Predictable adaptive trajectories of sexual coloration in the wild: evidence from replicate experimental guppy populations

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Supporting Information: Tables S1–S3 and Figures S1–S3

AUTHOR CONTRIBUTIONS

D.N.R and D.J.K jointly conceived the study, its rationale and experimental design, and wrote the paper. All authors contributed to data processing and/or analysis and to editing the final manuscript.

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DATA ARCHIVING

Data are to be lodged in Dryad repository (lodgement detail to be advised).

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The question of whether populations evolve predictably and consistently under similar selective regimes is fundamental to understanding how adaptation proceeds in the wild. We address this question with a replicated evolution experiment focused upon male sexual coloration in guppies (*Poecilia reticulata*). Fish were transplanted from a single high predation population in the Guanapo River to four replicate, guppy-free low predation headwater streams. Two streams had their canopies thinned to adjust the setting under which male coloration is displayed and perceived. We assessed evolutionary divergence using second-generation lab-bred offspring of fish sampled four to six years following

translocation. A prior experiment of the same design, performed in an adjacent drainage, resulted in the evolution of more extensive orange, black and iridescent markings. We however found evidence for expansion only in structural coloration (iridescent blue/green), no change in orange, and a reduction in black. This response amplifies earlier findings for Guanapo fish, revealing that trajectories of color elaboration differ among drainages. We also found that color phenotypes evolved more greatly at the thinned-canopy sites. Our findings support the predictability of sexual trait evolution in the wild, and underscore the importance of signaling conditions and ornamental starting points in shaping adaptive trajectories.

KEY WORDS: experimental evolution, iridescence, color ornament, sexual selection, visual ecology

Introduction

Sexual competition generates sexual selection and often drives phenotypic divergence between males and females (Darwin 1874). When the premium on mating success is high, selection can favor extravagant traits such as weapons, ornaments and the behaviors that express them. In part because mate competition is a zero-sum game, theory predicts that such traits will evolve to costly levels among members of the competitive sex (Zahavi 1975). Costliness is conceptualized as the net viability consequence of increased marginal investment to sexual traits/behaviors; that is, the extent to which a sexual phenotype deviates from what would be otherwise optimum for viability (Grafen 1990; Rowe and Houle 1996). Practically, costs are levied via avenues defined by the particulars of species ecology. This may include the re-appropriation of limited resources (i.e., resource allocation), sacrifices in foraging opportunity or efficiency (i.e., resource acquisition) and/or an elevated risk of injury or death. Individuals are variously able to bear such costs (Zahavi 1975; Grafen 1990; Rowe and Houle 1996), which generates a distribution of sexual phenotypes that in-turn sets the parameters of local mate competition (Maynard Smith 1982). The strength of viability selection is thought to be balanced against the intensity of sexual selection, which exemplifies the trade-off considered to shape traits dedicated to mating competition.

Studies of Trinidadian guppies have informed the evolutionary consequence of variation to this trade-off in the wild (Endler 1980, 1983; Reznick et al. 1990; Carvalho et al. 1996; Ghalambor et

al. 2004). Guppies are small freshwater fish with a promiscuous mating system in which females choose mates based upon ornamental coloration (detailed below). Highly ornamented males are attractive to females but also more vulnerable to predation (Endler 1978, 1983). Their most dangerous predator is the pike cichlid, *Crenicichla frenata* (formerly: *alta*; Endler 1978, 1991), a diurnal, visually-oriented piscivore that partially overlaps with guppies across their range in Trinidad. Empirically, it is possible to manipulate the balance between natural and sexual selection by translocating guppies above upstream dispersal barriers such as waterfalls that exclude *C. frenata* (and other predators; (Haskins et al. 1961; Endler 1980, 1983; Winemiller et al. 1990; O'Steen et al. 2002). Establishing such populations elegantly manipulates a defined vector of natural selection while largely preserving the natural setting of socio-ecological dynamics. It facilitates the evolution of more elaborate (i.e., visually conspicuous) male ornamentation because selection due to female mating preferences can then prevail more strongly. Phenotypic divergence following translocation has been established in as few as two years (Endler 1980), which encompasses up to 5 generations of population recruitment (Reznick et al. 1997).

Guppy translocation experiments were conceived first-and-foremost to manipulate the strength of selection levied by visual predation in the wild. Longitudinal studies have informed how adaptation subsequently proceeds across a breadth of traits, including life history phenotypes in both sexes (Reznick and Bryga 1987; Reznick et al. 1990). In accord with theory, translocated populations have consistently tracked towards a "slower" life history strategy (*sensu* Stearns 1992). This is a syndrome characterized by delayed maturity at a larger size, the production of fewer, larger offspring, and a reduction in overall investment per reproductive bout. Translocated populations also vary demonstrably in behavioral traits, such as predator inspection (Magurran et al. 1992) and escape ability (O'Steen et al. 2002). Evolutionary responses in these traits broadly parallel those established for male coloration (e.g., Endler 1983), yet they are simpler to interpret. This is due to the complex, multicomponent nature of guppy ornamentation, its context of visual perception (Endler 1991; Grether et al. 2004), and because breeding value is ultimately a function of the contemporary distribution of alternative male phenotypes. Each of these points is expanded below.

(1) Sexual color phenotypes invariably comprise multiple elements (Candolin 2003; Grether et al. 2004), that may each vary in their appearance to predators, prey and conspecifics due to male display behavior (e.g., White et al. 2015). Male guppies exhibit complex ornamental

phenotypes comprising a mosaic of up to ten discrete elements (Endler 1978, 1980, 1991; Endler and Houde 1995). The main features are (a) red/orange/yellow markings – hereafter "orange", (b) iridescent ultraviolet/blue/green – hereafter "iridescence", and (c) black or brownish-black markings – hereafter "black". Each feature is controlled by different colorants situated in discrete dermal layers. Orange arises from carotenoid and pteridine pigments in the uppermost xanthophore layer (Grether et al. 2005), iridescence from optical microstructures in the iridophore layer, and black due to melanin pigment in the basal melanophore layer (Grether et al. 2004). These elements appear in diverse configurations across the lateral flank and caudal fin, and influence overall pattern conspicuousness due to their relative size and/or positioning (Endler 1978). This might for example include variation in the adjacency or demarcation of contrasting colors – such as black outlines around orange or iridescent patches, or via overall color diversity (*sensu* Brooks and Endler 2001b). Genetically, color variation is known to have a complex allosomal basis comprising many X- and Y-linked genes and linkage complexes ("supergenes"; Haskins et al. 1970);

(2) Signal conspicuousness is ultimately a matter of perception (Kemp et al. 2015). Aside from variation in perceptual systems themselves, the appearance of color signals will be strongly influenced by variation in viewing conditions. Stream habitats constitute the perceptual "theatre" in which sexual and natural selection are expected to shape the evolution of guppy color phenotypes. Two critical features of this theatre are ambient illumination and viewing background. The former will be largely determined by canopy closure and weather (Endler 1992, 1993a, b). The latter will depend upon substrate characteristics and other in-stream features such as vegetation, leaf litter and woody debris. A third feature is signal attenuation, which is influenced by the transmission medium. This is less important for guppies that occupy shallow clear-water streams (Endler 1991), but any discoloration due to tannins or suspended sediment will modify the appearance of signals, backgrounds and the relationship between the two (Endler and Houde 1995; Ehlman et al. 2015);

(3) Male breeding value will depend upon how females in the population rate attractiveness (Brooks and Endler 2001b), but also upon the contemporary distribution of alternative male phenotypes. To the first point, although female choice proceeds via the appraisal of entire male color patterns, evidence suggests that constituent elements are differentially favored in populations from different Trinidadian watersheds (Endler and Houde 1995). Orange spots

are for example near-universally favored, whereas preferences for black spots and iridescent blues, violets and green/bronze may be positive, neutral or even negative in some populations (see further below). Second, female guppies have long been known to favor novelty, expressed either for rare color components or unusual arrangements of existing components (Farr 1977; Hughes et al. 1999). This casts male attractiveness as a dynamic quality, and invokes a role for sexual selection in maintaining rare phenotypes via negative frequency-dependence (Hughes et al. 2013). Other studies have supported genetic covariance between the expression of specific male color traits and the strength of female preference for them (Houde and Endler 1990; Houde 1994; Brooks 2000). Covariance of this nature is predicted to arise from the establishment of linkage disequilibrium under "run-away" sexual selection (Fisher 1930). In the context of a translocation experiment, these and similar features of genetic architecture may promote founder effects if (for example) assortative mating among individuals with particular linkage complexes promote disproportionate contributions to early population recruitment.

The sum of these considerations imply numerous avenues for ornamental evolution in guppy populations founded under relaxed predation risk (Endler and Houde 1995). The primary evolutionary-genetic considerations are founder effects, localized adaptation (due to the visual and ecological theatre of introduction sites), and genetic architecture. The latter possibility encompasses epistatic variance arising from linkage (e.g., Haskins et al. 1970; Houde 1994; as above) as well as pleiotropic influences upon the competitive expression of male color elements (Endler 1995). Beyond these factors, however, the reduced effective size (N_e) of translocated populations makes them susceptible to genetic drift. The relative strength of drift is theoretically expected to increase as a mathematical consequence of decreasing N_e (Falconer 1981), and empirical work has convincingly demonstrated how it can overpower adaptation in small populations (Rich et al. 1979). Drift will have particular importance for guppy ornamentation because many color genes are sex-linked (Haskins et al. 1970), and N_e for sex-linked genetic variation is theoretically one-quarter of that autosomal variation (Falconer 1981).

Given such complexity, understanding the factors that shape ornamental trajectories in guppies has been hindered by a lack of controlled replication in the wild. Existing efforts to address replicability in this context are limited to two sources. The first is Endler's (1980) comprehensive set of experiments on wild and captive Trinidadian populations. This work demonstrated that

captive pond populations manipulated for exposure to *Crenicichla* evolved very similar color phenotypes to those translocated in the wild (i.e., from a downstream reach of the Aripo River to *Crenicichla*-free upstream site). Notably, the captive pond experiment drew upon fish from 11 different rivers across Trinidad and Venezuela, which were carefully admixed for several generations in order to maximize genetic variation and disrupt linkage complexes. The concordance between Endler's field and captive populations argues for a strong and potentially generalizable adaptive trajectory.

The second insight comes from comparison of the Aripo River result with that seen in an analogous translocation conducted in the El Cedro River, an adjacent tributary of the Guanapo drainage (Kemp et al. 2009). Males in both experiments evolved more elaborate ornamentation once introduced to upstream *Crenicichla*-free sites. They however varied in how color elaboration was achieved; that is, the relative extent to which orange, black and iridescent elements evolved. Interestingly, differences in male ornamentation among extant source populations appeared to become amplified in the translocated populations. Whereas males in the Aripo experiment evolved larger orange and black markings and more numerous iridescent spots (Endler 1980), El Cedro males evolved more extensive and brighter blue-violet and green iridescence but reduced coverage of black and orange (Kemp et al. 2009). Both experiments support an underlying role for adaptation, yet their contrast implies that specific adaptive trajectories were driven by initial vectors of sexual selection and/or existing genetic architecture, if not differences among newly-faced signaling environments. The broader question is to what extent ornament elaboration is driven by pre-existing selective regime and/or genetic architecture, the environmental context of selection, or stochastic effects such as genetic drift. Given that these factors are likely to have complex interactive effects upon color patterns (Cole and Endler 2015; Fuller and Noa 2010), insight into this question will ultimately require controlled experimental replication in the wild.

Here we report data arising from the first comprehensively replicated wild translocation study in Trinidadian guppies, designed and implemented to also achieve a manipulation of the ambient signaling environment. Briefly, fish from a single downstream location of the Guanapo River, where they exist with *C. frenata* and other predators, were translocated to four previously guppy-free upstream sites (**Figure 1**). Two of these sites were subject to sustained overhead canopy thinning to manipulate both the intensity and spectral quality of ambient illumination, and hence the visual setting of male display (*sensu* Endler 1993a). The two other sites retained their natural

dense canopy cover. This design effectively imposed a common change of visual predation upon replicate populations drawn from a common genetic background, but with a nested manipulation of the visual 'theatre' of color-based mate choice.

We first aimed to test whether fish across all translocated populations evolved more elaborate ornamentation (gauged via the coverage of key color elements; Endler 1978, 1980). This was simply to assess the general signature of adaption, and to provide an initial basis for comparison against the Aripo and El Cedro experimental outcomes (Kemp et al. 2009). Given that the El Cedro and Guanapo Rivers are co-tributaries, we expected that potential commonality in preference profiles would drive our experimental populations along a similar trajectory of color elaboration; that is, chiefly towards a greater predominance of iridescent blue/green (sensu Kemp et al. 2009). By contrast, the limited existing knowledge of preference itself within the Guanapo (Endler and Houde 1995) predicts that elaboration should proceed largely via increases in orange and bronze-green coverage, potentially accompanied by a reduction in black. We next sought to assess differences in ornamental trajectories in and among translocated populations. This encompassed a contrast among environmental treatments (i.e., intact-versus-thinned canopies), and then among the two replicate populations nested within each treatment. These contrasts sought to isolate the impact of relaxed predation and canopy manipulation as brokers of adaptation versus the potential effects of founder genotypes, genetic architecture (i.e., linkage and/or pleiotropy) and drift. We sampled source site fish immediately prior to translocation to estimate the starting phenotype, and then at intervals across the ensuing 5 – 6 years. The rationale for our a-priori predictions is described further alongside their basis for testing in the "statistical analysis" section below.

Methods

WILD EXPERIMENTAL PROTOCOL

Fish from the Guanapo source site (hereafter "G") were introduced among upstream sites (**Figure 1**) in two stages. In March 2008, 38 males and 38 females were introduced to the Lower Lalaja (hereafter in abbreviation: "LL") and Upper Lalaja ("UL"). A year later, 50 males and females were introduced to the Taylor ("T") and 63 of each sex to the Caigual ("C"). In each case, Guanapo fish were collected as juveniles and reared to maturity in single sex groups. Adults were first allowed to mate in the laboratory in groups of 4 – 5 randomly chosen males and females per tank. Males and females from each tank were then introduced into different streams.

Introduced females therefore carried the stored sperm of one set of males (arising from laboratory mating), but could subsequently also be inseminated by a different set of males. This ensured that effective population size (N*e*) at each site could exceed that predicted from simply the number of introduced fish, and was done to reduce the potential for founder effects (the rationale is further discussed by Lopez-Sepulcre et al. 2013).

One of the two paired streams in each stage of introduction (i.e., the Upper Lalaja and Taylor, respectively) was subject to experimental canopy thinning. This was achieved by removing riparian trees/shrubs or their overhanging foliage that would otherwise shade the watercourse, and was repeated annually to maintain the thinned canopy effect. We validated the effect of canopy thinning by quantifying total ambient irradiance at all experimental sites starting ca. 12-months prior to introduction and extending ca. 12-months after introduction. Measures were recorded continuously across daylight hours in units of lumens/m^2 (Lux) using Hobo® data loggers [Onset Computer Corp., Bourne, Massachusetts; refer to Kohler et al. (2012) for further detail upon these methods]. It is important to note that Lux is a human-biased measure, and therefore imperfectly estimates absolute quantum irradiance and the full spectrum available for guppy vision (as discussed in detail by Endler 1993a). We therefore use the aggregate mean of daily data logged between September 2008 and 2009 as an index of relative light intensity across sites arising due to the canopy thinning treatment. These data are also the basis of the photosynthetically active radiation (PAR) estimates presented in Figure 3 of Kohler et al. (2012).

FISH SAMPLES

Fish were sampled from the Guanapo source site in 2008 (immediately prior to the first introductions), and then in 2010, 2013 and 2014. Fish were sampled from the experimental sites in 2013 and 2014. In all cases, juveniles were collected and then reared to maturity in single sex groups under a common laboratory environment. The collection protocol involved sampling immature individuals from each and every pool at each site, to ensure representation of genetic diversity, and yielded 40-50 individuals for each population. Given that the probability of survival to adulthood is only around 20 % at these sites, our sampling effort was also sensitive to potential population impacts. Upon reaching maturity, sampled males and females from each locality were then randomly paired to produce an F1 generation. This generation was raised under the same conditions as their parents, with color assessed for fish once they reached adulthood. Our approach to rearing immature-collected parents in a "common garden"

environment (including a controlled diet), then collecting data from offspring that were reared under the same conditions ensured that our assessments could identify genetic differences in male coloration.

PHOTOGRAPHY PROTOCOLS

Specimens were photographed using standard procedures (detailed in Kemp et al. 2008) using a Canon EOS 600D digital camera fitted with an EF 100 mm f/2.8 fixed macro lens. Briefly, each specimen was anesthetized with a pH-buffered solution of ethyl 3-aminobenzoate methane sulfonic acid salt (MS-222) then laid upon moistened card (see below) atop a Leitz UT-4 multi-axis crystallography stage. The camera was situated overhead at 90° relative to the stage at a standard working distance of 200 mm. Illumination was provided by a light emitting diode source situated 45° dorsally in the transverse plane, which emitted light across a spectral range from 375 to 725 nm. The stage was rotated to tilt each specimen's head slightly downward such that the lateral flank was parallel relative to the camera's focal plane. Glare was minimized by pipetting small amounts of water onto the specimen immediately prior to image capture.

Both sides of each fish were photographed, first against a matte black background (black card stock), and then against a matte white background (artist's watercolor paper). We used both black and white backgrounds because the former affords accurate assessment of structural color (i.e., iridescent violet/blue/cyan/green), whereas the latter affords accurate assessment of orange and black spots (see **Supporting Information** for exemplar images against each background). The camera's field-of-view included a 20 mm graduated scale, and a 20 x 10 mm composite standard divided equally among filter paper and black cardstock.

COLOR ASSESSMENT

Raw format images (Canon Raw 2nd Edition [*.CR2] files) were first imported into Adobe Photoshop Elements v14.1 and adjusted for minor exposure differences according to the white/black standard contained in each image. Body and color patch areas were quantified using the "polygon" tool in Motic Images Plus v2.0.

Following extensive precedents (e.g., Endler 1980, 1983; Brooks and Endler 2001a; Millar et al. 2006; Kemp et al. 2008, 2009), we sought to quantify the areal coverage of four main elements: (1) black spots, (2) orange spots, (3) iridescent blue and (4) iridescent green. Whereas the former two elements are discrete and easily demarcated, the iridescent (structurally-colored) markings are not always so. Our classification of blue versus green markings was necessarily limited by their "human-visible" appearance in static photographs. These elements result from similar platelet microstructures (Grether et al. 2004), and as typical for colors borne from multilayer interference, both indicate changes in peak hue and reflectance intensity with changes in viewing orientation. This complicates the discrete categorization of blue and green, and in fact these markings tend to grade into each other across the flank via intermediate hues best described as "cyan". Consistent with this, the coverage of these two components are negatively correlated across the flank (**online Tables S1-S2**). We therefore recognize a degree of overlap in the classification of blue and green, and treat these elements separately as well as jointly in our analyses.

Body and color patch areas were assessed for each specimen's left and right flank using blind-coded images randomly sorted with regard to population, individual and year. Measures were strongly correlated across flanks (Pearson's correlation coefficients: black $r = 0.880$; orange $r = 0.945$; blue $r = 0.832$; green $r = 0.949$; body area $r = 0.985$ [N = 206; $P < 0.001$ in all cases]) and we therefore averaged values for each individual.

WHOLE COLOR PATTERN APPRAISAL

We analyzed variation at the whole-phenotype level using two approaches. First, we calculated an index of color diversity (*D*), which expressed the evenness of contribution by black/orange/blue/green). Following Endler (1978; pp. 347), this was derived from the expression:

$$D = \frac{1}{\sum P_i^2}$$

Where P_i represents relative coverage for each of $i = 4$ elements.

Second, we explored color phenotype constitution using an ordination approach based on the Maxwell triangle (Maxwell 1860). Individual phenotypes were represented in 2-dimensional

Cartesian space according to their lateral coverage of black-versus-orange-versus-iridescence relative to total ornamented area (i.e., the sum of all elements). This generated effect sizes for whole-phenotype variation while aiding interpretability because the vertices of the triangle correspond directly to coverage of each color class. Representation of phenotypes in two-dimensional space required pooling the blue and green markings (and hence their "cyan" intermediates) to derive a single value for iridescent coverage. Cartesian (x,y) coordinates for individual phenotypes within the triangle were calculated via the formulae:

$$x = \sqrt{\frac{1}{2}}.(Irid - Blk) \qquad y = \sqrt{\frac{2}{3}}.\left(Ora - \frac{Irid + Blk}{2}\right)$$

Where "*Blk*" = black, "*Ora*" = orange, and "*Irid*" = blue + green.

We plotted individuals and means for each population/year. Phenotypic difference between any two points within the triangle was calculated as Euclidean distance (*d*) according to the Pythagorean Theorem:

$$d(x,y) = \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2}$$

STATISTICAL ANALYSIS

We first assessed color change at the Guanapo source site between 2008 (before upstream introduction) to 2014 (when latest measures were obtained from all sites). Here we used a General Linear Model (GLM) with year treated as a continuous variable. For this contrast – and in all GLMs subsequently described – we conducted a multivariate analysis that included the lateral coverage of all four color elements simultaneously, as well as separate univariate analyses for each element, color diversity (*D*), and body size. Significance of multivariate models was assessed using Wilks lambda (λ) for multiple *F*-values (Everitt and Dunn 1991). We elected not to use a repeated-measures design because unique samples were drawn each year and multiple generations of reproduction occur per-annum (Reznick et al. 1997).

Data obtained in 2013 and 2014 spanned all study sites, which for experimental populations represented either four and five years (Caigual and Taylor) or five and six years (Upper and Lower Lalaja) following upstream introduction. Our main analysis consisted of a nested factorial

GLM with a "hanging" control group, which is based upon Type IV sums of squares. The hanging control refers to the obvious absence of nested canopy replicates at the Guanapo source site. Model effects included year (2013-14), canopy treatment (control/intact/thinned), year x treatment, and population (with the two replicate sites nested within each experimental treatment). This design achieves balance for the amount of time since introduction among experimental canopy treatments, because fish were introduced to one site in each case (i.e., Lower Lalaja [intact] and Upper Lalaja [thinned]) 12-months prior to introducing fish to their paired replicate site (the Caigual and Taylor, respectively).

Given that it is unclear whether selection would influence ornament area in absolute or size-relative terms (Endler 1980), we assessed both possibilities by conducting two sets of GLMs. These differed only via the inclusion of body area as a covariate in models of size-relative coverage. In all cases, outcomes were identical at $\alpha = 0.05$ to those obtained from a factorial Year x Population model based on Type I sums of squares, which supports the robustness of our conclusions.

Each GLM generated omnibus tests for all three main effects, plus the year x treatment interaction. Based upon the experimental design/rationale, we also planned four orthogonal contrasts to address specific hypotheses regarding evolutionary divergence among treatments and sites:

1. **Control versus all introduction sites** (G vs. [LL+UL+C+T]); designed to test for common change due to the overall effect of lower predation pressure (and any effects due to correlated ecological changes such as increased overall population density);
2. **Intact versus thinned introduction sites** (LL+C vs. UL+T); designed to test for overall divergence among low predation fish due to environmental manipulation; that is, whether populations founded under thinned canopies evolved in different ways to those founded under intact canopies;
3. **Among replicate intact populations** (LL vs. C); designed to test differences among experimental populations subject to unaltered (natural) overhead canopy conditions;
4. **Among replicate thinned populations** (UL vs. T); as per the above contrast for the two thinned-canopy experimental populations.

We supplemented this analysis with a GLM that included mean daily ambient light levels across the four experimental sites as a continuous predictor variable (which effectively substituted for both treatment and population terms in earlier analyses). This approach treated the four experimental sites as random replicates of varied canopy coverage according to their differences in ambient illumination.

Analyses were performed with Statistica™ version 7.0 (Statsoft, Tulsa, USA). Body and color area variables were normalized using the natural log-transformation. Means are quoted with standard deviations unless otherwise specified.

Results

VARIATION OVER TIME AT THE SOURCE SITE

Source site (Guanapo) fish indicated no linear trend in body size from 2008 to 2014 ($F_{1,67}$ = 0.975, P = 0.33; **Table 1**). There was however evidence for variation in multivariate color phenotype over this timeframe (Wilks λ = 0.861, $F_{4,64}$ = 2.59, P = 0.045). Single-trait analysis indicated a decrease in absolute black spot cover ($F_{1,67}$ = 7.80, P < 0.01), an increase in iridescent green ($F_{1,67}$ = 4.82, P < 0.05), but no difference in lateral coverage of orange spots ($F_{1,67}$ = 0.009, P = 0.92) or iridescent blue ($F_{1,67}$ = 0.027, P = 0.87). Equivalent results were gained (at α = 0.05) for analyses of size-relative coverage (see **Supporting information**), which follows logically from the lack of body size variation across years. Male ornament constitution – that is, the relative coverage of black-versus-orange-versus-iridescent blue/green – varied little across sampling years (i.e., 0.023 > d > 0.065; **Figure 3a**), a result that we expand upon further below.

DIVERGENCE OF EXPERIMENTAL POPULATIONS

We examined the divergence of experimental phenotypes from the source population and among/within canopy treatments using 2013 and 2014 data (**Table 1**). This represents 4 – 6 years following upstream translocation. GLM results are outlined below for both absolute and size-controlled ornament coverage, and detailed respectively in **Tables 2–3**. Notably, omnibus main and interactive effects of sampling year proved non-significant in all models, which validated the pooling of 2013 and 2014 data for our planned contrasts.

Overall translocation (predator) effects

Our first planned contrast assessed the divergence of all experimental populations from their 2013-14 Guanapo (source population) contemporaries. Overall, experimental fish were significantly larger at maturity and exhibited greater coverage of blue and green (see contrast 1 in **Table 2**). Although the strength of population divergence varied (explored further below), male ornamentation generally tracked towards relatively greater coverage of iridescence following translocation (**Figures 2 & 3**). Importantly, the significant color differences persisted once population variation in body size was controlled (**Table 3**), meaning that the increased ornamentation of experimental populations did not arise simply due to increased body area.

Environmental (canopy) effects

Our next planned contrast assessed divergence among the different experimental treatments, and hence explicitly addressed the role of canopy modification. Fish from intact-canopy populations were smaller on average than their thinned-canopy counterparts. The two groups also diverged in multivariate color phenotype, a difference again evident in both absolute and size-controlled analysis (refer to planned contrast 2 in **Tables 2–3**). In terms of absolute coverage, intact canopy fish exhibited greater black, whereas thinned canopy fish exhibited greater iridescent blue and green. Controlling for body size (**Figure 2**) revealed significant deviation among treatments in black only, although the effect for green fell at the critical significance level (i.e., $P = 0.051$; **Table 3**). Color pattern constitution (**Figure 3b**) tracked more consistently towards greater relative iridescent coverage in the two thinned-canopy populations.

Replicate (within-canopy treatment) effects

Our remaining contrasts assessed the conformity of phenotypic responses among replicates of each canopy treatment (i.e., contrasts 3 and 4 in **Tables 2–3**). These revealed significant color variation only in the extent of blue iridescence among the two intact-canopy populations, with greater coverage in Lower Lalaja fish (**Figure 2**). In phenotype space (**Figure 3a**), Lower Lalaja fish evolved in the direction of greater iridescent coverage over twice as much as Caigual fish (i.e., $d = 0.159$ versus $d = 0.073$). By contrast, thinned-canopy replicates varied only in body size, which was greater in the Taylor population (**Table 1**).

The effect of ambient illumination

We supplemented the above models by analyzing color variation purely as a function of mean ambient illumination across the four experimental sites. Light intensity predicted significant variation in multivariate color phenotype, both when calculated as absolute coverage (Wilks λ = 0.772, $F_{4,94}$ = 6.93, $P < 0.001$) and body size-relative coverage (Wilks λ = 0.830, $F_{4,93}$ = 4.74, $P <$ 0.005; **Table 4**). In terms of absolute color patch size, light intensity was negatively related to black coverage but positively related to blue. Analysis of size-relative color coverage revealed a significant effect only upon the extent of black (**Table 4**; **Figure 4**).

Discussion

The manipulative study of adaptation in natural populations is challenging if not untenable for most animals. Guppy translocation experiments have proven exceptional in this regard, and support a paradigm for adaptation under a shifting tension between sexual and natural selection (Endler 1980, 1983; Reznick et al. 1990). At the broadest level, our findings strengthen this paradigm by demonstrating rapid elaboration in a sexual trait under relaxed predation. This reiterates the high selective consequence of predation for guppy populations in the wild (Endler 1978; Millar et al. 2006). More significantly, our replicate populations inform the paradigm by providing new insight into the consistency of adaptation and its key drivers such as signaling habitat. In discussing these points we draw upon the comparative basis offered by previous translocation experiments of the very same nature (e.g., Endler 1980, 1983; Kemp et al. 2009). Given the weight of evidence for mate choice in guppies, it is appropriate to focus upon female preference as the putative driver of male color evolution. We however also note that translocated males may have varied in courtship tactics (Gamble et al. 2003) and/or post-copulatory reproductive success (e.g., Evans et al. 2003, Devigili et al. 2015), and address those alternatives below.

ADAPTIVE TRAJECTORIES FOLLOWING TRANSLOCATION

The design of this study allows direct comparison with prior single-site translocation experiments in the Aripo and El Cedro Rivers (Endler 1980, 1983; Kemp et al. 2009), the latter being a co-tributary within the Guanapo drainage. Founding fish in all cases colonized similar small

headwater streams that previously lacked guppies and their main predator (*Crenicichla*), but supported resident populations of *Rivulus*. As outlined earlier, male ornamentation was seen to track different trajectories across these experiments (Kemp et al. 2009). The absence of replication in the wild however made it unclear whether this difference arose from adaptation versus stochastic factors such as founder effects and genetic drift. We discuss three ways in which our findings inform this question: (1) the role of initial biases in pre-disposing adaptive trajectories, (2) the influence of signal environment, and (3) the strength of adaptation versus drift.

The role of initial biases

Aside from the Caigual, we found that experimental populations clearly diverged from the average source phenotype via greater cover of blue and/or green, and in the case of thinned-canopy sites, a reduction in black (**Figure 2**). Elaboration of this nature largely parallels the pattern of evolution reported for the El Cedro experiment (Kemp et al. 2009), where the coverage of structural color expanded at the expense of black and orange. This means that four of the five experimental populations established thus far in the Guanapo drainage (across two different source sites) have tracked towards an average male phenotype dominated by iridescence. This trajectory varies from the pattern of evolution in both the Aripo translocation and its companion artificial stream experiment using admixed genetic stock (Endler 1980, 1983). In each case, *Crenicichla*-free populations evolved increased lateral coverage of all main color elements. Notably, this is also the phenotype characteristically seen across most natural guppy populations subject to low predation intensity (Endler 1978). Predator community composition can sometimes influence which colors are locally favored (Millar et al. 2006; Kemp et al. 2008), but comparative population data show little evidence that structural colors such as violet, blue, green and silver are either overtly favored or disfavored by reduced predation per-se. Together with the different experimental trajectories observed for Aripo versus El Cedro and Guanapo fish (for which high-versus-low predation communities were identical), this implies a selective background that particularly favors structural color in Guanapo populations.

Variation in male color patterns among extant guppy populations have been used to infer differences in female preference (Houde and Endler 1990; Houde 1994; Endler and Houde 1995). Such variation is thought to arise from (and hence reflect) population divergence in the genetic basis of female choice (Houde and Endler 1990). There is also evidence for linkage

between preference and trait genes (Houde 1994) in the manner predicted by Fisher's (1930) "run-away" model of sexual trait evolution. Genetic background should therefore determine at least initial responses in male color following translocation. Drawing from the inferential approach of Houde and Endler (1990), one means of addressing this is to ask whether our observed adaptive trajectories essentially "amplify" how source phenotypes were more (or less) exaggerated to begin with. Notably, the available comparative data do point to greater elaboration of iridescence and a relatively low per-body coverage of orange and black in Guanapo drainage populations (Houde and Endler 1990; Kemp et al. 2008, 2009). A more direct line of enquiry would however use knowledge of female preference itself. Such insight is presently limited to a single Guanapo population studied by Endler and Houde (1995). On average, females from this population expressed a bias against black, neutrality for blue/violet, and positive biases for orange, white and two iridescent elements: silver and bronze-green. This profile is partly consistent with our observed trajectories, especially for iridescent green (**Figure 2**). It would also predict an elaboration of orange, which we did not observe, but which may have occurred earlier in the study (see below). We also note that Endler and Houde's (1995) Guanapo sample came from a medium-predation site lacking *Crenicichla*, so the preferences expressed by their females may not represent those of our source population.

Further (albeit limited) insight into the role of initial biases comes from Gordon's et al. (2015) study of Upper and Lower Lalaja populations conducted just one year following translocation. Males from both sites displayed no appreciable change in black but an increase in orange pigmentation. Structural colors were not estimated, which precludes a full comparison with our data. Nevertheless, the initial increase of orange suggests that founding fish may well have possessed a preference for these markings, which would agree with Endler and Houde's (1995) data. More fundamentally, the contrast of male phenotypes in year 1 (Gordon et al. 2015) versus years 5-6 (this study) implies that initial trajectories of color evolution were not sustained. We do not consider this necessarily surprising. It is reasonable to expect a degree of "inertia" for the incumbent features of social dynamics, particularly if they are based upon complex genetic architectures. Color patterns and mate choice in guppies are regulated by Y- and X-linked genes and linkage complexes within the sexes (e.g., color "supergenes"; Haskins et al. 1970) as well as linkage disequilibrium across the sexes (i.e., between trait and preference genes; Houde 1994). The disruption of established linkage relationships by directional selection will require several generations of recombination to resolve, hence delaying the evolution of new adaptive optima.

In addition, reciprocal feedbacks between guppies and their newly colonized environments (e.g., El-Sabaawi et al. 2015) will modify the nature of selection over time. We know for example that experimental populations experienced rapid intrinsic rates of increase, which in-turn substantially altered the composition of resident algal communities (Bassar et al. 2017). This has direct relevance for orange spots because algae provide the carotenoid pigments needed to express such markings. Males may have therefore faced an increasing restriction in their ability to display more extensive orange ornamentation.

The role of light environment

The observed canopy treatment effects suggest a causal role of ambient signaling environment as a driver of evolutionary trajectory (*sensu* Endler 1992, 1993a,b). This result compliments findings from population-level comparative studies in color polymorphic fishes (Fuller 2002; Gray et al. 2008), as well as recent behavioral work in guppies (Cole and Endler 2015; Gamble et al. 2003; see further below). Canopy thinning would increase the amount of waterway exposed to direct sunlight throughout the day. Male guppies are known to preferentially court under low-light conditions around dawn and dusk (Archard et al. 2009), which corresponds to the inverse of diel *Crenicichla* activity (Endler 1987). In the absence of *Crenicichla*, it seems reasonable to expect that courtship activity would extend beyond this period. Even a moderate diel extension would modify the envelope of visual display conditions, chiefly via an increase in the intensity (and spectral quality) of illumination, and more so at thinned-canopy sites. It is therefore notable that males from these sites exhibited more extensive structural (iridescent) coloration (**Figure 3**). These markings are extremely reflective, but also highly sensitive to viewing orientation (Kemp et al. 2008, 2009), which means they often contribute strobe or "flashing" effects. As documented for birds (Dakin et al. 2016) and butterflies (White et al. 2015), such effects are greatly enhanced under direct sunlight, leading in particular to high background contrast and greater long-range visibility (Endler 1983). A greater opportunity to showcase iridescent flashes in full-sunlight may therefore explain why structural colors increased more greatly under thinned canopies. If so, the associated reduction in black would imply greater value in prioritizing background contrast over within-pattern contrast, that is, the capacity for long-range detection versus the display of a more diverse ornament at close-range.

Aside from its direct effect upon signaling environment, canopy treatment may have modified the context of sexual competition in ways that indirectly drove ornament evolution. We have

already noted how guppy population dynamics may have restricted the availability of resources such as algal-derived orange pigment. Canopy thinning is known to have influenced primary productivity (Kohler et al. 2012), which predicts a systematic treatment difference in the strength of such restriction. Our data do not support this. Orange coverage was in fact lowest at the site where light levels were highest (i.e., the Taylor; **Figure 4**), which is counter to expectation under a carotenoid-limitation hypothesis. A second possibility is that stream lighting influenced patterns of female responsiveness and/or male mating tactics. Relevant to this, Gamble et al. (2003) demonstrated increased female responsiveness to male displays and reduced rates of sneak-mating attempts under lighting conditions akin to midday woodland shade as compared with early/late and forest shade treatments. Woodland shade typifies more sparsely vegetated habitats when most illumination comes from a clear sky, whereas forest shade exists beneath closed canopies (Endler 1993a). We therefore consider woodland shade conditions more likely to exist at thinned-canopy sites. If Gamble's et al. (2003) findings are generalizable, then a greater precedence for display-based mating success could explain the more concerted rates of ornament evolution observed in the Upper Lalaja and Taylor. Finally, color could have evolved because it is genetically correlated with other fitness traits. Given that pre- and post-copulatory events contribute equally to male reproductive success (Devigili et al. 2015), one potential avenue lies in the features of sperm competitiveness. There is some evidence for genetic links between such features and male coloration (Evans et al. 2003; Evans 2010). Such evidence however remains inconsistent (see, for example, Boschetto et al. 2011; Locatello et al. 2006; Pitcher et al. 2007), so this and other possibilities regarding genetic architecture stand for future investigation.

Adaptation versus drift

Concerted evidence for color elaboration following upstream guppy translocation (Endler 1980; Kemp et al. 2009), coupled with the emergence of habitat effects (this study), argue for the power of adaptive change in small founder populations. Adaptation evidently proceeds in such populations even despite their likely reduction in heterozygosity, loss of rare alleles, and increased genome-wide linkage disequilibrium. Stochastic processes such as genetic drift could nevertheless influence the precise manner in which male ornamentation evolves to more elaborate states. For the current experiment, we can explicitly consider the potential for drift effects by estimating effective population size (N_e) using demographic data derived from monthly censuses starting from the beginning of each introduction (Arendt et al. 2014). The

harmonic means of N over time at each site (Falconer 1981) estimates N*e* ranging from 301 in the Caigual to 645 in the Upper Lalaja. Given the high promiscuity of guppies, multiple mating in both sexes and sperm storage (Lopez-Sepulcre et al. 2013), these values likely underestimate true N*e*. In any event, they fall well above the thresholds at which drift has been shown to influence adaptation in experimental populations (e.g., N < 20 in *Tribolium*; Rich et al. 1979). From this we conclude that site differences in ornamental change such as the relatively subdued Caigual response are unlikely to have resulted from genetic drift.

CONCLUSIONS

Our experiment represents a first in being a replicated study of evolution in a natural ecosystem that includes a manipulation of the light environment and hence the way that color is perceived and selected upon. There are two compelling features of our findings. The first is that male phenotypes evolved in a fundamentally different way under relaxed predation risk than previously seen in other streams, but in similar ways as reported for a prior experiment within the same drainage (Kemp et al. 2009). Comparative data upon male ornamentation and attractiveness to females (Houde and Endler 1990; Endler and Houde 1995) reveal a correspondence between male coloration and female preference. It thus becomes plausible, and testable, to propose that differences among rivers in how guppies adapt to life without predators are at least in part driven by the subtleties of ingrained female preference.

Second, our data implicate ambient illumination as a third main influence (after predation intensity and female preference) capable of shaping how male coloration evolves. Our results imply that guppies in streams with thinned canopies are experiencing more intense selection and hence evolving more rapidly. It will be interesting to see whether all populations ultimately reach a similar endpoint, or whether thinned-canopy populations attain a different equilibrium phenotype characterized by greater iridescent markings. Should the latter prove true, it would mean that existing conclusions based upon comparing high-versus-low predation sites may have in fact systematically underestimated the effect of predators upon male coloration. This is because high predation environments also tend to be wider streams with more open canopies and higher light levels (Reznick et al. 2001). Ambient light intensity and predation risk are therefore often confounded in the wild, but in a way that would mask the apparent impact of predation.

If thinned canopies cause male coloration to evolve to a different endpoint, then our system will also enable an examination of how female visual perception combines with male behavior and light environment to shape ornament evolution. Thus far it has been assumed that male guppies perform courtship displays primarily in the early morning and late afternoon (Archard et al. 2009; Endler 1987). However, drawing from the principles of sensory drive (Endler 1992, 1993a,b), we can use our habitat light manipulations to ask if the evolution of male coloration is integrated with female visual sensitivity and possibly changes in where and/or when males invest in display-based courtship. It will moreover become possible to address the extent to which these differences have a genetic basis, and whether female preference evolves as part of this dynamic interaction among predators, prey and the environment.

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Table 1: Summary of absolute phenotypic measures for male guppies derived from study populations between 2008 and 2014. All parameters are presented here ± 1 standard deviation, and all except color diversity were measured in units of mm^2.

Population	Year	N	Body area	Color element coverage				Color patch diversity (*D*)
				Black spots	Orange spots	Iridescent blue	Iridescent green	
Source (founding) site:								
G Guanapo								
Pre-introduction:	2008	16	53.0 ± 5.16	2.29 ± 0.617	3.27 ± 1.91	4.38 ± 1.70	0.915 ± 0.694	3.00 ± 0.406
Post-introduction:	2010	19	53.5 ± 5.54	2.21 ± 0.619	2.70 ± 1.18	3.26 ± 1.68	1.13 ± 2.40	2.91 ± 0.431
	2013	16	52.2 ± 8.38	1.81 ± 1.02	2.76 ± 1.19	3.35 ± 1.96	1.71 ± 0.992	3.15 ± 0.464
	2014	18	51.6 ± 5.27	1.73 ± 1.12	3.08 ± 1.31	4.55 ± 1.81	1.43 ± 1.17	2.94 ± 0.471
Introduction sites with intact canopies:								
LL Lower Lalaja	2013	4	58.6 ± 7.31	2.31 ± 1.24	3.50 ± 1.40	9.69 ± 3.94	1.05 ± 1.29	2.46 ± 0.527
	2014	15	58.3 ± 4.51	2.29 ± 1.50	3.02 ± 1.46	6.79 ± 2.29	2.36 ± 1.62	2.92 ± 0.644
C Caigual	2013	20	60.4 ± 5.50	1.75 ± 0.685	2.99 ± 1.06	4.46 ± 1.84	2.45 ± 1.73	3.11 ± 0.480
	2014	4	57.8 ± 2.52	2.55 ± 1.12	3.72 ± 0.34	4.78 ± 1.48	3.39 ± 2.11	3.48 ± 0.326

Introduction sites with thinned canopies:

UL Upper Lalaja	2013	17	61.0 ± 7.42	1.63 ± 0.808	3.07 ± 1.36	6.86 ± 3.92	3.29 ± 2.45	2.85 ±0.747
	2014	13	58.2 ± 3.65	1.68 ± 0.746	3.68 ± 1.70	6.33 ± 2.66	4.24 ± 3.30	2.94 ±0.563
T Taylor	2013	10	67.8 ± 7.67	1.45 ± 0.740	3.18 ± 1.83	7.33 ± 2.78	3.48 ± 3.33	2.70 ± 0.333
	2014	17	62.7 ± 6.95	1.17 ± 0.690	2.75 ± 1.09	8.11 ± 3.51	3.97 ± 3.72	2.54 ± 0.676

Table 2: GLM results for the analysis of absolute phenotypic trait size in 2013-14 fish.

Omnibus effects:	**All color traits (multivariate model)**	**Body size**	**Black spots**	**Orange spots**	**Iridescent blue**	**Iridescent green**	**Color patch diversity (*D*)**
Intercept*	Wilks λ = 0.111, $F_{1,126}$ = 249	$F_{1,126}$ = 27100	$F_{1,126}$ = 199	$F_{1,126}$ = 296	$F_{1,126}$ = 299	$F_{1,126}$ =56.5	$F_{1,126}$ =243
Year 2013 vs. 2014	Wilks λ = 0.966, $F_{4,123}$ = 1.09, P = 0.36	$F_{1,126}$ = 1.88, P = 0.17	$F_{1,126}$ = 0.042 P = 0.84	$F_{1,126}$ = 0.600 P = 0.44	$F_{1,126}$ = 0.856 P = 0.36	$F_{1,126}$ =1.58 P = 0.21	$F_{1,126}$ =0.093 P = 0.59
Treatment control vs. intact vs. thinned	Wilks λ = 0.588, $F_{8,246}$ = 9.35, P < 0.001	$F_{2,126}$ = 35.5 P < 0.001	$F_{2,126}$ = 3.41 P < 0.05	$F_{2,126}$ = 0.464 P = 0.63	$F_{2,126}$ = 19.3 P < 0.001	$F_{2,126}$ =6.26 P < 0.005	$F_{2,126}$ =5.04 P < 0.01
Population [LL vs. C] + [UL vs. T]	Wilks λ = 0.874, $F_{8,246}$ = 2.14, P < 0.05	$F_{2,126}$ =4.72 P < 0.05	$F_{2,126}$ =1.27 P = 0.28	$F_{2,126}$ = 0.711 P = 0.49	$F_{2,126}$ = 6.74 P <0.005	$F_{2,126}$ =1.77 P = 0.17	$F_{2,126}$ =5.57 P < 0.005
Year x Treatment	Wilks λ = 0.951, $F_{8,246}$ = 0.787, P = 0.61	$F_{2,126}$ = 0.008, P = 0.50	$F_{2,126}$ = 0.381 P = 0.68	$F_{2,126}$ = 0.102 P = 0.90	$F_{2,126}$ = 2.07 P = 0.13	$F_{2,126}$ =1.70 P = 0.19	$F_{2,126}$ =2.39 P = 0.10
Planned contrasts:							
1 Source vs. translocation sites G vs. [LL+C+UL+T]	Wilks λ = 0.652, $F_{4,123}$ = 16.4, ***P*** **< 0.001**	$F_{1,126}$ = 57.6 ***P*** **< 0.001**	$F_{1,126}$ = 0.052 P = 0.82	$F_{1,126}$ = 0.571 P = 0.45	$F_{1,126}$ = 31.3 ***P*** **< 0.001**	$F_{1,126}$ =9.87 ***P*** **< 0.005**	$F_{1,126}$ =2.31 P = 0.13
2 Intact vs. thinned sites [LL+C] vs. [UL+T]	Wilks λ = 0.831, $F_{4,123}$ = 6.26, ***P*** **< 0.001**	$F_{1,126}$ = 5.12 ***P*** **< 0.05**	$F_{1,126}$ = 8.82 ***P*** **< 0.005**	$F_{1,126}$ = 0.074 P = 0.79	$F_{1,126}$ = 4.75 ***P*** **< 0.05**	$F_{1,126}$ =5.85 ***P*** **< 0.05**	$F_{1,126}$ =5.21 ***P*** **< 0.05**

3	Among intact sites LL vs. C	Wilks λ = 0.918, $F_{4,123} = 2.74$, ***P* < 0.05**	$F_{1,126} = 0.110$ $P = 0.74$	$F_{1,126} = 0.032$ $P = 0.86$	$F_{1,126} = 0.131$ $P = 0.72$	$F_{1,126} = 10.7$ ***P* < 0.005**	$F_{1,126} = 3.51$ $P = 0.063$	$F_{1,126} = 7.61$ ***P* < 0.01**
4	Among thinned sites UL vs. T	Wilks λ = 0.951, $F_{4,123} = 1.60$, $P = 0.18$	$F_{1,126} = 9.34$ ***P* < 0.005**	$F_{1,126} = 2.51$ $P = 0.11$	$F_{1,126} = 1.29$ $P = 0.26$	$F_{1,126} = 2.82$ $P = 0.096$	$F_{1,126} = 0.034$ $P = 0.85$	$F_{1,126} = 3.54$ $P = 0.062$

Population abbreviations as per Table 1. Significant planned contrasts are highlighted in bold.
*All intercepts were significant at P < 0.001.

Table 3: GLM results for the analysis of size-relative lateral coverage of color elements for 2013-14 fish.

Omnibus effects:	**All color traits (multivariate model)**	**Black spots**	**Orange spots**	**Iridescent blue**	**Iridescent green**
Intercept	Wilks λ = 0.882, $F_{1,122} = 4.07$, $P < 0.005$	$F_{1,125} = 0.602$ $P = 0.44$		$F_{1,125} = 1.92$ $P = 0.17$	$F_{1,125} = 3.61$ $P = 0.06$	$F_{1,125} = 2.89$ $P = 0.09$
Year 2013 vs. 2014	Wilks λ = 0.942, $F_{4,122} = 1.88$, $P = 0.12$	$F_{1,125} = 0.050$ $P = 0.82$		$F_{1,125} = 0.547$ $P = 0.46$	$F_{1,125} = 1.77$ $P = 0.19$	$F_{1,125} = 2.36$ $P = 0.13$
Treatment control vs. intact vs. thinned	Wilks λ = 0.785, $F_{8,244} = 3.92$, $P < 0.001$	$F_{2,125} = 3.14$ $P < 0.05$		$F_{2,125} = 0.475$ $P = 0.62$	$F_{2,125} = 7.51$ $P < 0.001$	$F_{2,125} = 1.39$ $P = 0.25$
Population [LL vs. C] + [UL vs. T]	Wilks λ = 0.881, $F_{8,244} = 1.99$, $P < 0.05$	$F_{2,125} = 1.25$ $P = 0.29$		$F_{2,125} = 0.606$ $P = 0.55$	$F_{2,125} = 6.41$ $P < 0.005$	$F_{2,125} = 1.99$ $P = 0.14$
Year x Treatment	Wilks λ = 0.952, $F_{8,244} = 0.753$, $P = 0.64$	$F_{2,125} = 0.370$ $P = 0.69$		$F_{2,125} = 0.109$ $P = 0.90$	$F_{2,125} = 1.90$ $P = 0.15$	$F_{2,125} = 1.93$ $P = 0.15$
Body size (covariate)	Wilks λ = 0.813, $F_{4,122} = 7.00$, $P < 0.001$	$F_{2,125} = 0.033$ $P = 0.86$		$F_{2,125} = 0.049$ $P = 0.83$	$F_{2,125} = 9.76$ $P < 0.005$	$F_{2,125} = 4.94$ $P < 0.05$
Planned contrasts:						
1 Source vs. translocation sites G vs. [LL+C+UL+T]	Wilks λ = 0.857, $F_{4,122} = 5.10$, ***P* < 0.001**	$F_{1,125} = 0.007$ $P = 0.93$	$F_{1,125} = 0.558$ $P = 0.46$	$F_{1,125} = 9.28$ ***P* < 0.005**	$F_{1,125} = 1.95$ $P = 0.16$	
2 Intact versus thinned sites [LL+C] vs. [UL+T]	Wilks λ = 0.857, $F_{4,122} = 5.10$, ***P* < 0.001**	$F_{1,125} = 8.72$ ***P* < 0.005**	$F_{1,125} = 0.049$ $P = 0.83$	$F_{1,125} = 2.53$ $P = 0.11$	$F_{1,125} = 3.88$ $P = 0.051$	

3	Among intact sites LL vs. C	Wilks $\lambda = 0.911$, $F_{4,122} = 2.98$, ***P*< 0.05**	$F_{1,125} = 0.034$ $P = 0.85$	$F_{1,125} = 0.135$ $P = 0.71$	$F_{1,125} = 12.0$ ***P*< 0.001**	$F_{1,125} = 3.67$ $P = 0.070$
4	Among thinned sites UL vs. T	Wilks $\lambda = 0.967$, $F_{4,122} = 1.03$, $P = 0.39$	$F_{1,126} = 2.52$ $P = 0.12$	$F_{1,126} = 1.07$ $P = 0.30$	$F_{1,125} = 0.731$ $P = 0.39$	$F_{1,126} = 0.586$ $P = 0.44$

Population abbreviations as per Table 1. Significant planned contrasts are highlighted in bold.

Table 4: GLM results for the analysis of color as a function of mean ambient illumination across experimental sites.

	Black spots	Orange spots	Iridescent blue	Iridescent green
Absolute color coverage:				
Intercept	$F_{1,97} = 244$, $P < 0.001$	$F_{1,97} = 325$, $P < 0.001$	$F_{1,97} = 375$, $P < 0.001$	$F_{1,97} = 51.1$, $P < 0.001$
Ambient light	$F_{1,97} = 10.3$, ***P*< 0.005**	$F_{1,97} = 0.779$, $P = 0.38$	$F_{1,97} = 5.41$, ***P*< 0.05**	$F_{1,97} = 2.76$, $P = 0.10$
Size-relative color coverage:				
Intercept	$F_{1,96} = 0.572$, $P = 0.451$	$F_{1,96} = 1.00$, $P = 0.32$	$F_{1,96} = 0.424$, $P = 0.52$	$F_{1,96} = 125$, $P = 0.27$
Ambient light	$F_{1,96} = 9.31$, ***P*< 0.005**	$F_{1,96} = 0.676$, $P = 0.41$	$F_{1,96} = 2.76$, $P = 0.10$	$F_{1,96} = 1.19$, $P = 0.28$
Body area	$F_{1,96} = 0.0082$, $P = 0.93$	$F_{1,96} = 0.0006$, $P = 0.98$	$F_{1,96} = 2.95$, $P = 0.89$	$F_{1,96} = 2.29$, $P = 0.13$

Relationships for size-relative coverage are indicated in Figure 4. Significant effects are highlighted in bold.

Figure legends

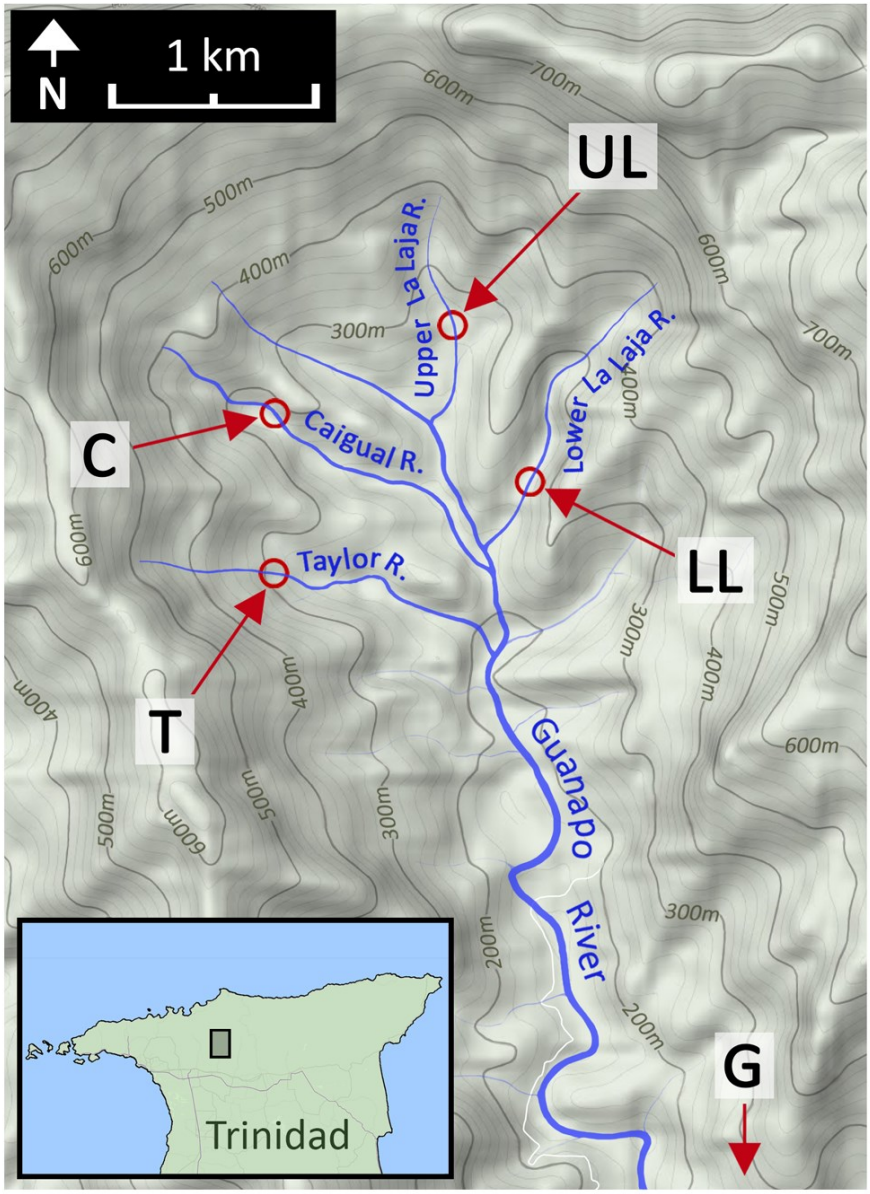

Figure 1

Map indicating the approximate location of the four upstream introduction sites. Guppies were introduced in either 2008 (LL & UL) or 2009 (C & T) from a single location on the Guanapo River, situated approximately 4 km downstream of the frame. Site abbreviations: "G" = Guanapo; "LL" = Lower Lalaja, and "C" = Caigual (intact canopy sites); "UL" = Upper Lalaja, and "T" = Taylor (thinned canopy sites).

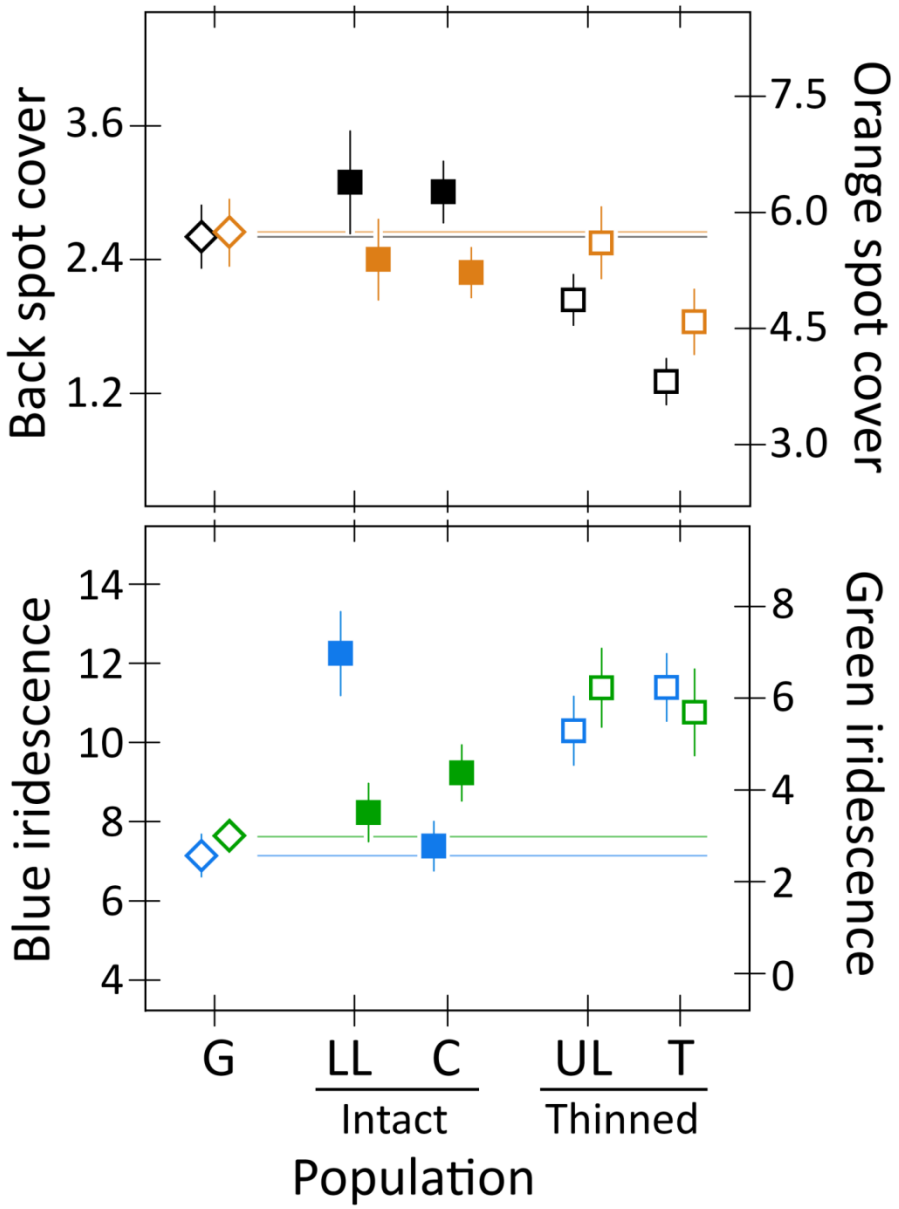

Figure 2

Lateral coverage of four primary color categories for fish measured in 2013-14, calculated as a percentage (%) of overall body area. Population means are indicated ± 1.0 standard error. Vertical axes are scaled to ± 1.5 the pooled standard deviation (calculated for fish across all populations) to enable direct comparison of relative effect size among color categories/panels. Mean source site values (Guanapo 2013-14) extend across each panel via the horizontal lines. Symbols are color-coded for each color element (black, orange, blue & green) and scaled to different Y-axes in each panel. Site labels are abbreviated as per Figure 1.

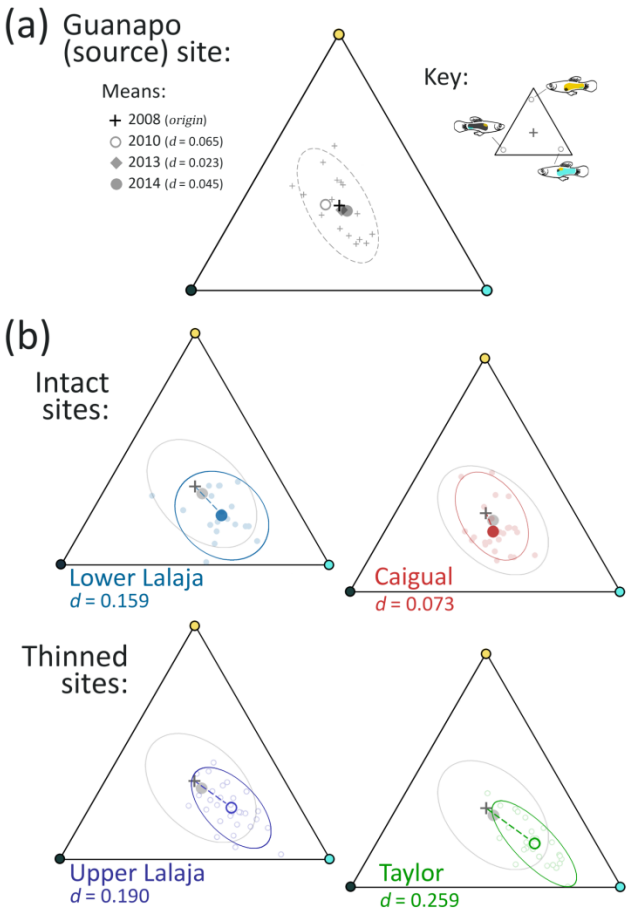

Figure 3

(a) Ordination of guppy color patterns at the Guanapo source site, spanning a timeframe from immediately prior to the upstream introductions (2008) to subsequent years at this site (2010, 2013 & 2014). The plot is an adaptation of the Maxwell triangle, whereby phenotypes are mapped according to the proportional coverage of orange (upper vertex) versus black (left vertex) versus iridescence (right vertex). The key image depicts the position of different stylized phenotypes. The main triangle is centered upon the 2008 mean phenotype (large cross), and indicates both individual fish (smaller crosses) and the 95 % confidence ellipse for this pre-introduction sample. Means for subsequent years at this site are plotted as depicted as larger symbols. **(b)** Ordination plots for color patterns among experimental populations in 2013-14. Each plot is centered upon the average phenotype for 2008 Guanapo fish (indicated as a cross), and shows the 2013-14 Guanapo mean as a halftone grey point. Individual and mean phenotypes for experimental populations are shown in arbitrary color with 95 % confidence ellipsoids. Mean divergence of each population from the 2008 Guanapo phenotype (*d*) is indicated by dashed lines.

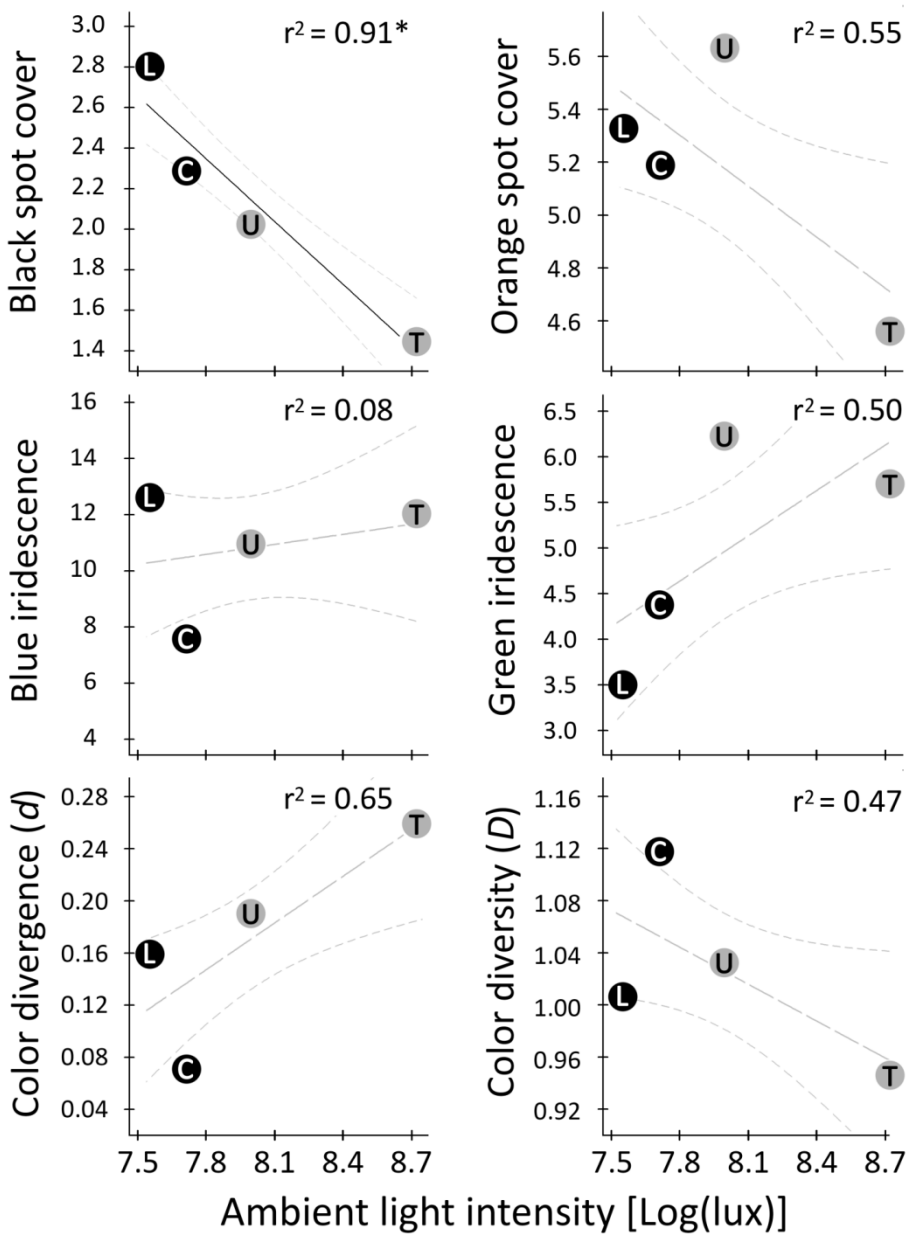

Figure 4

Relationships between mean ambient light level and mean color parameter values across the four experimental sites. Intact canopy sites are indicated as solid points ("L" = Lower Lalaja & "C" = Caigual) and thinned canopy sites indicated in halftone ("U" = Upper Lalaja & "T" = Taylor). Coverage of individual elements are represented as a percentage (%) of overall body area. Regression r^2 values are given for effect size, with best-fitting lines accompanied by 70 % confidence bounds. Color divergence (*d*; lower right panel) represents the difference from each population mean phenotype and the source site in 2008 (as shown in Figure 3). Non-significant regression lines are indicated in dashed halftone. *P<0.05.