

Male–male aggression is unlikely to stabilize a poison frog polymorphism

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Keywords:

colour polymorphism;
male–male competition;
negative-frequency-dependent selection;
Oophaga pumilio.

Abstract

Phenotypic polymorphism is common in animals, and the maintenance of multiple phenotypes in a population requires forces that act against homogenizing drift and selection. Male–male competition can contribute to the stability of a polymorphism when males compete primarily with males of the same phenotype. In and around a contact zone between red and blue lineages of the poison frog *Oophaga pumilio*, we used simulated territorial intrusions to test the nonexclusive predictions that males would direct more aggression towards males of (i) their own phenotype and/or (ii) the phenotype that is most common in their population. Males in the monomorphic red and blue populations that flank the contact zone were more aggressive towards simulated intruders that matched the local coloration. However, males in the two polymorphic populations biased aggression towards neither their own colour nor the colour most common in their population. In sympatry, the rarer colour morph gains no advantage via reduced male–male aggression from territorial males in these *O. pumilio* populations, and so male aggression seems unlikely to stabilize colour polymorphism on its own. More broadly, these results suggest that the potential for divergent male aggression biases to maintain phenotypic diversity depends on the mechanism(s) that generate the biases and the degree to which these mechanisms persist in sympatry.

Introduction

Phenotypic polymorphism, the coexistence of multiple distinct phenotypes within a species, is widespread in nature (Ford, 1945; Gray & McKinnon, 2007). Enumerating the evolutionary mechanisms that can maintain phenotypic polymorphisms against the homogenizing forces of drift and selection is key to understanding trait evolution and the potential role of polymorphism in speciation (Maynard Smith, 1962; Gray & McKinnon, 2007). Assortative mating can facilitate reproductive isolation, and numerous studies of polymorphic taxa

have demonstrated assortative female preferences that could result in assortative mating (Jennions & Petrie, 1997). However, assortative mating does not on its own maintain phenotypic polymorphism and may not be sufficient to drive speciation (Kirkpatrick & Ravigné, 2002; van Doorn *et al.*, 2004; Wellenreuther *et al.*, 2014). Male–male competition can also exert selection on phenotype and is often a key component of mate acquisition and thus mating patterns in the wild (Wong & Candolin, 2005; Hunt *et al.*, 2009). The potential for male–male competition to contribute to polymorphism maintenance and reproductive isolation has only recently received much attention (Seehausen & Schluter, 2004; Qvarnström *et al.*, 2012; Tinghitella *et al.*, In press).

The coexistence of multiple male morphs that differ morphologically and/or behaviourally has been

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observed in taxonomically diverse animals (Shuster, 2010). A common scenario is one where most males specialize in fighting, acquiring a territory and/or guarding females, and rarer 'alternative' morphs specialize in sneaking matings and/or mimicking females (e.g. water strider: Hayashi, 1985; bluegill sunfish: Gross, 1991; side-blotched lizard: Sinervo & Lively, 1996). In all these cases, the fitness of an alternative strategy is higher when it is relatively rare (it is under negative-frequency-dependent selection; Gross, 1991). Frequency-dependent selection can also occur even without morphs being restricted to males only or differing in mating strategy, specifically if males of any particular morph direct their aggression nonrandomly towards others (Mikami *et al.*, 2004; Seehausen & Schluter, 2004). When competition is stronger among phenotypically similar males, individuals expressing a rare phenotype enjoy the advantage of receiving, on average, less aggression than individuals of the common phenotype (Dijkstra *et al.*, 2010). In an African haplochromine cichlid species complex, males of most colour morphs are more aggressive towards rivals with similar nuptial coloration (Dijkstra & Groothuis, 2011). Consequently, this form of male–male competition has been hypothesized to facilitate the invasion of a novel phenotype and potentially stabilize a polymorphism (Dijkstra & Groothuis, 2011).

The opportunity to interact with phenotypically distinct morphs is common when lineages come into secondary contact, and the potential role of this phenomenon in the evolution of reproductive isolation will be especially important when these lineages are newly diverged. Among allopatric, phenotypically diverged populations, males are often more aggressive towards phenotypes typical of their population (Anderson & Grether, 2010; Bolnick *et al.*, 2016). Two potential mechanisms might explain such a pattern, both of which can generate negative-frequency-dependent selection among phenotypes upon secondary contact: (i) males act more aggressively towards males of their own phenotype ('own-type'; Dijkstra & Groothuis, 2011), and (ii) males act more aggressively towards the most common phenotype in the population ('familiarity'; Bolnick *et al.*, 2016). Simulated contact zones or experimental populations can provide valuable insight into the potential for differentiated male aggression biases to maintain polymorphism upon contact (Dijkstra *et al.*, 2010; MacGregor *et al.*, 2016). However, aggression patterns that arise in allopatry can shift in sympatry via selection (e.g. agonistic character displacement; Grether *et al.*, 2009) and/or nongenetic mechanisms (e.g. cross-generation learning; Verzijden *et al.*, 2008) that may not be expected to operate within short-term experimental assemblages. Natural transition zones provide unique opportunities to test whether and how patterns of aggression bias observed in allopatry are also observed in sympatry, and consequently the

extent to which male–male competition might stabilize polymorphisms in nature.

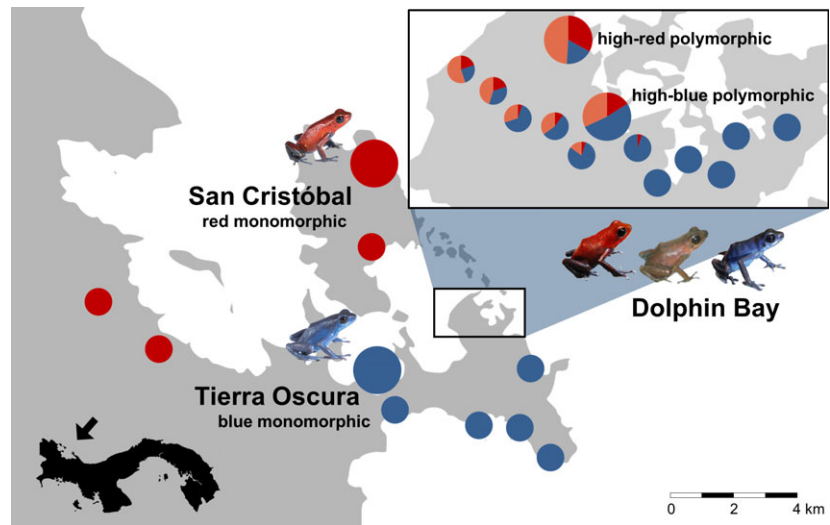
The strawberry poison dart frog (*Oophaga pumilio*) exhibits extreme colour polymorphism in and around the Bocas del Toro archipelago of Panama (Summers *et al.*, 2003). Coloration in males and females is qualitatively similar in this species (Summers *et al.*, 2003; Maan & Cummings, 2009) and is hypothesized to function both as an aposematic signal (Darst, 2006; Saporito *et al.*, 2007) and in inter- and intrasexual communication (Maan & Cummings, 2008; Crothers & Cummings, 2015). Here, we tested the hypothesis that colour-mediated male–male aggression could contribute to the stability of this colour polymorphism. We took advantage of a phenotypic contact zone between red (with blue legs)- and blue-coloured lineages of *O. pumilio* (Dugas *et al.*, 2015; Yang *et al.*, 2016). We studied two populations in the contact zone that differ in morph frequencies, and two monomorphic populations on either side (Fig. 1). We used simulated intrusions in the field to quantify the aggressiveness of territorial males towards a red or a blue rival, specifically testing whether males (i) bias aggression towards their own colour ('own-type'; Dijkstra & Groothuis, 2011) and/or (ii) bias aggression towards the most common colour in the population ('familiarity'; Bolnick *et al.*, 2016). Evidence that either of these mechanisms is operating in the contact zone would suggest that negative-frequency-dependent selection via male aggression biases could contribute to the dynamics of this sympatric polymorphism.

Materials and methods

Study species

Oophaga pumilio is a terrestrial frog that occupies lowland forests along the Caribbean side of Central America from Nicaragua to Panama. Throughout most of its range, this frog has a red body with blue or black limbs, but populations in the Bocas del Toro region of Panama display coloration spanning the visual spectrum (Summers *et al.*, 2003); males and females do not qualitatively differ in colour within populations (Maan & Cummings, 2009). Despite dramatic phenotypic differences, *O. pumilio* populations in this young archipelago (1–9 kya) are relatively undifferentiated at neutral microsatellite loci (Hauswaldt *et al.*, 2011) and there is no evidence of post-zygotic isolation, and so they are hypothesized to be one biological species (Summers *et al.*, 2004; Dugas & Richards-Zawacki, 2015). Most currently described colour morphs occur in allopatry (typically one morph per island), but two morphs are sympatric in a few documented cases (Wang & Shaffer, 2008; Richards-Zawacki & Cummings, 2011; Dugas *et al.*, 2015), and in one, morph frequency varies along a contact zone (Yang *et al.*, 2016).

Fig. 1 Map showing the transition zone between red and blue populations of *Oophaga pumilio* in the Bocas del Toro Archipelago, Panama. Pie charts show the relative red, blue and phenotypic intermediate morph frequencies at each location. We selected four populations with different morph frequencies (red monomorphic, high-red polymorphic, high-blue polymorphic, blue monomorphic). The four study populations are denoted by larger pie charts.



Male *O. pumilio* are territorial, defending territories through vocalization, visual displays, and physical combat (Bunnell, 1973; Gardner & Graves, 2005). Both sexes mate multiply, with females generally choosing a mate within a home range that overlaps several male territories (Pröhl & Hödl, 1999). Females are unlikely to mate with males not holding a territory (Meuche & Pröhl, 2005; Meuche *et al.*, 2012). Males advertising from higher perches are more likely to attract mates (Pröhl & Hödl, 1999), but all current evidence suggests that no aspect of male territory quality provides a direct benefit to females (Donnelly, 1989; Pröhl & Berke, 2001). If courtship is successful, the female lays a clutch in the leaf litter within a male's territory, where he may moisten it regularly (Weygoldt, 1980; Pröhl & Hödl, 1999). One parent, typically the female (Killius & Dugas, 2014), transports newly hatched tadpoles individually to water-filled leaf axils, and the female provisions her tadpoles with trophic eggs regularly until they complete metamorphosis (~45 days; Weygoldt, 1980; Dugas *et al.*, 2016).

Study populations

We tested the prediction that males of the rare (polymorphic populations) or novel (monomorphic populations) colour morph would experience less male–male aggression than males of the more common phenotype in four *O. pumilio* populations (Yang *et al.*, 2016; hereafter, red monomorphic, high-red polymorphic, high-blue polymorphic and blue monomorphic; Fig. 1). On Isla San Cristóbal ($9^{\circ}16'25.46''\text{N}$, $82^{\circ}15'12.74''\text{W}$), frogs are similar in coloration to ancestral populations, with red dorsal and ventral coloration and blue limbs (Wang & Shaffer, 2008; Fig. 1). On much of the Aguacate peninsula ($9^{\circ}11'47.65''\text{N}$, $82^{\circ}15'04.75''\text{W}$), frogs are entirely blue (Fig. 1). However, in the northern portion

of the Aguacate peninsula, near Dolphin Bay, red, blue and phenotypically intermediate frogs coexist. The red and intermediate phenotypes are most common at the tip of the peninsula, near Isla San Cristóbal (Fig. 1). We studied two populations in this polymorphic zone that differ in the frequencies of red, blue and intermediate frogs. At the 'high-red polymorphic' site, red frogs were almost twice as frequent as blue (33% red, 18% blue, 49% intermediate; $9^{\circ}13'15.70''\text{N}$, $82^{\circ}13'5.60''\text{W}$), and at the 'high-blue polymorphic' site, blue frogs were more than three times as frequent as red (16% red, 52% blue, 32% intermediate; $9^{\circ}12'42.48''\text{N}$, $82^{\circ}12'53.17''\text{W}$). Whereas intermediate morphs were common in both polymorphic populations, more of these intermediate animals fell towards the red end of the spectrum in the high-red population and more fell towards the blue end in the high-blue population (Y. Yang *unpublished data*; see Methods and Supporting Information for details). We therefore treated red as the most common coloration in the high-red population, even though the proportion of 'intermediate' morphs was greater than that of red.

Experimental design and protocol

We used simulated territorial intrusions to test the predictions that wild territorial *O. pumilio* males would be more aggressive towards (i) intruders with coloration similar to their own (Dijkstra & Groothuis, 2011) and/or (ii) intruders displaying the colour most common in the population (Bolnick *et al.*, 2016). As model intruders, we used 3D-printed plastic frogs (3D model from Turbosquid.com, printed by Shapeways, Inc., New York, USA; Crothers & Cummings, 2015). We used acrylic paint to match the reflectance of pure red and blue males and used a clear coat to mimic the reflective properties of moist skin (Fig. S1; *sensu* Dreher & Pröhl,

2014; Crothers & Cummings, 2015). Both movement and acoustic signals elicit aggression in poison frogs (Narins *et al.*, 2003; de Luna *et al.*, 2010), so we added full body rotation every 5 s by mounting the model on a radio-controlled servomotor hidden within a cylinder modelled to resemble a log, and played back local *O. pumilio* advertisement calls through a Bluetooth-enabled speaker (Omaker M4) (Fig. 2, Video S1). Call parameters across the red–blue transition zone do not differ among populations or colour morphs (M. L. Dye, unpublished data), so we used the same call in all trials (see Supporting Information for details). This design enabled us to control the stimuli and take notes from > 1.5 m away, a distance that does not disturb males (Staudt *et al.*, 2010; Gade *et al.*, 2016). All trials were recorded using a video camera (iCools Black Wifi Sports Camera) mounted on the stimulus apparatus.

We conducted behavioural assays between 700 and 1300 h, when *O. pumilio* males are most active (Graves, 1999; Graves *et al.*, 2005), locating target territorial males by their advertisement calls (Meuche & Pröhl, 2005). Upon locating a calling male, we placed the intruder stimulus apparatus within one metre of the perch from which the focal male was calling. The



Fig. 2 Experimental setup used in stimulated intrusion trials to quantify male aggression towards red- and blue-coloured opponents. (a) Hand-painted plastic models were mounted onto a radio-controlled servomotor hidden in a log-shaped cylinder. Advertisement calls were played through a Bluetooth speaker located behind the model. (b) Photograph of a blue territorial male attacking a red frog model during a trial. See also Video S1.

colour of the intruder stimulus was chosen haphazardly for each trial. Male *O. pumilio* defend ~ 3-m² core territories even in dense populations (Pröhl & Berke, 2001), so this model should always have been perceived as an intruder. We allowed the focal male to acclimate to the presence of the apparatus for 5 min or until it resumed calling, and then began audio playback and artificial movement of the intruder stimulus. We began behavioural observations (detailed below) after the focal male oriented towards the simulated intruder. If the focal male failed to orient to the model intruder within 15 min, we terminated the trial. Of the 372 males tested, 90% were responsive. When males were responsive ('track'; see below for operational definition), we ran trials for 10 min, or, if the male escalated the contest to the highest level of aggression ('attack'; see below), we extended or shortened the trial to allow 5 min of interaction after the first attack. Trials, then, could have ranged from 5 (immediate attack) to 15 (attack at minute 10) min (mean ± SD: 8.98 ± 2.00 min). This approach allowed us to quantify the intensity of the contest in individuals that did attack, but did not bias our likelihood of detecting other aggressive behaviours.

Following trials, we hand-captured the focal male and measured its SVL to the nearest 0.1 mm using a dial calliper. For colour quantification (see below), we took digital photographs (Panasonic DMC-TS5, Kadoma, Osaka, Japan) of the dorsum against an 18% grey standard (DGK Color Tools), manually setting white balance for each photograph. We marked each male with a toe clip (Funk *et al.*, 2005) so as to avoid resampling (if a male we observed turned out upon capture to be toe-clipped, we discarded this second observation) and released it near the point of capture. *O. pumilio* quickly resume territorial behaviour following release (Meuche *et al.*, 2012).

Male agonistic behaviours

A typical encounter between *O. pumilio* males involves an intruder approaching and calling to the resident, with the resident responding with calls and approaches. The contest can then either escalate to a physical fight (wrestling and pinning) or be resolved when one male (usually the intruder) shows submission (freezes in place or retreats and stops calling) before or upon the first attack by the winner (Baugh & Forester, 1994). In the current study, territorial males responded to the simulated intrusions similarly (Video S1). We recorded whether a male performed each of five aggressive behaviours that typically occurred in order (yes/no, not cumulative): (i) 'track', a male orienting towards and facing an intruder; (ii) 'approach', a male moving towards the model; (iii) 'calling' (typically accompanied an approach, but could occur any time); (iv) 'challenge' if a male continued an approach onto the stimulus

model's perch (log); and (v) 'attack', if the male wrestled with the moving model (see Video S1 for examples of each behaviour). As we continued to play calls and move the model frog after a focal male attacked, all trials mimicked a contest in which the intruder did not submit (i.e. an 'escalated contest' with physical fighting; Hsu *et al.*, 2006). For analysis, we treated each of the five behaviours as a binary response (yes/no) and additionally considered the number of attacks a focal male directed to the stimulus model during the 5 min that followed the first attack. If the focal male remained in contact with the stimulus model for > 5 s (i.e. attempting to pin or subdue the model), we tallied a new attack count every 5 s (the frequency at which we moved the model); doing so minimized differences between males that were able to maintain contact when the model was moved and those that were not.

We measured two additional parameters that we included in analyses as covariates. During trials, focal males sometimes interacted with nearby conspecifics (male–male combat or courtship). As these activities lowered the potential time a male could interact with the model, we included whether (yes/no) the focal male engaged in such interactions in all statistical models. Perch height of male *O. pumilio* is correlated with mating success (Pröhl & Hödl, 1999) and could influence the focal male's motivation to defend his territory. To account for this, we measured the height of the focal male's perch to the nearest cm with a flexible measuring tape and included this as a covariate in statistical models.

Defining colour morph in polymorphic populations

In these polymorphic populations, by-eye categorizations of males as blue, red or intermediate are equivalent to more quantitative measures of coloration, including visual models of colour space that take into account *O. pumilio* visual sensitivities (Dugas *et al.*, 2015). However, we were also interested in testing for nuanced relationships between focal male colour and its reaction to blue and red model intruders in the polymorphic population (e.g. asking whether redder males were more aggressive towards red intruders). For these analyses, we used digital photographs to generate objective colour scores for each focal male from polymorphic populations (detailed methods in Dugas *et al.*, 2015; Table S1 and Fig. S2).

Statistical analyses

In separate analyses, we modelled whether (yes/no) a territorial male (i) tracked, (ii) approached, (iii) called, (iv) challenged and (v) attacked the model intruder. We tested the main effect of population (red monomorphic, high-red polymorphic, high-blue polymorphic and blue monomorphic), intruder model colour (red or

blue) and their interaction. We used generalized linear models (GLMs) with binomial error structures, and Bonferroni-corrected for multiple comparisons after testing the five behaviours. In the subset of males that attacked the frog model (hereafter, 'escalated contests'), we similarly tested the main effect of population, intruder colour and their interaction term on the intensity of attack ('attack' count during the 5 min following the first attack). Due to overdispersion when fitted with a Poisson distribution, we modelled attack counts using a negative binomial error structure. The prediction that population morph frequency is associated with aggression towards differently coloured intruders would be supported by a significant population \times intruder colour interaction. When this interaction was significant, we conducted separate *post hoc* analyses for each of the four focal populations, testing only the main effect of intruder colour; these analyses allowed us to ask whether differences were in the predicted direction.

Observations from polymorphic populations afforded us the additional opportunity to test the prediction that focal males would direct more aggression towards intruders of their own colour. We reran the follow-up models described above in polymorphic populations, testing (in addition to intruder colour) the effect of focal male colour (red, blue or intermediate) and the interaction term. The prediction that males would bias their aggression towards intruders of their own colour would be supported by a significant male colour \times intruder colour interaction followed by *post hoc* analyses for each male colour confirming that the differences were in the predicted direction. Because male colour in polymorphic populations is not discrete, we also reran the models after replacing the by-eye colour categories with quantitative colour scores generated from photographs (PC1 and PC2; Table S1 and Fig. S2).

For all statistical models described above, we included perch height and whether the male interacted with nearby conspecifics (yes/no) during the observation period as covariates. Because conspecific interactions were negatively associated with aggression in several models (see Results), we also reran all models using a data set that excluded trials in which the focal male interacted with conspecifics (Supporting Information: Additional results: trials without conspecific interactions).

All analyses were performed in R 3.2.3 (R Core Team, 2015). We used the 'glm' and 'glm.nb' function in the stats package (R Core Team, 2015) to fit the GLMs. We tested the significance of main effects, interaction terms and covariates using a likelihood ratio test with the 'ANOVA' function in the car package (Fox & Weisberg, 2011), which compares overall model fit with and without a particular effect. *Post hoc* Tukey pairwise comparisons of the significant main effects were made using the 'glht' function in the multcomp package (Hothorn *et al.*, 2008).

Results

We tested a total of 372 males (after discarding 15 trials due to resampling; see Table S2 for a breakdown of sample sizes). Males from all four populations responded aggressively to both red and blue intruder models. Of the 372 males tested, 335 (90%) tracked, 320 (86%) approached, 312 (84%) called, 252 (68%) challenged and 142 (38%) attacked the model intruder.

Does male aggression pattern vary with population morph frequency?

In our initial GLMs modelling whether (yes/no) a territorial male (i) tracked, (ii) approached, (iii) called or (iv) challenged the model intruder, the population \times intruder colour interaction term was always nonsignificant, as were the main effects of population and intruder colour (Table 1), suggesting no effect of colour on these aggressive behaviours. However, there was a significant population \times intruder colour interaction on whether a territorial male attacked the model intruder (Table 1). In these models, perch height was nonsignificant (Table 1). Males that interacted with conspecifics had lower aggression levels, but only with respect to the likelihoods of challenge and attack (yes/no; Table 1; challenge: $\beta \pm SE = -1.01 \pm 0.27$; attack: $\beta \pm SE = -0.82 \pm 0.26$).

When we modelled each population separately and considered all males, regardless of interaction with conspecifics during the trial, territorial males in the monomorphic red population were more likely to attack a red (12/21) than a blue (4/27) model intruder (GLM, $LR\chi^2 = 7.60$, d.f. = 1, $P = 0.006$; Fig. 3a). Similarly, males in the monomorphic blue population were more likely to attack a blue (14/23) than a red (7/22) model intruder (GLM, $LR\chi^2 = 4.16$, d.f. = 1, $P = 0.041$; Fig. 3a). Males in the high-red polymorphic population, on the other hand, were equally likely to attack a red (23/72) and a blue (26/68) model intruder (GLM, $LR\chi^2 = 1.25$, d.f. = 1, $P = 0.264$; Fig. 3a). Males in the high-blue polymorphic population were also equally

likely to attack a red (24/68) and a blue (32/71) model intruder (GLM, $LR\chi^2 = 1.24$, d.f. = 1, $P = 0.266$; Fig. 3a). In none of these four follow-up models was perch height associated with the likelihood that a focal male would attack (GLM, all $LR\chi^2 < 2.32$, all $P > 0.128$); interaction with conspecifics (yes/no) was negatively associated with attack likelihood in one population (GLM, high-red: $LR\chi^2 = 9.00$, d.f. = 1, $P = 0.003$, $\beta \pm SE = -1.16 \pm 0.40$) but not the other three (all $LR\chi^2 < 2.71$, all $P > 0.100$).

Considering only the subset of males that attacked the model intruder, there was no effect of population, intruder colour or the interaction term on the number of attacks (GLM, population: $LR\chi^2 = 2.00$, d.f. = 3, $P = 0.573$; intruder colour: $LR\chi^2 = 1.60$, d.f. = 1, $P = 0.206$; interaction term: $LR\chi^2 = 1.08$, d.f. = 3, $P = 0.781$; Fig. 3b). Both perch height and interaction with conspecifics were nonsignificant in this model.

When we reran the analyses with the subset of observations in which the focal male did not interact with a conspecific during the trial, we found an overall similar pattern to what is presented above (Table S8). The only exception is that in contrast to the original analyses, males in the high-red polymorphic population were more likely to attack a blue model intruder; in the high-blue polymorphic population, this trend was also present but nonsignificant (see Supporting Information: Additional results: trials without conspecific interactions for details).

Do males bias aggression towards intruders of their own colour in the polymorphic populations?

When considering all territorial males, regardless of interaction with conspecifics during the trial, neither the main effects of male colour and intruder colour nor their interaction was a significant predictor of the probability of attack in the high-red polymorphic population (Table 2). We then reran the model, replacing by-eye colour groups with quantitative colour scores PC1 and PC2: there was no significant male \times intruder colour interaction nor any significant main

Table 1 Generalized linear models evaluating the influence of population (red monomorphic, high-red polymorphic, high-blue polymorphic and blue monomorphic), intruder colour (red and blue) and their interaction term on the likelihood of a territorial male to (i) track, (ii) approach, (iii) call at, (iv) challenge and (v) attack the frog model. Perch height and whether the male interacted with nearby conspecifics were included as covariates.

Parameters	d.f.	Track		Approach		Call		Challenge		Attack	
		$LR\chi^2$	P	$LR\chi^2$	P	$LR\chi^2$	P	$LR\chi^2$	P	$LR\chi^2$	P
Population	3	0.22	0.975	0.30	0.961	0.49	0.920	5.04	0.169	10.64	0.014
Intruder colour	1	0.05	0.825	0.54	0.464	0.21	0.648	2.30	0.129	1.05	0.305
Population \times intruder colour	3	0.70	0.873	0.63	0.889	1.56	0.669	6.58	0.087	12.87	0.005
Conspecific interactions	1	3.54	0.060	2.48	0.115	1.64	0.200	17.53	< 0.001	13.03	< 0.001
Perch height	1	0.28	0.599	0.77	0.381	0.13	0.720	3.12	0.077	1.33	0.250

Bolded P values denote significant effect(s). Significance level is at 0.01 (Bonferroni correction)

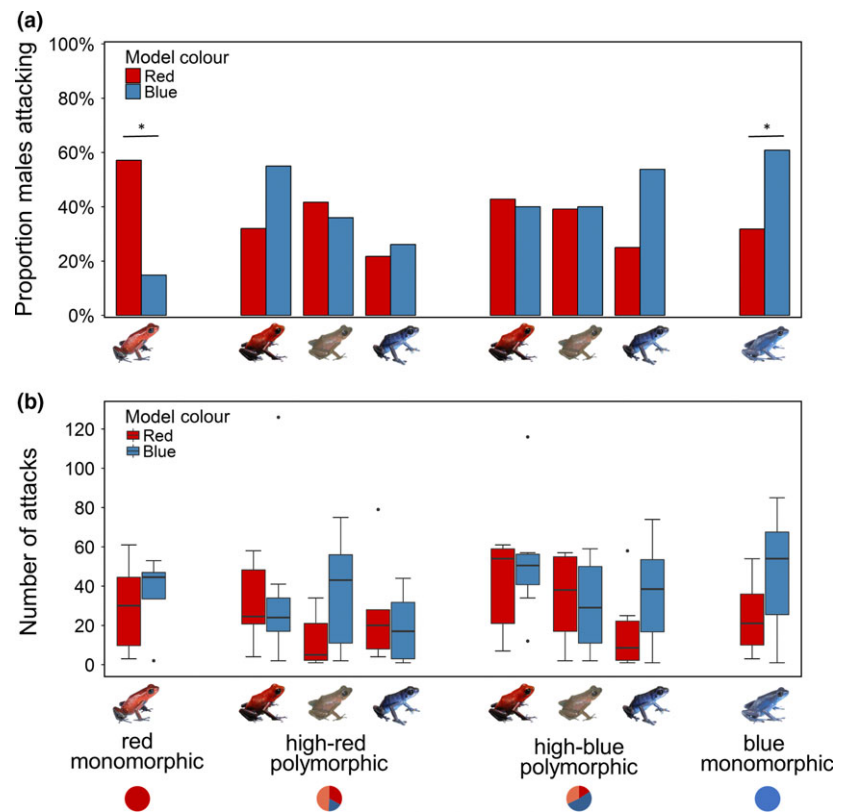


Fig. 3 (a) Proportion of males that attacked and (b) number of attacks on the intruder model (during 5 min following the first attack) when presented with a red or a blue model frog in four populations. Pie charts on the *x*-axis correspond to the morph frequencies in the four populations. Red, blue and phenotypic intermediate males from the two polymorphic populations are plotted separately.

Table 2 Generalized linear models evaluating the influence of male colour (red, intermediate and blue), model colour (red, blue) and their interaction term on the likelihood of a territorial male to attack the frog model in the two polymorphic populations. Perch height and conspecific interactions were included as covariates. GLMs with PC colour scores replacing by-eye male colour groupings are presented in Table S3. GLMs for the other four variables (likelihood to track, approach, call and challenge) are presented in Tables S4–S7.

Attack (y/n) Parameters	d.f.	High-red polymorphic		High-blue polymorphic	
		LR χ^2	<i>P</i>	LR χ^2	<i>P</i>
Male colour	2	2.84	0.241	1.07	0.585
Intruder colour	1	1.01	0.316	3.56	0.059
Male \times intruder colour	2	2.44	0.296	2.32	0.314
Conspecific interactions	1	10.21	0.001	2.47	0.116
Perch height	1	0.66	0.416	2.09	0.148

Bolded *P* values denote significant effect(s). Significance level is at 0.01 (Bonferroni correction)

effects (Table S3). In both models, perch height was unrelated to attack probability (Tables 2 and S3), whereas interaction with conspecifics (yes/no) was negatively associated with attack likelihood (Tables 2 and S3; by-eye grouping: $\beta \pm SE = -1.30 \pm 0.42$; colour PC scores: $\beta \pm SE = -1.07 \pm 0.42$).

In the high-blue polymorphic population, neither the main effects of male colour and intruder colour nor their interaction was a significant predictor of the probability of attack (Table 2). When we reran the model with male colour PC scores, there was no male \times intruder colour interaction (Table S3). However, the likelihood of attack was positively correlated with PC2 (a hue indicator that increases with male 'blueness'; Table S1), suggesting that bluer males were more aggressive than redder males. In both models, interaction with conspecifics was unrelated to attack probability (Tables 2 and S3), whereas focal males on higher perches were less likely to attack in the PC score model (Table S3; $\beta \pm SE = -0.024 \pm 0.010$).

When we reran the analyses with the subset of observations in which the focal male did not interact with a conspecific, we again found no significant interaction effect between male colour and intruder colour (the key prediction of the 'own-type' hypothesis) in either polymorphic population, using either by-eye colour groups or colour PC scores (Tables S9 and S10). Different from the analysis of the full data set, our models that included focal male colour showed a main effect of intruder colour in the high-blue, but not the high-red polymorphic population. Regardless of its own colour, males in the high-blue (but not the high-red) population were more likely to attack a blue intruder. When we reran these models using colour PC scores

rather than by-eye category, we found this trend towards a higher likelihood of attacking a blue intruder in both polymorphic populations (see Supporting Information: Additional results: trials without conspecific interactions for details).

GLMs for the other four variables (likelihood to track, approach, call and challenge) are presented in Tables S4–S7. We did not detect any significant main effects or an interaction between male colour and model intruder colour in any of the models.

Discussion

Male–male competition is hypothesized to contribute to the stability of a polymorphism when a rare male phenotype has a fitness advantage over a common phenotype (Mikami *et al.*, 2004; Seehausen & Schluter, 2004). Territorial *O. pumilio* males in red and blue monomorphic populations were more aggressive towards the local coloration than the novel (allopatric) coloration, suggesting that colour is important in shaping male aggressive interactions. However, this pattern did not persist in sympatry: males in the two polymorphic populations did not bias aggression towards their own coloration ('own-type'), nor did they bias their aggression towards the more common colour in the population ('familiarity'). Instead, males in both the high-red and the high-blue polymorphic populations were more likely to attack a blue model intruder. Whereas aggression biases may facilitate the initial invasion of a novel *O. pumilio* phenotype, these biases appear to weaken and/or change in direction quickly as a second phenotype becomes more common. A related question remains whether males attend to the colour of residents when invading a territory/establishing a new territory as males of the rarer morph might also benefit if their territories are less likely to be invaded. Without any aggression-mediated fitness advantage for the rarer phenotype, male aggression seems insufficient to exert negative-frequency-dependent selection on *O. pumilio* colour morphs in sympatry.

Identifying the mechanism by which male aggression biases are formed may help to clarify how and why male aggression biases break down in sympatry. If the aggression bias is heritable (common in birds; McKinnon & Pierotti, 2010), alternative alleles might have been fixed in each monomorphic population. In this case, the lack of an aggression bias in the two polymorphic populations could be explained by recombination breaking down the association between male coloration and colour-biased aggression. Alternatively, if the aggression bias is plastic and shaped by experience (as assumed in the 'familiarity' hypothesis), our findings suggest that the threshold for rival recognition is relatively low: a phenotype need not be the most common to be recognized, and any bias disappears long before

morph frequencies approach parity. Because *O. pumilio* males interact with conspecific rivals on a daily basis, they are likely to learn from past agonistic interactions and adjust any aggression 'bias' accordingly (as in many other animals; Hsu *et al.*, 2006; Reichert & Quinn, 2017). Pertinent to this learning hypothesis, however, male *O. pumilio* do not reduce aggression towards known neighbours like other territorial animals are known to do ('dear enemy'; Ydenberg *et al.*, 1988; Bee, 2003; Gardner & Graves, 2005).

A third possibility is that colour bias is learned at an early age (i.e. sexual imprinting). Imprinting is common in the context of mate preference (Lorenz, 1935; Verzijden *et al.*, 2012) and may also shape the perception of rivals (e.g. tits: Hansen & Slagsvold, 2003; cichlids: Dijkstra *et al.*, 2008; but see Verzijden *et al.*, 2009). Depending on the modes of imprinting (e.g. parental vs. social), male colour and colour-biased aggression can be decoupled (although not as easily as genetic-based behaviours). The degree to which male aggression biases are genetic or plastic/learned in *O. pumilio* remains to be tested, but biparental care in this frog (males tending eggs and females feeding tadpoles, reviewed in Dugas, In press) provides ample opportunities for tadpoles to see adult colours. Male–male competition was recognized as a potential diversifying force only recently (Qvarnström *et al.*, 2012; Tinghitella *et al.*, In press), and the evolutionary consequences of whether aggression biases are genetic or learned remained to be explored.

Males from the two monomorphic populations we studied differed in their relative likelihoods of attacking red and blue intruders, but not in the pre-escalation display behaviours ('track', 'approach', 'challenge' and 'call'). This pattern could have arisen because intraspecific communication in *O. pumilio* is multimodal, with both acoustic and visual signals perceived in a sequential order (Dreher & Pröhl, 2014). If males use calls to identify a conspecific intruder from a further distance, whereas visual signals aid in closer-range assessments (Candolin, 2003), this could explain why aggression biases were only apparent after the focal male hopped on the log ('challenged'); at this point in the interaction, the model was not visually shielded by the complex forest environment (Willink *et al.*, 2013). On the other hand, we found that the intensity of attack (number of attacks) was not mediated by the intruder colour. In animal contests, aggression biases can influence the probability of contest escalation; however, if coloration does not predict fighting ability (i.e. the colour morphs are equally competitive), then colour biases may not influence the intensity of the contest once escalated (Hsu *et al.*, 2006). Although the two *O. pumilio* colour morphs in this study have not been directly tested for competitive ability, they did not differ in overall aggression level, body size (Y. Yang *unpublished data*) or call characteristics (M. L. Dye

unpublished data). Nevertheless, the majority of *O. pumilio* agonistic interactions in nature are resolved without escalation (~ 63% of 19 natural encounters; Meuche *et al.*, 2012), suggesting that colour-based differences in the likelihood of attack may be critical to a male's ability to hold/gain a territory. Moreover, females often leave the male they are courting if that male interacts with another male (Y. Yang, personal observation; H. Pröhl, personal communication). This suggests that even in cases where territories do not change hands, frequent agonistic interactions could be costly.

As in many other animals (Berglund *et al.*, 1996; Rojas, 2016), the dramatic differences in coloration among *O. pumilio* lineages appear also to be important in mediating female mate choice, with females often (but not always) expressing assortative preferences in laboratory trials (Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008, 2009; Richards-Zawacki & Cummings, 2011). Male–male competition in the wild can shape the extent to which these preferences actually contribute to reproductive isolation (Jennions & Petrie, 1997; Wong & Candolin, 2005). In the red–blue contact zone studied here, females from both monomorphic populations spend more time associating with males of the local colour morph, whereas red, blue and intermediate females from the polymorphic region all associate preferentially with red males (Yang *et al.*, 2016). Consequently, when a novel colour first invades a monomorphic population, male *O. pumilio* might simultaneously experience two, potentially opposing, selective forces: males of the rare colour morph may slip under the radar of competing males and enjoy an advantage in terms of gaining/defending territories, but they may also be less attractive as mates if females preferring the novel colour are also rare in the population.

Male–male competition could similarly limit females' access to preferred phenotypes if some male morphs are dominant over others (Dijkstra & Groothuis, 2011; Qvarnström *et al.*, 2012). Allopatric *O. pumilio* colour morphs differ in aggression level (Rudh *et al.*, 2013), and brighter males are more aggressive in one monomorphic population tested (Crothers & Cummings, 2015). Some of these differences in aggression level are reflected in contest dominance in a laboratory setting (Galeano & Harms, 2016). In this study, males that did not interact with conspecifics during the trial showed a trend towards attacking a blue model intruder more than a red one in both polymorphic populations, and bluer males were more likely to attack a simulated intruder (regardless of its colour) in one polymorphic population. Whereas these patterns were only evident in a subset of our analyses, and sometimes with only marginal statistical significance, they suggest that there may be asymmetries between the two colour morphs. Whether coloration is associated with any other measure of competitive ability

aside from aggression level in this contact zone warrants further investigation.

Whereas males in monomorphic populations were less aggressive towards novel phenotypes, males in the polymorphic populations bias aggression to neither their own colour nor the most common colour in the area. Broadly, this result indicates that the potential for divergent aggression biases to stabilize a polymorphism will depend upon whether and how quickly such biases break down as morph frequencies approach parity. Several avenues for future research can offer a more complete understanding of the role of male–male competition in phenotypic divergence: (i) identifying the proximate mechanisms that shape male colour-based agonistic behaviours, (ii) quantifying the fitness consequences of differential male aggression, (iii) deciphering the degree to which male–male competition constrains the expression of female preferences and (iv) exploring how inherited vs. learned male aggression biases may influence the likelihood and persistence of phenotypic divergence.

Acknowledgments

Mysia Dye, Simone Blomenkamp and Deyvis Gonzalez helped with experiments. Michael Jennions and two anonymous reviewers provided helpful comments that improved the quality of the manuscript. The Smithsonian Tropical Research Institute (STRI) provided logistical support, and we particularly thank Plinio Gondola (Bocas del Toro Research Station). This study was supported by a STRI short-term fellowship to YY and the National Science Foundation (Award No. 1146370 to CLRZ). The Panamanian National Authority for the Environment provided research, collection and export permission. This work is in compliance with IACUC (Tulane University Nos. 0382 and 0382R, University of Pittsburgh No. 15106566 and STRI No. 2014-0515-2017).

Authors' contributions

YY, MBD and CRZ conceived and designed the study. YY collected and analysed field data, carried out statistical analyses and drafted the manuscript. CRZ and MBD contributed to writing and revision of the manuscript. HJS and SM collected and analysed field data. All authors gave final approval for publication.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Photographs and the reflectance spectra of the hand-painted red and blue plastic models used as simulated intruders.

Figure S2 By eye color categories (“morph”) plotted on quantitative color space based on PC1 and PC2.

Table S1 Results of a principal components analysis of mean red, green, and blue color scores from the dorsum of *O. pumilio* from the two polymorphic populations.

Table S2 Sample size of responsive males of each male color/model intruder color combination in the four populations.

Table S3 Generalized linear models estimating the likelihood of attack in the two polymorphic populations, re-run from Table 2, replacing by-eye color groups (red, intermediate and blue) with color score PC1 and PC2.

Table S4 Generalized linear models evaluating the effect of male color (red, intermediate and blue), model intruder color (red, blue) and their interaction term on the likelihood of a territorial male to track the intruder model in the two polymorphic populations.

Table S5 Generalized linear models evaluating the effect of male color (red, intermediate and blue), model intruder color (red, blue) and their interaction term on the likelihood of a territorial male to approach the intruder model in the two polymorphic populations.

Table S6 Generalized linear models evaluating the effect of male color (red, intermediate and blue), model intruder color (red, blue) and their interaction term on the likelihood of a territorial male to call in the two polymorphic populations.

Table S7 Generalized linear models evaluating the effect of male color (red, intermediate and blue), model intruder color (red, blue) and their interaction term on the likelihood of a territorial male to challenge the intruder model in the two polymorphic populations.

Table S8 Generalized linear models estimating the likelihood of a territorial male to (i) track, (ii) approach, (iii) call at, (iv) challenge and (v) attack the intruder model. Re-run from Table 1 using a dataset that excluded all trials with conspecific interactions.

Table S9 Generalized linear models estimating the likelihood of attack in the two polymorphic populations, re-ran from Table 2 (main text) using a dataset that excluded all trials with conspecific interactions.

Table S10 Generalized linear models estimating the likelihood of attack in the two polymorphic populations, re-run from Table S3 using a dataset that excluded all trials with conspecific interactions.

Video S1 Examples of the five recorded aggressive behaviors (track, approach, call, challenge and attack) in simulated territorial intrusions.

Data and R file deposited at Dryad: doi:10.5061/dryad.9m0f7

Received 27 September 2017; revised 18 December 2017; accepted 9 January 2018