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FROM THE COVER

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Sensory-based quantification of male colour patterns in Trinidadian guppies reveals no support for parallel phenotypic evolution in multivariate trait space

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Abstract

Parallel evolution, in which independent populations evolve along similar phenotypic trajectories, offers insights into the repeatability of adaptive evolution. Here, we re-visit a classic example of parallelism, that of repeated evolution of brighter males in the Trinidadian guppy (*Poecilia reticulata*). In guppies, colonisation of low predation habitats is associated with emergence of 'more colourful' phenotypes since predator-induced viability selection for crypsis weakens while sexual selection by female preference for conspicuousness remains strong. Our study differs from previous investigations in three respects. First, we adopted a multivariate phenotyping approach to characterise parallelism in multitrait space. Second, we used ecologically-relevant colour traits defined by the visual systems of the two selective agents (i.e., guppy, predatory cichlid). Third, we estimated population genetic structure to test for adaptive (parallel) evolution against a model of neutral phenotypic divergence. We find strong phenotypic differentiation that is inconsistent with a neutral model but very limited support for the predicted pattern of greater conspicuousness at low predation. Effects of predation regime on each trait were in the expected direction, but weak, largely nonsignificant, and explained little among-population variation. In multitrait space, phenotypic trajectories of lineages colonising low from high predation regimes were not parallel. Our results are consistent with reduced predation risk facilitating adaptive differentiation, potentially by female choice, but suggest that this proceeds in independent directions of multitrait space across lineages. Pool-sequencing data also revealed SNPs showing greater differentiation than expected under neutrality, among which some are found in genes contributing to colour pattern variation, presenting opportunities for future genetic study.

KEYWORDS
coloration, colour pattern analysis, guppy, parallel evolution, *Poecilia reticulate*, pool-sequencing

1 | INTRODUCTION

Parallel evolution is defined as the repeated, independent evolution of similar phenotypes under similar selection regimes in multiple independent populations within closely related lineages (Bolnick et al., 2018; Schluter et al., 2004). Notable examples include the repeated emergence of stream and lacustrine morphotypes in sticklebacks (McKinnon et al., 2004; Schluter et al., 2004), and of thin and thick shelled habitat-specific forms of periwinkle (Butlin et al., 2014). This phenomenon allows us to ask questions about the predictability of evolution. Does adaptation frequently recapitulate the same phenotypic "solutions" to the same selective challenges? Also, what are the genetic pathways and processes involved? However, characterizing evolutionary trajectories of complex multivariate phenotypes as being either parallel or not is too simplistic. In reality, any two trajectories may be more or less aligned in phenotypic space such that the degree of parallelism lies on quantitative continuum (Bolnick et al., 2018; Stuart et al., 2017). By characterizing the distribution of (multivariate) phenotypes within and among-lineages, we can quantify parallelism, testing for similarity in both magnitude and direction of evolutionary trajectories over ecological transitions. We can also assess the importance of parallel (adaptive) evolution within the context of other processes contributing to phenotypic divergence (e.g., drift). Here, we apply this approach to a well-known instance of putatively parallel evolution, that of replicated divergence in male colour patterns across predation regimes in the Trinidadian guppy (*Poecilia reticulata*) (Endler, 1980; Reznick et al., 1996).

Animal coloration and pattern traits serve many functions including signalling and crypsis (Cuthill et al., 2017) and have long been used to test predictions about the role of predation in driving parallel evolution (Allender et al., 2003; Houde, 1997; Steiner et al., 2009). High predation risk should select for less "conspicuous" colours and patterns (Endler, 1978, 1980; Young et al., 2011; Martin et al., 2014), but testing this may be sensitive to how colour phenotypes are quantified. Specific colour traits chosen for analysis often vary across studies even within species, while quantitative measures based on human perception (Martin et al., 2014) or RGB information (van Belleghem et al., 2018; Montenegro et al., 2019) may sometimes lack ecological relevance. The latter concern arises because colour signals may (co)evolve with the visual systems (and downstream behaviours) of receiver species (i.e., the sensory drive hypothesis; Endler, 1992). For instance, flower traits have coevolved with hymenopteran vision (Dyer et al., 2012), and variation in opsin gene sequence and expression is linked to colour polymorphism in African cichlids (Seehausen et al., 2008). While this means that human vision could misrepresent colour phenotypes as perceived by relevant selective agents (e.g., conspecific mates, predators), recent advances have improved our ability to model colour variation under different visual systems (Stevens et al., 2007; Endler et al., 2018; Troscianko & Stevens, 2015; van den Berg et al., 2020). Nevertheless, challenges remain such that colour and pattern phenotypes are necessarily complex and multivariate. Chromatic (e.g., colour) and achromatic (e.g., luminance) aspects of a colour signal are commonly considered, but continued reliance on univariate analyses means that the consequences of trait combinations may be overlooked (Endler & Mappes, 2017). Because of these challenges, there has been a call for increased use of spatiochromatic phenotyping approaches that integrate variation in colour with pattern (spatial arrangement) (Endler & Mielke, 2005; Endler et al., 2018; van den Berg et al., 2020).

A more general limitation of many previous studies has been the relatively infrequent use of population genetic data (but see Steiner et al., 2009; Kratochwill et al., 2018). Colour traits are important targets of selective processes, but it does not follow that all divergence among populations (whether exhibiting parallelism or not) maps adaptively to local selection regime. Gene flow can sometimes preclude phenotypic divergence between populations despite differences in selection (Nosil et al., 2009; Räsänen & Hendry, 2008), while genetic drift could cause (nonadaptive) phenotypic divergence that masks parallelism (De Lisle & Bolnick, 2020; Stuart et al., 2017). Fortunately, patterns of molecular genetic differentiation, specifically genome-wide F_{ST}, can be used to construct null models against which to test for and isolate the phenoptypic signal of local adaptation (e.g., Pascoal et al., 2016, 2017; Whitlock & Guillaume, 2009). There are important caveats to this however, for instance when using phenotypic variation as a proxy for quantitative genetic variation (Pujol et al., 2008), or when "isolation by adaptation" scenarios are plausible (e.g., Funk et al., 2011; Nosil et al., 2008). Nonetheless, molecular genetic data provide an important opportunity to nuance expectations of phenotypic structuring among populations hypothesised to have undergone parallel evolution. In some cases, notably where dense marker data are available, they can also be used to probe the genetic basis of phenotypic differentiation - parallel or otherwise - among populations (Elmer & Meyer, 2011; Gautier et al., 2018).

In this study, we revisit the well-documented case of putatively parallel evolution of colour in the Trinidadian guppy by combining novel colour phenotyping methods, with estimation of phenotypic divergence among-populations in multivariate trait space and use of population genomic data. Guppies have provided important insights about many evolutionary processes (Endler, 1980; Houde, 1997; Reznick, 1997; Magurran, 2005) with the highly variable male colour patterns receiving particular attention. Male phenotypes are subject to antagonistic sexual and viability selection; females prefer more conspicuous male phenotypes but these also confer greater predation risk. In many rivers in the northern mountain range of Trinidad, upstream dispersal of piscivores, notably the pike cichlid *Crenacichla frenata*, is limited by barrier waterfalls. Downstream habitats are thus characterised by high predation risk (HP), relative to upstream low predation (LP) sites. Repeated colonization of LP sites and transplants of guppies from HP to LP sites have been associated with phenotypic shifts towards brighter, more colourful males (Endler, 1983; Gordon et al., 2015; Millar et al., 2006). This is presumably because the costs of being conspicuous (i.e. predation risk) are relatively lower in LP populations while the benefits (i.e., attractiveness to females) remain (Haskins et al.,1961; Endler, 1980; Houde, 1997).

We stress that these patterns of among-population variation in male guppy colour, and their interpretation with respect to predation risk have proven qualitatively robust (Millar et al., 2006). However, the diversity of phenotyping methods used across studies has also limited quantitative comparisons of the extent, and direction of (multivariate) evolution across lineages. Phenotyping methods have included scoring the presence/absence, number, size and position of particular colour patches (Endler, 1978; Gotanda et al., 2019), spectral measurements (Kemp et al., 2018), and spatial pattern approaches (Endler, 2012; Endler et al., 2018). It is also widely acknowledged that the ecological context is more nuanced than described above (Endler & Houde, 1995; Karim et al., 2007; Kemp et al., 2009). Predation risk varies within HP and LP contexts not just between them (Endler, 1995), but canopy cover and light environment also differ between upstream and downstream sites, and frequency-dependent selection also maintains variation within populations (Hughes et al., 2013; Olendorf et al., 2006). Finally, not all predictions are fully upheld by experiments. For example, low predation risk is thought to allow the evolution of larger colour spots on the body (Haskins et al., 1961). However, in a recent study, parallel increases in the relative area and number of melanic (black) spots as well as their size occurred across mesocosms lacking predators, but other colour traits changed inconsistently (Gotanda et al., 2019).

Here, we assess the extent of parallel evolution in male guppy colour patterns across repeated instances of LP colonisation from HP ancestors. We implemented the quantitative colour pattern analysis (QCPA) phenotyping approach (van den Berg et al., 2020) to model multivariate phenotypic divergence as perceived by the two main hypothesized selective agents: namely the guppy (sexual selection) and the Trinidadian pike cichlid (viability selection). Our specific goals were: (i) to determine whether this phenotyping approach recapitulates the expected finding that LP guppies are more "conspicuous" than HP guppies across rivers, (ii) to adopt a geometric perspective (following e.g., Bolnick et al., 2018) to quantify the extent of "parallelism" in multivariate trait space, (iii) to evaluate whether the conclusions differ with respect to the two visual systems modelled, and (iv) to assess patterns of phenotypic differentiation (parallel or otherwise) in the context of population genetic structure. We used genomic information obtained via pool-sequencing (hereafter, Pool-seq, Schlötterer et al., 2014) to estimate genome-wide population genetic differentiation. The population genomic data also allowed us to (v) conduct genome wide scans with genetic regions associated with phenotypic divergence, and thus conduct a preliminary investigation into the genomic basis of colour patterns in the wild.

2 | MATERIALS AND METHODS

2.1 | Sampling of wild fish

Wild guppies (n = 388 males) were sampled from 16 sites across seven rivers in the Northern Mountain Range of Trinidad by seine nets (Table 1) in March 2017. One HP and one or two LP sites were sampled in each river except the Paria (which contributed a single LP site). Fish were transported to the University of West Indies (UWI, St Augustine, Trinidad), housed in 200 L aquaria and allowed to acclimate to laboratory conditions for 5–7 days. A subset of males from each population were photographed (Table 1). Subsequently, different individuals (n ~100 per population) from 12 of the 16 sites were shipped live to the University of Exeter (Cornwall, UK). Although primarily for genetic investigations outwith the current study, paternal sibships produced from these wild-caught fish were used in a pilot to validate the present phenotyping strategy (see Supporting Information Appendix A for details and results of validation pilot).

2.2 | Phenotyping of male colour traits

Visible and UV-spectrum photography (Figure 1) were carried out using a Samsung NX1000 camera converted to full spectrum, fitted with a Nikkor EL 80 mm lens out on live, unsedated fish housed in a custom designed UV-transparent water filled chamber, as detailed in Appendix A. After photography, fish were humanely euthanised in a lethal dose of buffered MS-222 and tissue preserved in RNAlater (Invitrogen). Photographs were analysed using the QCPA framework, which quantifies carotenoid and structural-based colour traits using images and species-specific visual system parameters (see www.empiricalimaging.com and van den Berg et al., 2020). In addition to chromatic and achromatic signals, QCPA allows incorporation of spatial information using the geometric arrangement colour patches (with discrimination of adjacent patches based on the receptor noise estimate ΔS; Vorobyev & Osorio, 1998). This has ecological relevance because in behavioral trials female guppies prefer males that are more conspicuous in colour pattern, or have higher discriminant values (Sibeaux et al., 2019). We quantified colour variation over the whole body (excluding head, tail and fins), using four complementary traits: saturation boundary strength $sat_{\Delta S}$, luminance boundary strength $lum_{\Delta S}$, chromaticity boundary strength $chrom_{\Delta S}$ and chromaticity boundary strength variation $CoV_{\Delta S}$ (Endler et al., 2018). Together, these measures represent the chromatic (saturation and hue), achromatic (luminance), and spatial (size and position of colour pattern elements) properties of colour patterns. We used whole body trait measures for several reasons. First, specific pattern elements (i.e. colour patches at particular body locations) differ greatly within and among-populations, making it problematic to compare phenotypes from variable discrete pattern elements. Second, female guppies probably select males based on groups of colour patches not single elements (Cole & Endler, 2015). Finally, past genetic studies on colour patterns have focused on individual elements, whereas here our study takes a novel and more holistic approach at patterning.

For each photograph, the four traits were scored under two visual system parameter sets: conspecific (guppy) and a predator (pike cichlid, *Crenacichla frenata*) vision models fully detailed in Supporting InformationAppendix A. The parameters sets included user specified values of (i) receiver spectral sensitivities, (ii) receiver visual acuity, and (iii) physical distance at which the receiver might be from a male guppy. The use of two visual systems reflects our

TABLE 1 Description of collection site (drainage, river, and regime type) and their GPS coordinates

Drainage	River	Regime	Site code	Lat (N)	Long (W)	n_{males}
Caroni	Aripo	High	AH	10° 39' 02"	61° 13' 26"	26
Caroni	Aripo	Low	AL	10° 41' 08"	61° 13' 56"	29
Caroni	Aripo	Low	AL2	10° 41' 10"	61° 13' 30"	23
Caroni	Guanapo	High	GH	10° 39' 28"	61° 15' 13"	26
Caroni	Guanapo	Low	GL	10° 42' 42"	61° 16' 01"	5
Caroni	Guanapo	Low	GL2	10° 42' 20"	61° 15' 48"	26
Northern	Marianne	High	MH	10° 45' 54"	61° 18' 15"	25
Northern	Marianne	Low	ML	10° 44' 58"	61° 17' 13"	23
Northern	Marianne	Low	PML	10° 46' 40"	61° 18' 04"	28
Northern	Paria	Low	PL	10° 44' 57"	61° 15' 54"	24
Northern	Yarra	High	YH	10° 47' 23"	61° 21' 12"	24
Northern	Yarra	Low	YL	10° 44' 25"	61° 19' 17"	26
Oropouche	Quare	High	QH	10° 39' 53"	61° 11' 35"	27
Oropouche	Quare	Low	QL	10° 40' 33"	61° 11' 49"	26
Oropouche	Turure	High	TH	10° 40' 32"	61° 09' 54"	26
Oropouche	Turure	Low	TL	10° 41' 31"	61° 10' 18"	24

Note.: Sample size denotes fish that were photographed.
Abbreviations: Lat, latitude; Long, longitude; n_{males}, sample size.

view that perception of male coloration may well differ between receiver species imposing selection (i.e., a pattern that is more conspicuous to a female guppy may not always be so to predator). Our approach did not allow us to incorporate spectral transmission in an aqueous environment, and thus this parameter was not included. Note that *Crenacichla* is used because it is widely viewed as the most important selective visual predator in guppies. However, it should be noted that this species is not found in the rivers on the Northern slope of the Northern Mountain range, and so is not actually present in two of the high predation sites (Yarra, Marianne) included in this study. In these rivers, the major predator is thought to be the spiny cheek sleeper (*Eleotris Pisonis*), although little is known about its visual system or whether it is an important agent of selection on colour phenotypes. Thus, we acknowledge that the *Crenicichla* vision model probably provides as an imperfect proxy for predator vision overall.

We have previously validated the suitability of the QCPA phenotyping pipeline for detecting genetic variation in male colour and patterns under both visual models (see Appendix A) with photographs of males from known paternal sibships. Data analysis confirmed moderate to high sire level repeatabilities (analogous to heritabilities) for all traits under both visual models as expected (since male colour is partly Y-linked; Lindholm & Breden, 2002). This confirms the QCPA approach effectively characterises predominantly genetically meaningful phenotypic variation, though plasticity could also contribute to some degree. Importantly, (paternal) family correlations (r_F) between homologous traits defined under alternative vision models (e.g., $chrom_{\Delta S}$ based on guppy and cichlid vision models) are positive, but also significantly less than +1 in three of four cases. Using r_F as an estimate of the additive genetic correlation (Astles et al., 2006), indicates homologous metrics defined under the two vision models are genetically distinct (if correlated) traits.

2.3 | Generation of Pool-Seq data

To characterise population genetic structuring, we used a Pool-seq approach to obtain allele frequency estimates (Gautier et al., 2013; Schlötterer et al., 2014). While this approach is cost effective, available funding and tissue samples limited inclusion to 12 of the 16 wild populations (with guppies from the Turure (TH and TL) and Guanapo (GL1 and GL2) rivers excluded). With these exceptions, genomic DNA samples were obtained for 40 fish (20 males; 20 females) per population, using a Qiagen DNAeasy kit (Qiagen Co). For each sample, the concentration and purity of the genomic DNA were measured using a Nanodrop spectrophotometer and q-bit (Thermo Fisher Scientific), respectively. Purity was further checked on a 1% agarose gel before sex-specific DNA pools were created for each population (n = 24; 12 populations by two sexes). Pools, each containing equal DNA concentration from 20 individual fish, were sequenced at the Earlham Institute (Norwich). Barcoded DNA paired end libraries with read sequence size of approximately 150 bp were prepared using Illumina Truseq HT library prep and sequenced on two lanes using Illumina NovaSeq (Illumina Inc.). Raw paired-end reads were checked for quality using FastQC, and adapters were verified and removed using cutadapt. To investigate among population differences, the raw reads of male and female pools for each population were merged. Reads were then mapped to the reference guppy genome (Guppy_female_1.0+MT, GCA_000633615.2, Künster et al., 2016) with BWA mem (Li & Durbin, 2010) using default parameters to generate initial BAM files. Aligned reads were then sorted, marked for duplicates and indexed, to generate final BAM files using SAMTOOLS v1.9 (Li et al., 2009). All BAM files were merged to create a mpileup file (i.e., samtools mpileup –f), which was subsequently filtered for indels and then used to generate a sync file (mpileup2sync.jar; base

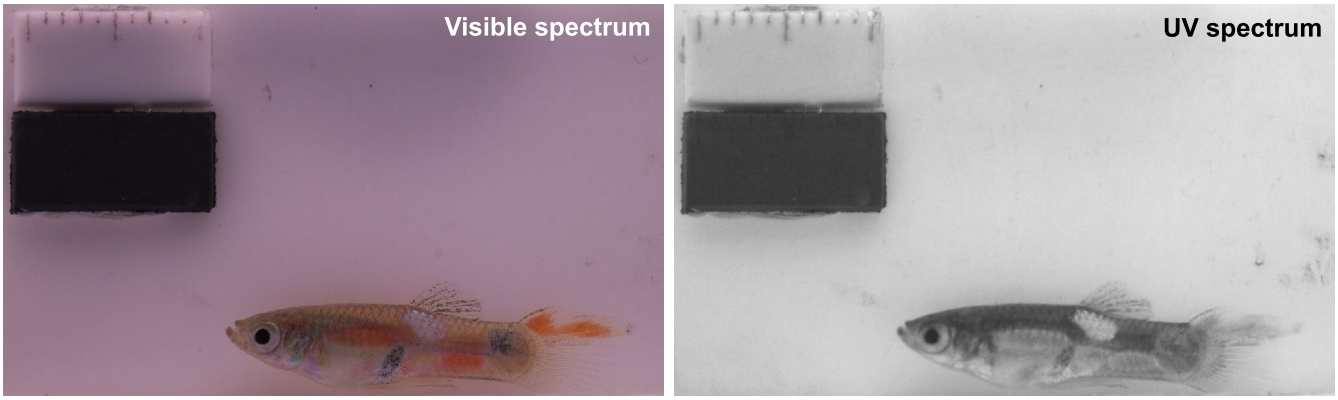

FIGURE 1 Example of photography setup using calibrated photography using visible and UV spectrum (greyscale). Black and white blocks are 5% and 95% reflectance standards, respectively

quality >20 or --min-qual 20) containing allelic frequency information for every population using PoPoolation2 (v1.201) (Kofler et al., 2011). A clean sync file was then obtained after indel removal (filter-sync-by-gtf.pl), and subsequently used for downstream population genomic analyses.

2.4 | Data analysis

The phenotypic data set comprised measures of eight response variables ($sat_{\Delta S}$, $lum_{\Delta S}$, $chrom_{\Delta S}$, $CoV_{\Delta S}$ x 2 visual system models) for 388 male guppies representing 16 distinct populations (6 HP and 10 LP) from seven rivers. The genomic data comprised of 24 pools of DNA-seq data (12 populations × 2 sexes), whose summary statistics are described in Table S1. In brief, each pool generated between 362–599 M reads with a mean Phred score of 35.8 and 96× depth of coverage. Between 88%–98% of raw reads were successfully mapped to the guppy female reference genome assembly. Unless otherwise stated, all statistical analysis was done in R (R Core Team, 2019) with ASReml-R (Butler et al., 2017) used to fit mixed models.

2.4.1 | Testing effects of population and predation regime

We first plotted trait means to check whether differences between populations and or predation regimes were visually apparent. Next, we formally tested for variance among populations (V_{Pop}) by fitting univariate mixed models to each trait. Each model included a random effect of population and a fixed effect of predation regime. This means that the estimate of V_{Pop} is conditional on regime, which should be interpreted as the among-population variance not explained by regime. Before fitting the models, we divided all observed values by the corresponding trait standard deviation to obtain standard deviation units (sdu) for analysis. This simplifies subsequent interpretation as each trait has unit variance on this scale, thus the estimate of V_{Pop} can be interpreted as an intraclass correlation unadjusted for fixed effects. Following standard practice, we also estimated the adjusted population level repeatability (R_{Pop}) for each trait by expressing V_{Pop} as a proportion of total variance conditional on the fixed effect. Statistical inference on the fixed effect was by Wald *F*-tests, and we used likelihood ratio tests (LRT) to assess the significance of the random effect. For the latter, we assumed that twice the difference in log-likelihoods between the full model and reduced version with no random population effect is distributed as a 50:50 mix of χ^2_0 and χ^2_1 (Stram & Lee, 1994). Note we subsequently denote this mix of distributions as $\chi^2_{0,1}$.

2.4.2 | Estimation of D and pairwise phenotypic distances between all population pairs

We used a multivariate mixed model to estimate among-population variance for the multivariate phenotype as defined by all eight traits. This is estimated as the among-population variance-covariance matrix (D). The model fit was also used to generate predictions of pairwise distance between population mean phenotypes in multivariate trait space. The mixed model included a random effect of population was included on each trait but no fixed effects (except intercepts). D is then estimated as a covariance matrix that contains among-population trait variances on the diagonal and among-population among-trait covariances off the diagonal. Note that the former are expected to differ from the V_{Pop} estimates generated by our univariate models since they are no longer conditional on any *regime* effects. To help interpretation, we scaled all covariances to among-population correlations (r_{Pop}) between all trait pairs. We assume approximate 95% confidence intervals (CI) of r_{Pop} ±1.96 standard error (SE).

We then used the fitted multivariate model to predict pairwise phenotypic distances between multivariate population means by extracting the best linear unbiased predictions (BLUP) of trait means by population. From these, we calculated the 16 × 16 (since $n_{population}$ = 16) distance matrix (E) among populations in n_{trait}-dimensional trait space. Each element of E corresponds to the Euclidian distance between the multivariate phenotypic means of a population pair. We used all eight traits (E_{all}), but also generated

the matrices based on guppy (E_{gvm}) and cichlid (E_{cvm}) vision model traits respectively. This allowed us to determine whether general conclusions about among-population divergence were sensitive to the vision model(s) used to define phenotype, noting again that that pike cichlids were not present at two of our high predation sites. In reality, there is uncertainty in the statistical predictions of the population mean phenotypes, and so E_{all}, E_{gvm} and E_{cvm} represent point estimates of the distance matrices. Here, we use these matrices to visualise patterns of among-population divergence only and note that subjecting them to further (robust) statistical inference would be inappropriate. Specifically, neighbour-joining trees based on E_{all}, E_{gvm} and E_{cvm} were plotted to visually assess any tendency to cluster phenotypically by regime. For each population pair, we also plotted the distance based on the guppy vision model against the corresponding cichlid distance to assess whether (i) a relationship between $\boldsymbol{E}_{gvm}$ and E_{cvm} was apparent, and (ii) phenotypic distances tended to be lower for within- versus across-regime population pairs.

2.4.3 | Testing for association of phenotypic and genetic distance

For the subset of 12 populations with molecular as well as phenotypic data, we used the sync file to estimate genome-wide differentiation among populations using the fixation index (F_{ST}). F_{ST} was calculated for single nucleotide polymorphisms (SNPs) in 50k windows using fst.sliding.pl implemented in PoPoolation2 (parameters: –min-count 2 –min-coverage 4 –max-coverage 80 –pool-size 80 –window-size 50,000 –step-size 50,000), which calculates F_{ST} following Hartl and Clark (1997). The resultant among-population F_{ST} matrix was compared to phenotypic distance estimates in two ways. First, we used Mantel tests to assess the correlation with phenotypic distance (represented by E_{all}, E_{gvm} and E_{cvm} subsetted to the 12 populations with SNP data). Second, following Pascoal et al. (2017) we refitted univariate mixed models to each of the eight response variables, but this time with no fixed effects and a modified random effect structure to total V_{Pop} (i.e., not conditional on regime) into two components: one attributable to divergence under neutral processes (i.e. drift; $V_{Pop.N}$); and another attributable, under some assumptions, to divergence under selection (from predation, sexual selection and/or unknown selective agents; $V_{Pop.S}$).

This second approach, follows the same premise as Q_{ST}-F_{ST} comparisons (Leinonen et al., 2013) and asks whether there is more quantitative phenotypic divergence among-populations than expected from levels of (putatively) neutral molecular divergence. To implement it, we ran models with two random effects of population identity, one which covaries among populations according to their molecular 'relatedness' structure, and a second which is uncorrelated across populations. For the former, we assume phenotypic covariance between fish sampled from populations *i* and *j* that is attributable to neutral population effects is equal to $S_{ij} \cdot V_{Pop.N}$ where S_{ij} is genome-wide 'similarity' between the populations. Since F_{ST} increases wih dissimilarity we define $S_{ij} = 1 - F_{ST_{i,j}}/F_{ST_{max}}$, where $F_{ST_{max}}$ is the highest observed pairwise F_{ST} among the populations sampled. This simply scales S_{ij} from a maximum of 1 (when I = j) to 0 (for the most differentiated pair of populations). To test whether trait variation among populations is greater than expected under neutral divergence alone, we compared the model with both variances to one where $V_{Pop \cdot S}$ is absent using LRT (Pascoal et al., 2016, 2017). Two caveats to this method should be noted: first, it assumes that to a first approximation genome-wide F_{ST} measures neutral differentiation (i.e., from drift and gene flow); second, since we are modelling phenotypic variation in wild caught fish rather than in a common-garden experiment, any contributions to population divergence from phenotypic plasticity are likely to be partitioned into $V_{Pop \cdot S}$ (Pujol et al., 2008).

2.4.4 | Testing for parallelism among replicate population pairs

Phenotypic analyses described above are blind to the ancestral versus derived status of HP and LP population pairs within rivers. Therefore, we also adopted recent methods that use phenotypic change within lineages to quantify parallelism (De Lisle & Bolnick, 2020). We assumed that within each river, an HP and LP pair can be viewed as representing a lineage in which the HP is ancestral. This assumption yielded nine lineages among the 16 populations with phenotypic data. Fish from the Paria are excluded as there is no HP site, but the Aripo, Guanapo, and Marianne rivers each contribute two lineages (i.e.. HP-LP comparisons). We describe the approach briefly, using notation from De Lisle and Bolnick (2020) and referencing their equation numbers.

First, we calculated, the n_{trait} row by $m_{lineage}$ column data matrix X, where each element of X represents $\Delta z_{n,m}$ (the difference in mean standardized phenotype (z) between HP and LP populations for trait n in lineage m; Equation 3). Each row of X thus represents the vector of phenotypic change from HP to LP within a lineage in multi-trait space. X was used to calculate C, the (m × m) among-lineage correlation matrix of phenotypic change vectors which was then subject to eigen decomposition (following Equations 5 and 6). To mirror our investigation of the among-population distance matrices (i.e., E_{all}, E_{gvm} and E_{cvm}), we calculated and decomposed C matrices based on $sat_{\Delta S}$, $lum_{\Delta S}$, $chrom_{\Delta S}$ and $CoV_{\Delta S}$ determined using both vision models (C_{all}), but also using guppy (C_{gvm}) and cichlid vision model (C_{cvm}) traits, separately.

In the case that multivariate trajectories are perfectly parallel across all lineages, the first eigenvector of C should explain all variation and all lineages would load on this vector with the same sign (but differing magnitudes if the extent, rather than direction, of phenotypic change differed among lineages). In contrast, a uniform distribution of eigenvalues (meaning low among-lineage correlations of change vectors), and/or a mixture of positive and negative lineage-specific loadings on the first eigen vector is expected under nonparallelism. As suggested by De Lisle and Bolnick (2020), for the case of fewer traits (here 4 or 8 depending on the version of C) than lineages

(here 9), we generated a null distribution of *m* eigenvalues by simulating 1000 random vectors representing evolutionary change under independence (among lineages) of multivariate phenotypic trajectories. While acknowledging that power to reject the null (no parallelism) may be low, observed eigenvalues were then compared to the simulated distribution. An eigenvalue of C greater than the 95% confidence intervals of the simulated values is taken as statistical support for parallelism.

2.4.5 | Genotype-phenotype association mapping from pool-seq data

Leveraging the pool-seq data, we tested for relationships between allele frequencies at SNP loci and phenotypic variation (colour metrics: $sat_{\Delta S}$, $lum_{\Delta S}$, $chrom_{\Delta S}$ and $CoV_{\Delta S}$) and predation regime (high vs. low). We did this using genome-wide scans for association in BayPass (Gautier, 2015). This method identifies loci more with higher than expected differentiation among populations based on the *XtX* statistic (Günther and Coop, 2013), and also tests associations between SNPs and population-specific covariables (while accounting for background population structure from drift and gene flow). Since direct selection on male coloration is necessarily sex-limited, we elected to use SNP data from male pools only for these analyses (rather than combined male and female data used for genome wide F_{ST} estimates). Major and minor allele counts for each SNP were calculated for each population from the previously compiled sync file using the snp-frequency-diff.pl script with the following parameters (–min-coverage 75 –max-coverage 200). Using custom awk scripts, allele counts were extracted and formatted for BayPass's input genotype file (Gautier, 2015). SNPs from the male pools that were mainly on known linkage groups (LGs) were obtained (ca. 5,977,803 SNPs) and then subsampled to yield about 100 k SNPs along the whole genome (mean =95,911 ± 61.2 SNPs from known LGs only, corresponding to about 4,170 per LG) to generate about 100 subdata sets. Subsets were then analysed individually using the BayPass core and standard covariate models. The resulting population covariance matrices of allele frequencies (interpretable as similarity matrices) were generated under the core model and checked for consistency across all data subsets by calculating the distance among matrices (hereafter FMD) using the fmd.dist function (Figure S1; Table S2). The mean FMD among all pair wise comparison was 0.2 (with $r > 0.99$ for all pairwise comparisons).

To identify SNPs that deviated from neutral expectations we used *simulate.baypass*. This uses the population covariance matrix to simulate "pseudo-observed data sets" (POD), assuming SNP neutrality, consistent with the demographic history. This allows a null distribution of the *XtX* to be generated, the 99% quantile of which was used as a significance threshold (median $XtX > 21.2$) to determine whether observed SNPs might be under selection. We then used the BayPass IS covariate models and resultant Bayes factor (BF) to test for association between SNPs and population level male colour traits (and/or predation regime). Evidence for significant association between SNP and a tested covariate was based on BF > 30 db (Gautier, 2015).

Finally, we checked whether SNPs identified as deviating from neutrality and/or associated with a covariate showed enrichment for gene ontology (GO) categories and functions. We extracted the corresponding identity of genes or nearby genes, which were blasted against the zebrafish genome (*Danio rerio* NCBI Refseq GCF_000002035.6, Howe et al., 2013) for annotated orthologues. The annotated orthologues were run in the gene ontology resource (http://geneontology.org/; Ashburner et al., 2000; The Gene Ontology Consortium, 2019).

3 | RESULTS

3.1 | Effects of predation regime and among-population variance for male traits

Mean values of $sat_{\Delta S}$, $lum_{\Delta S}$, $chrom_{\Delta S}$ and $CoV_{\Delta S}$, varied considerably among populations and between vision models within populations (Figure 2a–d). Overall, trait means were higher in LP than HP guppies (all populations combined) at all eight traits as predicted. However, looking at within river comparison the pattern was less compelling. Arguably, only $lum_{\Delta S}$ under the guppy vision model is strongly suggestive of a consistent pattern, with higher means found for low predation populations in all rivers except Guanapo. In Guanapo, both low predation populations actually had less luminance on average than the high predation population (Figure 2b). More generally, across the other traits and vision models, there was little evidence for a consistent directional shift in mean between HP-LP comparisons where witin-river comparisons were available. Qualitatively, traits defined under the guppy vision model tended to be less variable among populations and to yield higher (more conspicuous) values for $chrom_{\Delta S}$ and $sat_{\Delta S}$ relative to cichlid vision. The opposite pattern is seen for $lum_{\Delta S}$ and $CoV_{\Delta S}$.

Univariate mixed models confirmed this pattern, but provided limited statistical support for differences in trait means between HP and LP (Table 2). Positive *regime* coefficients for all traits indicated a tendency to higher conspicuousness at LP sites; but across the eight models, only $lum_{\Delta S}$ under the guppy vision model was significant (coefficient (SE): 0.83 (0.37) sdu, $p < .04$; Table 2). A moderately large, yet nonsignificant, effect (+0.57 sdu) was estimated for $chrom_{\Delta S}$ under the cichlid model, while the average estimated effect size of regime was +0.328 sdu. Support for strong among-population differentiation, over and above any regime effects was unequivocal; LRT comparisons to reduced models with no population effect yielding $p < .001$ for all traits (Table 2). Population level (conditional) repeatability R_{Pop} ranged from 30% to 71% with an average of 54%. R_{Pop} estimates were very close to V_{Pop}, indicating that *regime* explained little of the among-population differentiation (if regime explained large amounts of trait variance, then in these models $V_{Pop} + V_R$ would <1 leading to a systematic finding of $R_{Pop} > V_{Pop}$).

3.2 | Among-population differentiation of multivariate phenotype

The **D** matrix (estimated without conditioning on regime) yielded V_{Pop} estimates very similar to (conditional) estimates obtained from univariate models (Table 3). The correlation structure within **D** was not universally positive (which is the expectation if populations varied along a simple axis from greater to less conspicuousness; Table S3). Population-level correlations between homologous traits defined using guppy and cichlid vision models were positive but significantly less than +1 (assuming an upper 95% confidence interval of r_{Pop} + 1.96 SE). Specifically r_{pop} (SE) were estimated as 0.65 (0.16), 0.39 (0.23), 0.28 (0.24) and 0.09 (0.28) across the two models for $sat_{\Delta S}$, $lum_{\Delta S}$, $chrom_{\Delta S}$, and $CoV_{\Delta S}$, respectively.

Phenotypic distance matrices derived from the multivariate mixed model fit provided little support for clustering of populations by regime in multitrait space (Figure 3, Table S4). Using distance defined from all traits at once and guppy vision models, there was arguably some trend visible. For instance, the upper right portion of Figure 3a contains a grouping of 6 LP populations with 1 HP whereas Figure 3b has a grouping of six LP and no HP. However, this pattern is not seen using just the traits subsets from the cichlid (Figure 3c) vision model. Moreover, under the simple prediction that population mean phenotypes cluster by regime, we would also see shorter average pairwise distances between populations within- versus across-predation regimes. However, no such pattern was evident (Figure 4). If it was, and higher phenotypic distances were found for low-high predation (LH) comparisons relative to low-low (LL) or high-high (HH) the red ellipse (LH points) in Figure 4 would be shifted to higher values on x and/or y axes than the blue ellipses.

Two further points emerge from Figure 4; first, there is no apparent relationship between the pairwise population distance estimates defined by the two vision models. Population pairs that are more distinct with respect to guppy vision are not generally more distinct under the cichlid vision model. This is consistent with the absence of strong positive r_{POP} between homologous traits noted above. Second, the 95% confidence ellipse of pairwise distances for HH comparisons is smaller and shifted to lower values (on both axes) relative to HL or LL comparison. This pattern implies that there may be lower phenotypic variance among high predation populations that among low predation populations. To investigate further, we fitted post-hoc multivariate mixed models (described Table S5) to regime specific data subsets and compared total phenotypic variance among-populations (calculated as the trace of regime specific D matrices). Point estimates corroborated

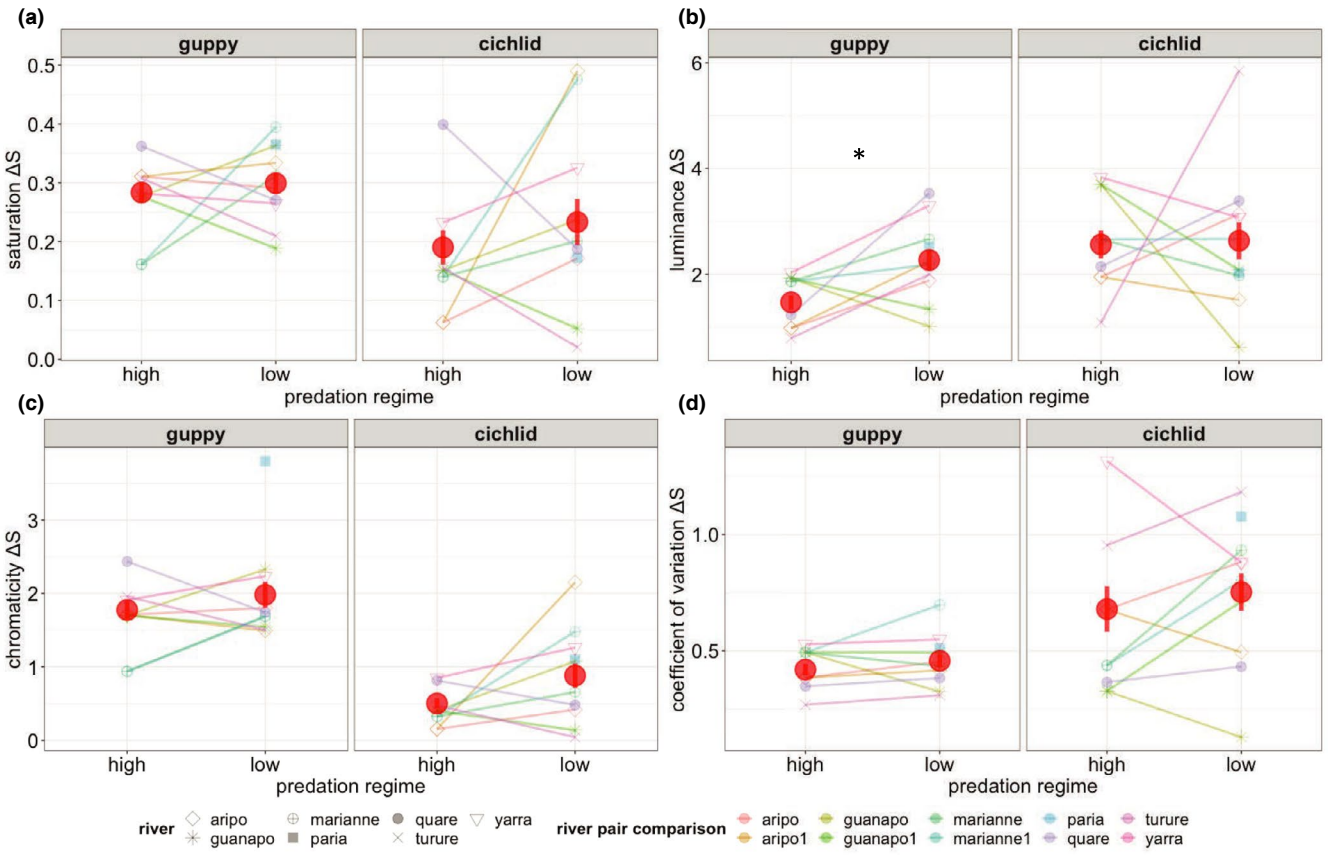

FIGURE 2 Distribution of population mean trait values (prior to conversion to sdu) by predation regime and population under guppy and pike cichlid vision models for (a) colour saturation ($sat_{\Delta S}$), (b) luminance ($lum_{\Delta S}$), (c) chromaticity ($chrom_{\Delta S}$), (d) coefficient of variation in chromaticity ($chrom_{\Delta S}$). Lines link populations of differing regime within rivers. Also shown are the overall trait means by predation regime (red circle) with standard error. Asterisk (*) denotes significant difference in overall mean between regimes. Note that Paria (light blue square) does not have a corresponding high predation population

TABLE 2 Estimated regime (low vs. high predation) and population (random) effects from univariate mixed models of each trait inferred by *F*-tests

		Fixed effects				Random effects			
Vision model	**Trait**	***Regime* (SE)**	**F**	**DF**	***p*-value**	**V_{Pop} (SE)**	**R_{Pop} (SE)**	**$X^2_{0,1}$**	***p*-value**
Guppy	$sat_{\Delta S}$	0.148 (0.353)	0.177	1,13.9	.681	0.439 (0.177)	0.418 (0.1)	150	<.001
	$lum_{\Delta S}$	0.833 (0.366)	5.171	1,13.7	.04	0.484 (0.192)	0.539 (0.1)	204	<.001
	$chrom_{\Delta S}$	0.271 (0.434)	0.39	1,14	.543	0.689 (0.267)	0.638 (0.091)	307	<.001
	$CoV_{\Delta S}$	0.230 (0.302)	0.58	1,13.8	.459	0.307 (0.130)	0.301 (0.091)	80.5	<.001
Cichlid	$sat_{\Delta S}$	0.270 (0.449)	0.361	1,14	.557	0.744 (0.286)	0.713 (0.08)	403	<.001
	$lum_{\Delta S}$	0.054 (0.401)	0.018	1,13.7	.896	0.578 (0.230)	0.514 (0.101)	186	<.001
	$chrom_{\Delta S}$	0.570 (0.433)	1.728	1,14	.21	0.690 (0.266)	0.682 (0.085)	357	<.001
	$CoV_{\Delta S}$	0.245 (0.400)	0.375	1,13.2	.551	0.556 (0.231)	0.512 (0.106)	136	<.001

Note.: Among-population variance (V_{Pop}) is conditional on fixed effects and was tested by likelihood ratio test.

R_{Pop} denotes population-level repeatability.

Abbreviations: $chrom_{\Delta S}$, chromaticity; $CoV_{\Delta S}$, coefficient of variation; $lum_{\Delta S}$, luminance; Trait: $sat_{\Delta S}$, saturation.

our interpretation of Figure 4, the trace of D_H being 65% of the trace of D_L. However, unsurprisingly given the number of populations in each regime, our trace estimates were characterized by high uncertainty and the difference is not statistically significant.

3.3 | Phenotypic differentiation is not consistant with neutral genetic divergence

Genome-wide pairwise F_{ST} comparisons revealed strong genetic structuring by river and drainage (Figure 5; Table S6). This result was expected and is consistent with previous studies (e.g., Fraser et al., 2015; Suk & Neff, 2009; Willing et al., 2010). There was almost no correlation between molecular differentiation and estimated phenotypic distance (Mantel test of F_{ST} matrix correlation with E_{all} $r = -0.049$, $p = .608$; with E_{gvm} $r = 0.026$, $p = .426$; and with E_{cvm} $r = -0.033$, $p = .569$). A neutral model of among population divergence was also rejected (at $\alpha = 0.05$) for six out of eight traits using our mixed model strategy to partition V_{Pop} into components attributable to neutral ($V_{Pop.N}$) and selective ($V_{Pop.S}$) components (Table 3). The proportion of trait variance explained by the latter was considerably higher than by the former for most traits. Under the cichlid vision model, $sat_{\Delta S}$ and $chrom_{\Delta S}$ proved exceptions to this pattern, with the more complex model offering no improvement to model fit and $V_{Pop\cdot S}$ being bound to zero. Thus, with these two exceptions, genome wide-molecular divergence among populations cannot readily explain the phenotypic differentiation structure.

3.4 | Evolution of guppy colour pattern is nonparallel across lineages

The among-lineage correlation matrix of phenotypic change vectors C had a highly skewed distribution of eigenvalues (Table 4).

TABLE 3 Estimates of among-population repeatabilities (R_{Pop}) derived from univariate mixed models that partition total population effects into components attributable to putative neutral ($R_{Pop.N}$) versus selective ($R_{Pop.S}$) processes

Vision model	**Trait**	**R_{Pop} (SE)**	**$R_{Pop.N}$ (SE)**	**$R_{Pop\cdot S}$ (SE)**	**$X^2_{0,1}$**	***p*-value**
Guppy	$sat_{\Delta S}$	0.385 (0.109)	0.000 (-)[a]	0.385 (0.109)	3.787	.026
	$lum_{\Delta S}$	0.414 (0.144)	0.073 (0.083)	0.342 (0.196)	4.537	.017
	$chrom_{\Delta S}$	0.654 (0.136)	0.031 (0.092)	0.623 (0.208)	4.95	.013
	$CoV_{\Delta S}$	0.240 (0.089)	0.000 (-)[a]	0.240 (0.089)	9.651	.001
Cichlid	$sat_{\Delta S}$	0.418 (0.109)	0.418 (0.109)	0.000 (-)[a]	0	.5
	$lum_{\Delta S}$	0.364 (0.108)	0.000 (-)[a]	0.364 (0.108)	5.286	.011
	$chrom_{\Delta S}$	0.400 (0.108)	0.400 (0.108)	0.000 (-)[a]	0	.5
	$CoV_{\Delta S}$	0.495 (0.114)	0.000 (-)[a]	0.495 (0.114)	8.581	.002

Note.: Also shown are LRT comparisons to a reduced model in which all among-population variance is assumed to have a neutral basi.

Abbreviations: $chrom_{\Delta S}$, chromaticity; $CoV_{\Delta S}$, coefficient of variation; $lum_{\Delta S}$, luminance; $sat_{\Delta S}$, saturation.

[a]Note that note that negative variance components estimates are not permitted in the model fitting. Where the estimation algorithm repeatedly hits the boundary condition for a given component, the variance and corresponding repeatability estimates are fixed to zero and obtaining a SE is not possible.

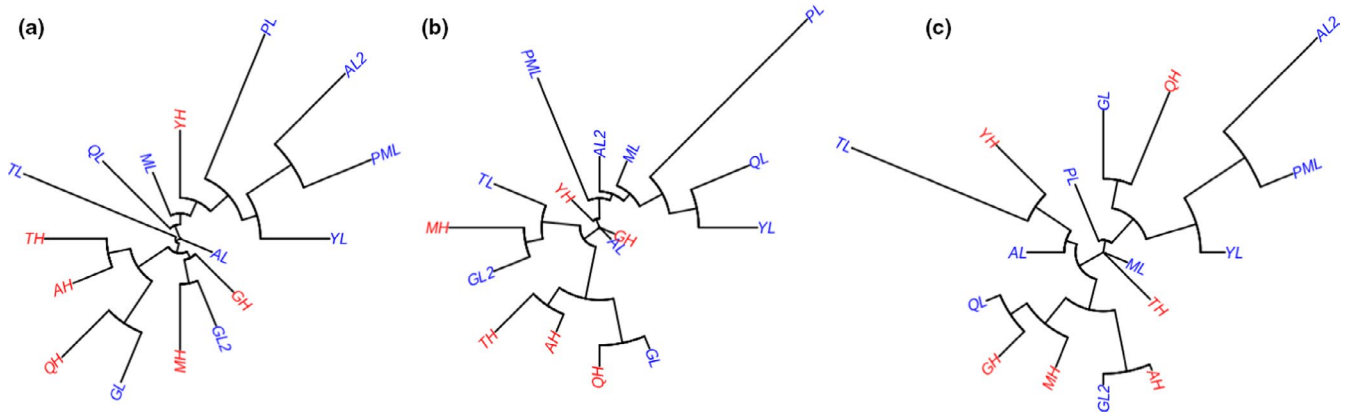

FIGURE 3 Neighbour joining trees of populations based on estimated phenotypic distance matrices in n-dimensional trait space. Tree shown are determined using (a) all eight colour measures, (b) the four traits derived from the guppy-and (c) the four traits derived from the cichlid-vision model. Red and blue labels denote high and low predation regime populations, respectively

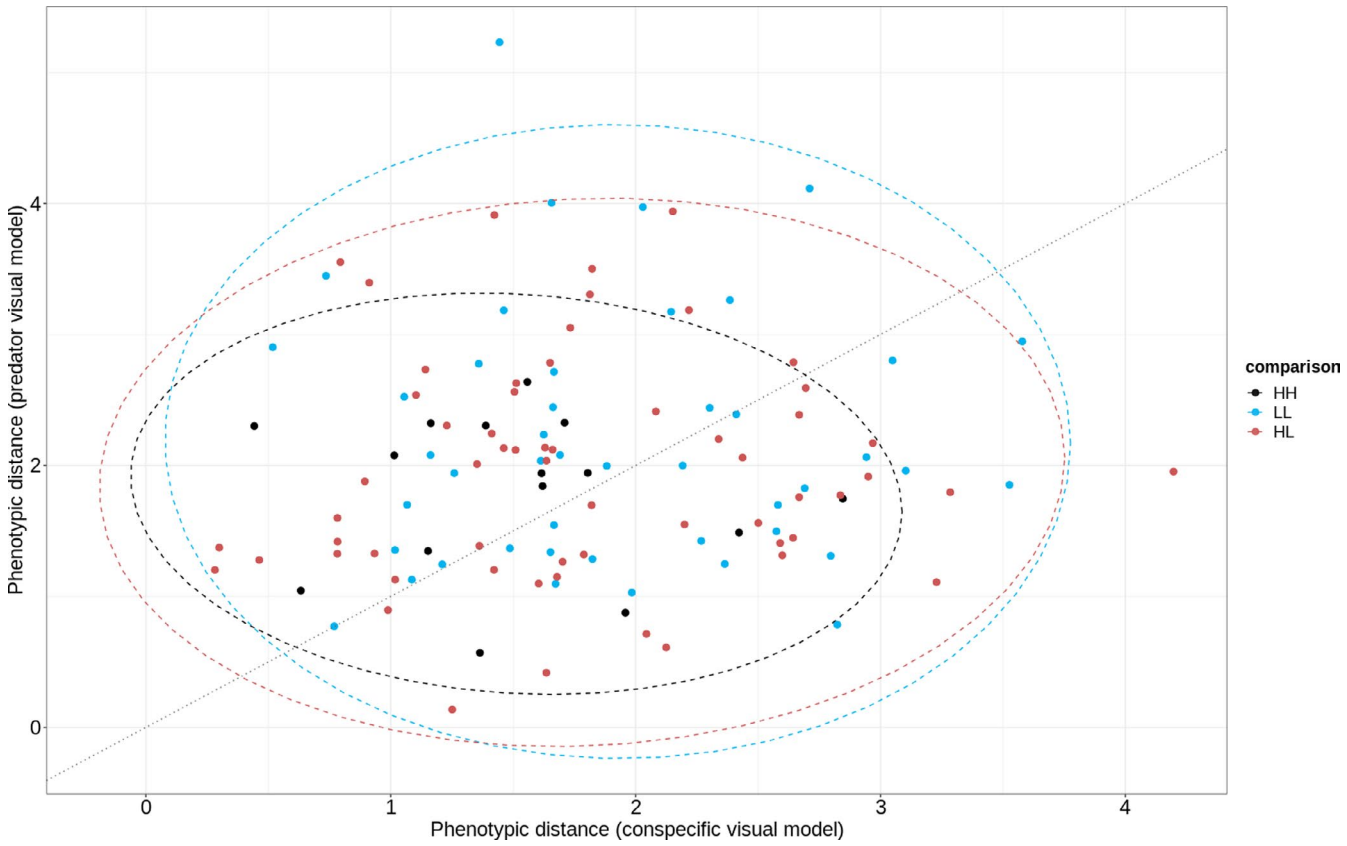

FIGURE 4 Between-population distance estimates based on guppy (x-axis) and cichlid (y-axis) vision models. Each point denotes a single pairwise population comparison. Red points denote cross-regime distances between a high and a low predation site, and black and blue points denote pairwise distances within predation regimes (black for high, light blue for low), Ellipses illustrate approximate 95% confidence limits of the distributions and the 1:1 line is also shown

Although this skewing is expected under parallel evolution generally, we cannot infer that here. Specifically, since the rank of C is limited to the smaller of n (traits, here either 4 or 8) and m (lineages, here 9), all three 9 × 9 C matrices will necessarily be rank deficient, meaning that eigenvalues of zero are inevitable and creating skew that is not biologically informative. Moreover, the first eigenvectors were not particularly dominant, as would be expected under strong parallelism. Specifically they capture 55%, 51% and 40% of $C_{gvm,}$ C_{cvm} and $C_{all,}$ respectively. Furthermore, lineages loaded on this first eigenvector with a mixture of positive and negative signs in all three cases, which is contrary to the prediction under paralellism.

The structure of the C matrices was thus not consistent with parallel phenotypic evolution among these lineages in 8-trait space, or in either of the 4-trait spaces. Random vectors provided no evidence

that the first eigenvalues were larger than expected under a null model of independent evolutionary trajectories across lineages (Figure S2). The second eigenvalues of C_{gvm} and C_{all} were greater than 95% of simulated values, which suggested some overdispersion and thus deviation from complete independence. However, lineages also loaded on this second eigenvector with a mix of signs too (in all three C matrices) so we cannot interpret this as parallel evolution in a direction defined by the second axis of C. We suspect that this signal of nonindependence arises because data from single high predation populations in Aripo, Guanapo, and Marianne rivers each contributed to the vectors of phenotypic change for two lineages (i.e., HP vs. LP comparisons within-river).

3.5 | Genome-phenotype association analysis reveals that differentiated SNPs are found in genes related to cell morphogenesis and neural development.

We detected 85 SNPs more differentiated among populations than expected under neutrality at the 0.01% POD significance threshold (Figure 6). Of these SNPs, detected using the BayPass core model, 61 and 24 were located within genic and nongenic regions, respectively (summarized in Table 5). Some of the genes have general functions in cell morphogenesis (e.g., adhesion), projections (e.g., dendritic) and interaction, and are orthologous to zebrafish genes previously documented in patterning and pigmentation (e.g., *xpc*, *rpl*, *rilp*, *netrins*) (Baxter et al., 2019). Some were also within genes involved in neural development, such as sensory axon guidance (e.g., *ptprfa*). Using orthologous zebrafish genes, our GO analysis found no enrichment for any gene ontology category.

We found a total of 195 SNPs associated with a tested covariate (using the BayPass IS model) using a BF threshold >30 db (colour traits only: guppy vision model = 76; cichlid vision model = 101; predation = 17 for both models, summarized in Table S7). Interestingly, there was no overlap with the 85 differentiated SNPs identified under the core model. Taken at face value, this suggests SNPs identified by the core model as being more differentiated than expected are not related to colour pattern differences among populations. SNPs significantly associated with covariates in the IS model were scattered throughout the genome (not clustered on specific chromosomes), and found within both nongenic and genic regions (~41% in nongenic regions: n_{gvm} = 40; n_{cvm} = 46). With a single exception (LG22: 9903588; associated with both measures of $sat_{\Delta S}$), SNP-colour trait associations differed between between the guppy and cichlid visual perceptual models, consistent with the finding that traits defined under the two models have different genetic underpinnings. As with the *XtX*-based results from the core model, many SNPs associated with covariates under the IS model were found in genes previously implicated with cell morphogenesis and neuronal functions (e.g., *hapln2*; Table S7). There was no enrichment of genes related to any functional processes using putative genes identified

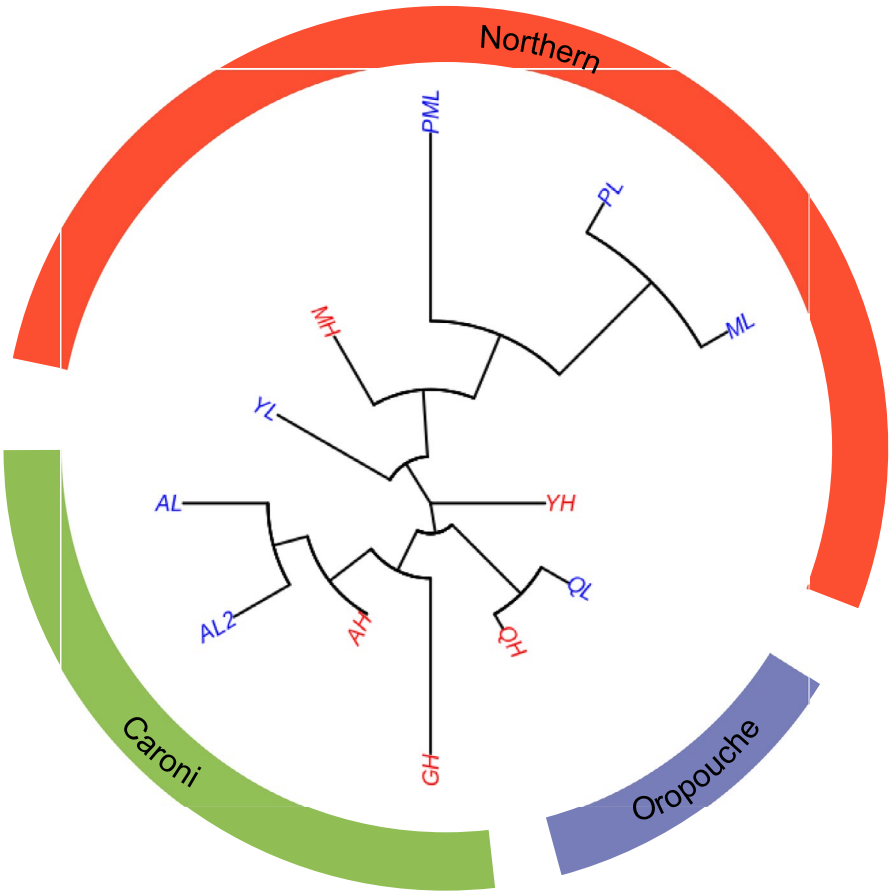

FIGURE 5 Neighbour joining tree based on whole-genome population F_{ST} distance, clustering by rivers, then drainages, consistent with the independent evolution of each replicate. Branch length represents F_{ST}. Populations: AH, Aripo High; AL, Aripo Low; AL2, Aripo Low 2; GH, Guanapo High; MH, Marianne High; ML, Marianne Low; PML, Petite Marianne; PL, Paria; QH, Quare High; QL, Quare Low; YH, Yarra High; YL, Yarra Low. Blue denotes Low predation regime, whereas Red High

TABLE 4 Spectral decomposition for among independent lineages for phenotypic parallelism

(a) Guppy-vision colour										
		Eigenvectors								
		q1	***q2***	***q3***	***q4***	***q5***	***q6***	***q7***	***q8***	***q9***
	Eigenvalues (expected upper 95% CI)	5.06	3.25	2.13	1.26	0	0	0	0	0
	Eigenvalues (observed)	4.97	3.38*	0.4	0.26	0	0	0	0	0
	% variance explained	0.55	0.38	0.04	0.03	0	0	0	0	0
Lineage	Aripo	−0.43	0.08	−0.13	0.5	0.72	0	0	0	0.17
	Aripo1	−0.41	0.17	0.29	−0.3	0.05	−0.01	0.71	−0.23	−0.25
	Guanapo	0.35	0.29	−0.46	−0.18	0.21	−0.56	0.32	0.28	0.04
	Guanapo1	0.18	−0.48	−0.2	0.45	−0.07	0.18	0.4	0.24	−0.5
	Marianne	−0.07	0.52	−0.32	−0.16	0.08	0.44	−0.26	0.1	−0.56
	Marianne1	0.01	0.5	0.47	0.51	−0.29	−0.26	−0.02	0.32	−0.12
	Quare	−0.42	−0.16	0.01	−0.28	−0.08	0.13	0.01	0.8	0.21
	Turure	−0.38	−0.28	−0.04	−0.1	−0.02	−0.61	−0.38	−0.07	−0.49
	Yarra	−0.4	0.14	−0.56	0.24	−0.58	−0.03	0.12	−0.21	0.23
(b) Cichlid-vision colour										
		Eigenvectors								
		q1	***q2***	***q3***	***q4***	***q5***	***q6***	***q7***	***q8***	***q9***
	Eigenvalues (expected upper 95% CI)	5.06	3.19	2.12	1.26	0	0	0	0	0
	Eigenvalues (observed)	4.59	2.42	1.85	0.14	0	0	0	0	0
	% variance explained	0.51	0.27	0.21	0.02	0	0	0	0	0
Lineage	Aripo	−0.16	0.56	−0.25	0.19	0.57	0	0	0	0.48
	Aripo1	−0.43	0.2	0.1	−0.42	0.32	−0.37	0.21	−0.12	−0.54
	Guanapo	−0.38	−0.34	−0.17	−0.32	−0.03	−0.29	−0.17	0.61	0.35
	Guanapo1	0.09	−0.5	−0.44	0.03	0.22	−0.37	−0.08	−0.59	0.12
	Marianne	−0.28	0.02	−0.59	−0.15	−0.29	0.43	0.53	−0.06	−0.01
	Marianne1	−0.41	0.26	−0.18	0.02	−0.36	0.09	−0.72	−0.26	−0.11
	Quare	0.44	0.08	−0.1	−0.76	0.2	0.32	−0.26	−0.05	0.06
	Turure	0.33	0.44	−0.14	−0.16	−0.5	−0.59	0.16	−0.03	0.17
	Yarra	−0.31	−0.06	0.54	−0.25	−0.16	0.09	0.18	−0.44	0.54
(c) Combined										
		Eigenvectors								
		q1	***q2***	***q3***	***q4***	***q5***	***q6***	***q7***	***q8***	***q9***
	Eigenvalues (expected upper 95% CI)	3.66	2.57	1.87	1.36	0.96	0.62	0.33	0.11	0
	Eigenvalues (observed)	3.63	2.97*	1.06	0.71	0.42	0.11	0.09	0.01	0

(Continues)

TABLE 4 (Continued)

(c) Combined										
		Eigenvectors								
		q1	*q2*	*q3*	*q4*	*q5*	*q6*	*q7*	*q8*	*q9*
	% variance explained	0.4	0.33	0.12	0.08	0.05	0.01	0.01	0	0
Lineage	Aripo	−0.15	−0.5	−0.25	−0.17	0.37	−0.41	−0.27	0.36	0.36
	Aripo1	−0.41	−0.24	0.27	−0.04	0.53	0.53	0	0.09	−0.36
	Guanapo	−0.37	0.38	0.01	−0.25	0.03	0.28	−0.39	−0.32	0.57
	Guanapo1	0.23	0.37	−0.08	−0.66	0.44	−0.27	0.11	−0.14	−0.28
	Marianne	−0.38	−0.04	−0.49	−0.38	−0.5	0.11	−0.13	0.2	−0.39
	Marianne1	−0.47	−0.09	−0.31	0.14	0.11	−0.22	0.62	−0.45	0.08
	Quare	0.29	−0.4	0.08	−0.5	−0.19	0.39	0.44	0	0.35
	Turure	0.32	−0.4	−0.32	0.06	0.07	0.14	−0.39	−0.64	−0.19
	Yarra	−0.25	−0.28	0.65	−0.24	−0.29	−0.41	−0.14	−0.29	−0.14

*Indicates dimension that is significantly above 95% threshold.

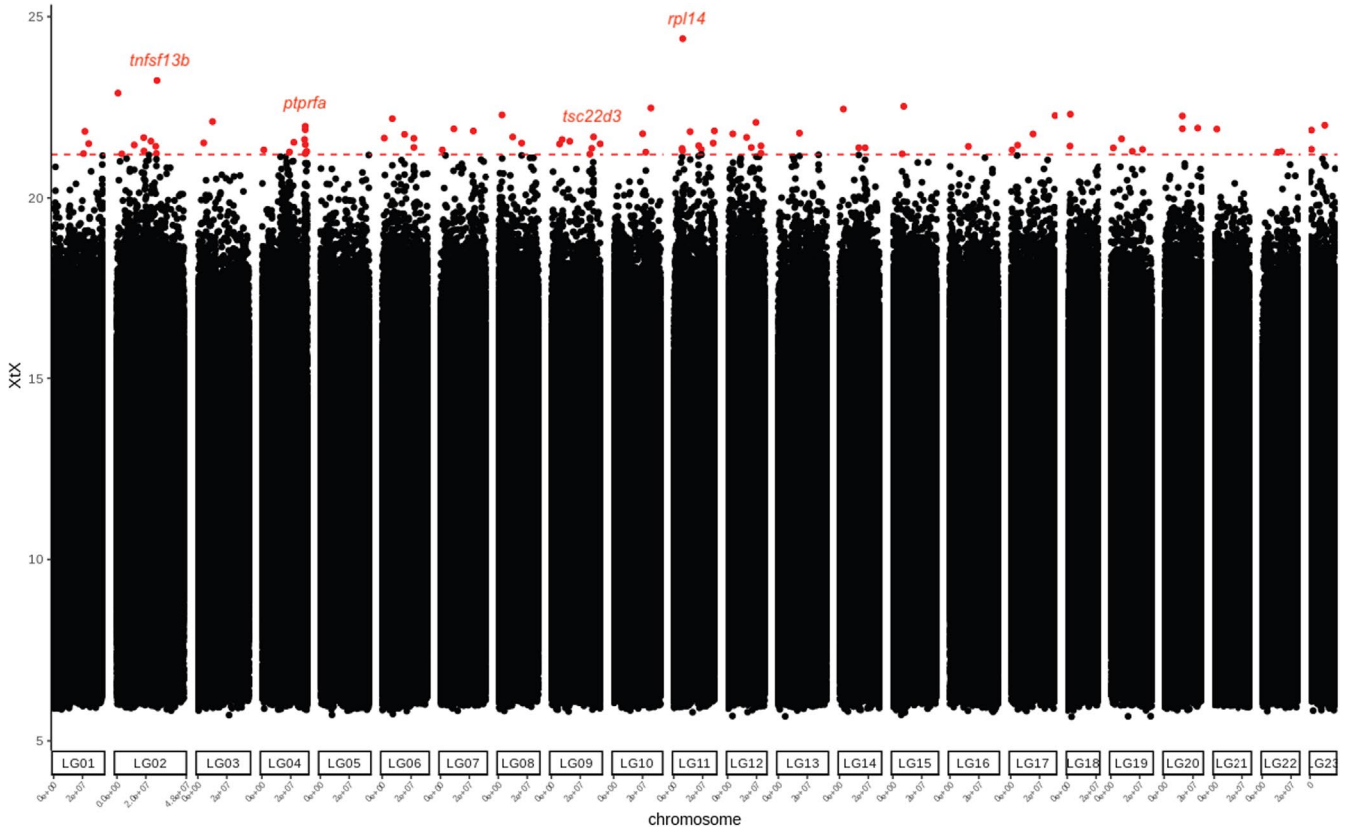

FIGURE 6 Manhattan plot of genome-wide differentiation among 12 male populations of guppies. The red dotted line and points indicate the 0.01% (21.2 *XtX*) threshold and significantly differentiated SNPs, respectively. Red dots denote significantly differentiated SNPs. Labels above dots denote putative candidate genes implicated cell morphogenesis linked to colour patterns

from SNPs associated with traits under the guppy visual model. However, we found enrichment of genes for nervous system development, anatomical structure morphogenesis, and cellular process using SNPs associated with the traits defined usng the cichlid visual model ($p < .0001$, Table S8). Interestingly, some of the nongenic SNPs with elevated BF values were near genes that have known roles in teleost patterning (e.g., *bnc2*, *cdh11*, for full list see Tables S9 and S10).

4 | DISCUSSION

Using colour traits defined by the visual systems of two major agents of selection on colour in male guppies (conspecifics, cichlid predator), we found an overall tendency towards higher, more conspicuous, phenotypic means in LP guppies as predicted. However, (i) trait means are not systematically higher at LP for within-river comparisons, (ii) statistical support for predation regime effects is weak, and (iii) regime effects explain little of the total among-population differentiation. Moreover, when modelling colour variation in multivariate phenotypic space we find (iv) little support for clustering of populations by predation regime, and (v) no evidence for parallel evolution of lineages. Nevertheless, putatively neutral patterns of genome-wide molecular differentiation did not readily explain the among-population phenotypic structure. This suggests that adaptive evolutionary processes have caused divergence of male colour among populations. Here, we first discuss these phenotypic patterns and their interpretation in relation to the evidence for parallel evolution of brighter male coloration across the high to low predation ecological transition in guppies. We then comment on the results of our genomic analyses that revealed some SNPs differentiated among populations in (or close) genes implicated in pigmentation, patterning and neuronal development in other fish species.

4.1 | Patterns of among-population phenotypic differentiation

Traits derived from the novel QCPA phenotyping pipeline offer only qualified support for the longstanding view that guppies from LP populations are particularly colourful and conspicuous (Endler, 1980; Haskins et al., 1961; Millar et al., 2006). Thus, trait means are higher at LP overall, but this is not a statistically robust pattern. Nor is it found consistently across the HP to LP transition within rivers. Although the lack of stronger patterns may seem surprising, we note that multiple previous guppy studies have also reported similarly inconsistent differences across regimes within rivers using transplant (Dick et al., 2018; Kemp et al., ,2009, 2018) and predator-manipulation experiments (Gotanda et al., 2019). More generally, we stress that our conclusions relate to the phenotype as defined here from the QCPA pipeline, and it is not our contention that previous studies using different colour traits are incorrect. Rather we suggest that different ways of quantifying colour variation may yield different, but hopefully complementary, patterns and insights. Colour differentiation between populations is strong using the QCPA phenotyping approach (approximately half of all phenotypic variance is among-populations), but predation regime does not explain much among-population variance (in single traits or multivariate phenotype). This conclusion is further supported by the finding that populations do not obviously cluster by predation regime in multitrait space, regardless of whether this space is defined in eight dimensions (using all traits defined from guppy and cichlid visual models) or 4 (i.e., using just guppy or just cichlid perception). Nor does correlation structure in the population variance-covariance matrix (**D**) support the presence of a single major axis of variation running from low (i.e., low trait values) to high conspicuousness.

In principle, these results could occur even with parallel evolution of replicate lineages across the HP-LP transition, if recent coancestry, drift and/or ongoing gene flow between populations are masking the phenotypic signature of parallelism. We think multiple lines of evidence argue against this possibility. First, among the 12 populations with SNP data we found no correlation of pairwise genome wide F_{ST} values and phenotypic distance. Second, our use of mixed models to partition among-population variance suggest not only that neutral processes are insufficient to explain among-population differentiation, but also that adaptive divergence explains most variation (discussed further below). Third, and perhaps most importantly, when isolating the phenotypic change from HP to LP within rivers, the inferred directions of phenotypic evolution are not parallel among lineages. This analysis assumes that river can be used as a proxy for lineage (sensu De Lisle & Bolnick, 2020), but that appears reasonable based on present and previous population genetic analyses (Willing et al., 2010; Blondel et al., 2019).

Thus, viewing male colour as a complex multivariate phenotype seen through the vision systems of biological agents of selection, we find no quantitative support for parallel evolution across the HP to LP regime transition. Rather, our results suggest that – in this phenotypic space – colour patterns evolve approximately independently in each river. However, the fact that genome wide molecular genetic structure does not predict phenotypic structuring argues against the idea that divergence of colour patterns could be primarily neutral. How can these results be reconciled with our existing understanding of colour evolution in guppies? In addition to predation regime, there are many among-population differences in local habitats that could lead to divergent selection regimes (e.g., substrate type, food sources, canopy cover and light environment). However, we also note that the lack of parallelism, coupled to apparently greater variation among LP than among HP populations, is actually consistent with the widely-held view that reduced selection pressure from visual predators facilitates evolutionary divergence of male traits by female choice. In guppies, the evidence that females prefer more conspicuousness male patterns that also increase predation risk is abundant (reviewed in Houde, 1997). However, this (univariate) conceptualisation of female choice masks the fact that, in multitrait space, there may be many different directions that increase conspicuousness to females.

We cannot conclude from this study that female choice is the major driver of among-population differentiation in male phenotype, although we consider it a plausible hypothesis. The extent to which female choice drives Fisherian runaway evolution of traits that are “arbitrary” (in the limited sense of not under viability selection) versus “costly” has been extensively debated (Kokko et al., 2002). However, even if females do prefer costly male phenotypes in all populations, this need not translate into closely aligned vectors of sexual selection on multivariate phenotype. Indeed, Endler and Houde (1995) demonstrated substantial geographic variation

TABLE 5 Location (LG and bp position) of significantly differentiated SNPs among populations and putative underlying genes

LG	Position	Median XtX	Annotated gene	Gene description	Zebrafish orthologue
LG01	20679153	21.22	ctnna2	Catenin (cadherin-associated protein), alpha 2	ctnna2[a]
LG01	21630340	21.84	na		
LG01	24267902	21.50	na		
LG02	735314	22.89	na		
LG02	3366912	21.21	mid1	Midline 1	mid1[b]
LG02	11968486	21.46	na		
LG02	18552459	21.67	LOC103480034	Teleost multiple tissue opsin 2b	tmtops2b
LG02	18738097	21.30	LOC103480073	DCN1, defective in cullin neddylation 1, domain containing 2a	dcun1d2a
LG02	23502075	21.57	LOC103481844	Potassium voltage-gated channel, subfamily H, member 3	kcnh3[b]
LG02	26822612	21.43	LOC103457726	Kalirin-like	kalrnb
LG02	27317080	21.23	na		
LG02	27737287	23.24	tnfsf13b	TNF superfamily member 13b	tnfsf13b[a]
LG03	4026992	21.52	na		
LG03	10136707	22.11	LOC103462047	UPF0469 protein KIAA0907 homologue	Uncharacterised
LG04	1388450	21.33	LOC108166264	Collagen alpha−1 chain-like	col24a1
LG04	19135875	21.27	LOC103464057	Sec1 family domain-containing protein 2	scfd1
LG04	22158698	21.54	LOC103463697	Uncharacterised	Uncharacterised
LG04	29560803	21.61	elavl4	ELAV like neuron-specific RNA binding protein 4	elavl4[a]
LG04	30040468	21.89	LOC103464223	Protein tyrosine phosphatase receptor type Fa	ptprfa[a]
LG04	30061932	21.98	LOC103464223	Protein tyrosine phosphatase receptor type Fa	ptprfa[a]
LG04	30108556	21.22	LOC103464223	Protein tyrosine phosphatase receptor type Fa	ptprfa[a]
LG04	30110320	21.47	LOC103464223	Protein tyrosine phosphatase receptor type Fa	ptprfa[a]
LG04	30492932	21.26	LOC103464192	Epidermal growth factor receptor substrate 15-like 1	eps15l1a
LG04	31086053	21.28	pde6d	Phosphodiesterase 6D, cGMP-specific, rod, delta	pde6d
LG06	1451327	21.65	dusp6	Dual specificity phosphatase 6	dusp6
LG06	7012709	22.19	tmem117	Transmembrane protein 117	tmem117
LG06	15480429	21.76	polg	Polymerase (DNA directed), gamma	polg
LG06	21928581	21.39	LOC103466341	Netrin−4-like	ntn4[a]
LG06	21928809	21.65	LOC103466341	Netrin−4-like	ntn4[a]
LG07	1303358	21.33	na		
LG07	9267800	21.91	xpc	Xeroderma pigmentosum, complementation group C	Xpc[b]
LG07	22765503	21.85	dcaf1	DDB1 and CUL4 associated factor 1	DDB1- and CUL4-associated factor 1-like
LG08	2558818	22.29	LOC103468161	Carbonic anhydrase-related protein 10-like	ca10a[c]
LG08	9964866	21.68	clg8h16orf72	Linkage group 8 C16orf72 homologue	zgc:66160
LG08	16159265	21.51	lfng	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	lfng[c]
LG09	5667793	21.49	na		
LG09	7471619	21.61	LOC103469702	Protein kinase, AMP-activated, beta 1 noncatalytic subunit, b	prkab1b

(Continues)

TABLE 5 (Continued)

LG	Position	Median XtX	Annotated gene	Gene description	Zebrafish orthologue
LG09	12715576	21.56	LOC103469911	Uncharacterised	zgc:152968
LG09	26617339	21.21	LOC103470491	Transient receptor potential cation channel, subfamily M, member 3	trpm3[c]
LG09	27956069	21.37	na		
LG09	29207832	21.68	dtx1	Deltex 1, E3 ubiquitin ligase	dtx1[d]
LG09	33808366	21.49	prdm6	PR domain containing 6	prdm6
LG10	20059227	21.77	na		
LG10	22172121	21.26	LOC103471396	TSC22 domain family, member 3	tsc22d3
LG10	25748269	22.48	na		
LG11	5785615	21.36	LOC103472064	Uncharacterised	uncharacterised
LG11	6043719	21.31	trio	Trio Rho guanine nucleotide exchange factor	Trio[c]
LG11	6221092	24.40	rpl14	Ribosomal protein L14	rpl14[d]
LG11	11324398	21.83	LOC103472245	Calcitonin receptor	calcr
LG11	17282316	21.44	na		
LG11	19054623	21.34	LOC103472636	Uncharacterised	uncharacterised
LG11	27311868	21.51	LOC103472993	Cyclic GMP-AMP synthase-like	uncharacterised
LG11	28122311	21.85	LOC108166719	Uncharacterised	uncharacterised
LG12	3570482	21.77	LOC104373253	Macrophage mannose receptor 1-like	rilpl1
LG12	13100673	21.67	LOC103473585	Matrix metallopeptidase 17a	mmp17a
LG12	16344388	21.39	na		
LG12	19606315	22.08	na		
LG12	23073848	21.24	LOC103474004	Immunoglobulin superfamily, member 9a	igsf9a[d]
LG12	23074083	21.44	LOC103474004	Immunoglobulin superfamily, member 9a	igsf9a
LG13	14610669	21.79	tusc5	Trafficking regulator of GLUT4 (SLC2A4) 1a	trarg1a
LG14	2734379	22.45	na		
LG14	13371933	21.39	na		
LG14	17753953	21.38	med13	med13	med13a
LG15	6448226	21.22	na		
LG15	7538997	22.53	na		
LG16	12769623	21.42	pianp	PILR alpha associated neural protein	pianp
LG17	963224	21.33	LOC103478874	TOX high mobility group box family member 4 a	tox4a
LG17	4792866	21.46	lrp8	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	lrp8
LG17	15468027	21.77	na		
LG17	30749000	22.27	slc39a6	Solute carrier family 39 member 6	slc39a6
LG18	1955592	21.43	LOC103480181	Neurexin−2-beta-like	uncharacterised
LG18	2195221	22.31	na		
LG19	1718516	21.38	rab11fip3	RAB11 family interacting protein 3 (class II)	rab11fip3[a]
LG19	7450869	21.63	LOC103481309	Uncharacterised	uncharacterised
LG19	14860722	21.29	ttyh2	Tweety family member 2	ttyh2
LG19	21916746	21.34	LOC108167282	Uncharacterised	uncharacterised
LG20	12634650	22.26	LOC103482504	Uncharacterised	uncharacterised
LG20	12638410	21.91	LOC103482504	Uncharacterised	uncharacterised
LG20	23266788	21.93	ptprn2	Protein tyrosine phosphatase receptor type N2	ptprn2
LG21	1839279	21.90	LOC103457165	AT rich interactive domain 1B (SWI1-like)	arid1b[a]

(Continues)

TABLE 5 (Continued)

LG	Position	Median XtX	Annotated gene	Gene description	Zebrafish orthologue
LG22	10792343	21.27	na		
LG22	13499891	21.28	kiz	Kizuna centrosomal protein	kiz
LG23	1038549	21.87	LOC103459116	Protein FAM180A-like	FAM180A
LG23	1038658	21.34	LOC103459116	Protein FAM180A-like	FAM180A
LG23	10260495	22.01	na		

Note.: Shaded cells indicate nongenic regions within which SNPs are found. Superscript number indicates putative function of gene (see Note).

[a]Cell/neural projection.

[b]Pigmentation/ion-transport.

[c]Neural processes.

[d]Cell morphogenesis.

in female preferences for colour patches, for example, black, orange, and colour contrast. Among-individual (female) differences in preference, a prerequisite for heritable variation that would allow trait-preference coevolution, have also been demonstrated (Brooks, 2002; Brooks & Endler, 2001). Lastly, we note that frequency dependent selection, with females preferring novel male phenotypes is well documented in guppies (Hughes et al., 2013), will generally impose vectors of directional selection that differ in space (i.e., across populations) as well as time (i.e., shifting as initially rare phenotypes become more common).

Our conclusion that predation regime has no consistent effect on the direction of evolution in multivariate trait space holds irrespective of the vision model(s) used to define phenotypes. We fully acknowledge that the *Crenicichla* vision model will imperfectly capture phenotypic variation perceived by fish predators; pike cichlids are absent from two sites while other piscivorous species are present at all sites. However, given qualitatively similar findings based on guppy vision traits alone, we consider it unlikely that this limitation is impacting our conclusion. In a more quantitative sense, the finding of population level correlations significantly less than +1 between homologous traits illustrates the wider potential for QPCA to offer new insights. What this means is that conclusions about among-population differences based on guppy vision will not necessarily hold true if we consider them from the perspective of a different viewer (in this case a cichlid). More simply, at the level of an individual encounter, it may be dangerous to assume a particular male phenotype that is conspicuous to a prospective mate is necessarily conspicuous to a (cichlid) predator.

Here, guppy colour patterns appear generally more conspicuous in the chromatic channel (saturation and chromaticity) for conspecifics, whereas the pike cichlid is more sensitive to achromatic (luminance) elements (Weadick et al., 2012). Such differences highlight the value of adopting colour measures appropriate to the visual systems of hypothesised selective agents (Endler, 1978; Endler & Mielke, 2005) and have implications for the way we the evolution of colour signals with multiple receiving species. We also acknowledge however, that that our study is naive to any abiotic factors (e.g., substrate, water colour, light transmission, and canopy cover) that may affect in situ perception of phenotype by guppies and/or their predators (Marshall et al., 2019). Local abiotic conditions are an explicit part of the wider sensory drive hypothesis (Endler, 1980, 1992), and Kemp et al. (2018) found that canopy cover influenced the relative abundance of iridescent versus melanic colour patches in guppies. While we have no evidence to suggest this, it is at least possible that conditioning among-population (or lineage) variance on abiotic covariates would reveal greater support for parallel evolution in the colour phenotype as measured here.

4.2 | Insights from genome-scans and SNP associations

Our pool-sequencing data provide several genetic insights that complement the phenotypic analyses. First, based on detected associations with the phenotypic traits defined here, male colour patterns are probably highly polygenic with genes distributed across the genome rather than being restricted to the sex chromosomes or otherwise clustered. Associated SNPs are found both outside and within genic regions and involve cis-acting elements. For instance, a SNP (LG:31463600) associated with differences in guppy-specific saturation was found in an intergenic region near basonuclin 2 (*bnc2*), a gene implicated in the maintenance of extracellular environments within which pigment cells driving patterning reside (Lang et al., 2009). Third, the minimal overlap between SNPs associated with homologous traits defined under the two vision models is consistent with the population level phenotypic correlations being less that one, suggesting that homolgous traits defined under different models are genetically distinct. However, since colour genetics have often been studied using human visual perception (Tripathi et al., 2009), it also highlights the possibility that ecologically-important genes may have been previously overlooked. Conversely, we did not detect any trait-SNP association consistency with colour genes previously identified in guppies, that is, *csf1ra* and *kita* (Kottler et al., 2013). This may again be explained by our choice of defined phenotype; focusing here on measures of overall body patterns and/or conspicuousness (as perceived by selective agents), rather than

features of individual pigmented patches (as perceived by humans). Fourth, no SNPs significantly associated with tested covariables were actually among the set identified as being significantly more differentiated than expected under neutrality in the core BayPass model. Taken at face value, this could indicate that greater (adaptive) genetic divergence among populations has occurred for specific traits or aspects of phenotype (e.g., life history) that were not quantified in this study.

To our knowledge, this is the first study to investigate among-population natural variation in male guppy patterns at the genomic level. Consequently some brief, and necessarily tentative, comments on specific SNPs identified are perhaps warranted. Among those more differentiated than expected under neutrality, several SNPs were detected in the protein tyrosine phosphatase receptor of types *Fa* (*ptprfa*) and N2 (*ptprn2*). Basic functions of these genes include cell proliferation and epithelial cell-cell adhesion. They are considered to counteract tyrosinase kinase receptors (Xu & Fisher, 2012), which play known roles in melanocyte and melanophore development (*Kita* and *cKit*, respectively; Alexeev & Yoon, 2006; Patterson & Parichy, 2019). It is not known if tyrosine phosphatase receptors carry a direct pigmentation function, although this is plausible given their documented involvement in cell differentiation, oncogenic-events, and sensory guidance in the skin (Wang et al., 2012). Several SNPs deviating from neutral expectations were also identified in less well-known genes including *polg* and *trio*, which are part of regulatory networks influencing melanocyte differentiation (Park et al., 2018; Seberg et al., 2017). Among SNPs associated with tested covariates (trait means and predation regime), we also find several in (or near) genes with known roles in fish colour. For instance, one SNP association with guppy vision $sat_{\Delta S}$ was near *ablim3*, a gene thought to be involved in pigment cell movement in fishes (Ahi et al., 2020). Another was found within *cx30.3*, part of a family of genes implicated in zebrafish skin development and pattern formation (Tao et al., 2010; Irion et al., 2014).

5 | CONCLUSION

Trinidadian guppies ostensibly represent a classic example of parallel (or convergent) evolution, with repeated divergence in male colour patterns between upstream and downstream populations due partly to predation conditions. Here, using a novel approach to characterising colour variation, we find that the well-described tendency for greater conspicuousness under low predation holds qualitatively true, when we analyse single traits defined under guppy and predator vision model. However, statistical support for this is weak, and repeated colonisation of low predation habitats has not lead to the parallel evolution of conspicuous phenotypes when these are characterised in quantitative, multivariate, phenotypic space. Instead, we suggest that colonisation of LP habitat may reduce selective constraints on phenotypic space imposed by predation, facilitating population-specific divergence under local selection regimes. Together, our results are not inconsistent with the widely-held hypothesis that reduced selection by predators allows male colour traits to evolve under sexual selection imposed by female choice. However, if so, the common assertion that female choice in guppies selects for brighter or more conspicuous males may mask the fact that, in multivariate trait space, colour is evolving in rather different directions across populations and lineages.

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CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

Lengxob Yong and Alastair J. Wilson designed the study with input from Darren P. Croft and Jolyon Troscianko. Lengxob Yong, Darren P. Croft and Alastair J. Wilson performed the research. Jolyon Troscianko and Indar W. Ramnarine contributed analytical tools. Lengxob Yong and Alastair J. Wilson analysed the data. Lengxob Yong and Alastair J. Wilson wrote the manuscript with input from Darren P. Croft, Indar W. Ramnarine, and Jolyon Troscianko.

DATA AVAILABILITY STATEMENT

Phenotypic data, SNP data sets, R and Java scripts have been made available on Dryad (doi.org/10.5061/dryad.s1rn8pk83), and genomic data (raw fastq files) are under ENA Project Accession PRJEB45804.

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SUPPORTING INFORMATION

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