Behav Ecol Sociobiol* **62**:439–445.

Roberts NS, Mendelson TC, 2017. Male mate choice contributes to behavioural isolation in sexually dimorphic fish with traditional sex roles. *Anim Behav* **130**:1–7.

Rodriguez RL, Ramaswamy K, Cocroft RB, 2006. Evidence that female preferences have shaped male signal evolution in a clade of specialized plant-feeding insects. *Proc R Soc Lond B* **273**:2585–2593.

Rowland WJ, 1984. The relationships among nuptial coloration, aggression, and courtship in male three-spined sticklebacks, *Gasterosteus aculeatus*. *Can J Zool* **62**:999–1004.

Rowland WJ, 1989. The effects of body size, aggression, and nuptial coloration on competition for territories in male threespine sticklebacks *Gasterosteus aculeatus*. *Anim Behav* **37**:282–289.

Rowland WJ, 1994. Proximate determinants of stickleback behavior: an evolutionary perspective. In: *The Evolutionary Biology of the Threespine Stickleback*. Oxford: Oxford University Press. 297–344.

Rowland WJ, Bolyard AD, Halpern AD, 1995. The dual effects of stickleback coloration on rivals: manipulation of a graded signal using video playback. *Anim Behav* **50**:267–272.

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.

Seehausen O, Terai Y, Magalhaes IS, Carleton KL et al. 2008. Speciation through sensory drive in cichlid fish. *Nature* **455**:620–626.

Schindelin J, Arganda-Carreras I, Frise E et al. 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**:676–682.

Scordato ESC, 2017. Geographical variation in male territory defense strategies in an avian ring species. *Anim Behav* **126**:153–162.

Scott RJ, 2001. Sensory drive and nuptial colour loss in the three-spined stickleback. *J Fish Biol* **59**:1520–1528.

Scott RJ, 2004. Assortative mating between adjacent populations of threespine stickleback *Gasterosteus aculeatus*. *Ecol Freshw Fish* **13**:1–7.

Scott RJ, 2011. Reflectance characteristics are correlated with male condition in a population of threespine stickleback *Gasterosteus aculeatus* that has lost the species typical nuptial signal. *Environ Biol Fish* **91**: 287–294.

Scott RJ, Foster SA, 2000. Field data do not support a textbook example of convergent character displacement. *Proc R Soc Lond B* **267**:1–6.

Simmons LW, 2001. *Sperm Competition and Its Evolutionary Consequences in the Insects*. Princeton: Princeton University Press.

Tinbergen N, van Iersel JJA, 1947. "Displacement reactions" in the three-spined stickleback. *Behaviour* **1**:56–63.

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.

Tinghitella RM, Lackey ACR, Martin M, Dijkstra PD, Drury JP et al. Forthcoming. On the role of male competition in speciation: a review and research agenda. *Behav Ecol*.

Tyers AM, Turner GF, 2013. Signal and preference divergence among populations of the non-endemic basal lake Malawi cichlid fish *Astatotilapia calliptera* (Perciformes: *Cichlidae*). *Biol J Linn Soc*. **110**:180–188.

Vallin N, Qvarnström A, 2011. Learning the hard way: Imprinting can enhance enforced shifts in habitat choice. *I J Ecol* **2011**:287532.

van Iersel JJA, 1953. An analysis of the parental behavior of the male three-spined stickleback *Gasterosteus aculeatus* L. *Behaviour Suppl* **3**: 1–159.

von Hippel FA, 1999. Black male bellies and red female throats: color changes with breeding status in a threespine stickleback. *Environ Biol Fishes* **3**: 237–244.

While GM, Michaelides S, Heathcote RJP, MacGregor HEA, Zajac N et al., 2015. Sexual selection drives asymmetric introgression in wall lizards. *Ecol Lett* **18**:1366–1375.

Wickham H, 2017. Package "ggplot2". Create Elegant Data Visualizations Using the Grammar of Graphics. Available from: https://github.com/tidyverse/ggplot2/.

Winkelmann K, Genner MJ, Takahashi T, Rüber L, Sturmbauer C, 2014. Competition-driven speciation in cichlid fish. *Nat Commun* **5**.

Winter B, 2013. Linear models and linear mixed effects models in R with linguistic applications. arXiv: 1308.5499. Available at http://arxiv.org/pdf/1308.5499.pdf/.

Wootton RJ, 1976. *Biology of the Sticklebacks*. London: Academic Press.

Wong BBM, Candolin U, 2005. How is female mate choice affected by male competition? *Biol Rev* **80**:559–571.

Wong BBM, Candolin W, Lindström K, 2007. Environmental deterioration compromises socially enforced signals of male quality in three-spined sticklebacks. *Am Nat* **170**:184–189.

APPENDIX

Table A1. Relationship between after color measures, aggressive behaviors, and trial type on territory establishment by focal red or black males

	χ^2	df	P
Red focal males			
After red area* trial type	0.85	1	0.36
Aggressive behaviors * trial type	1.03	1	0.31
After red area	1.26	1	0.26
Aggressive behaviors	14.20	1	<0.001
Trial type	2.38	1	0.12
Black focal males			
After black area* trial type	0.35	1	0.55
Aggressive behaviors * trial type	1.25	1	0.26
After black area	0.56	1	0.45
Aggressive behavior	55.06	1	<0.0001
Trial type	0.41	1	0.52