Non-parallel transcriptional divergence during parallel adaptation

Running title: Transcriptional mechanisms of parallelism

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Abstract

How underlying mechanisms bias evolution toward predictable outcomes remains an area of active debate. In this study, we leveraged phenotypic plasticity and parallel adaptation across independent lineages of Trinidadian guppies (*Poecilia reticulata*) to assess the predictability of gene expression evolution during parallel adaptation. Trinidadian guppies have repeatedly and independently adapted to high- and low-predation environments in the wild. We combined this natural experiment with a laboratory breeding design to attribute transcriptional variation to the genetic influences of population of origin and developmental plasticity in response to rearing with or without predators. We observed substantial gene expression plasticity as well as the evolution of expression plasticity itself across populations. Genes exhibiting expression plasticity *within* populations were more likely to also differ in expression *between* populations, with the direction of population differences more likely to be opposite those of plasticity. While we found more overlap than expected by chance in genes differentially expressed between high- and low-predation populations from distinct evolutionary lineages, the majority of differentially expressed genes were not shared between lineages. Our data suggest alternative transcriptional configurations associated with shared phenotypes, highlighting a role for transcriptional flexibility in the parallel phenotypic evolution of a species known for rapid adaptation.

Key words: *Poecilia reticulata*, transcriptomics, phenotypic plasticity, adaptation, parallel evolution

Introduction

Phenotypic evolution is biased by the mechanisms that link genetic variation to phenotypic variation, i.e. the genotype-phenotype map (Alberch, 1991; Pigliucci, 2010). The complex nature of the genotype-phenotype map, the existence of 'many-to-one' mappings across hierarchical levels of biological organization, and the multigenic nature of complex traits make it challenging to assess how mechanistic properties shape evolutionary outcomes (Boyle, Li, & Pritchard, 2017; Potticary, Morrison, & Badyaev, 2020). A deeper understanding of these phenomena will further our insight into the forces limiting and potentiating the evolution of complex traits and deepen our knowledge of how organisms respond to novel and changing environments.

Studies focusing on the independent evolution of shared traits can reveal biases in mechanisms of adaptation by asking whether similar mechanism are associated with repeated, independent evolutionary transitions. Shared phenotypes arising in response to similar selection pressures are referred to as "parallel" or "convergent" depending on the evolutionary relatedness of populations and author preference (see Glossary). Different definitions have been discussed and debated elsewhere (e.g. Arendt & Reznick, 2008; Losos, 2011; Wake, Wake, & Specht, 2011), and we use "parallel" here following the terminology conventionally applied to our study system (see below).

Understanding whether shared phenotypes also share underlying genetic and molecular mechanisms is a long-stranding focus of evolutionary biology (reviewed in Bolnick, Barrett, Oke, Rennison, & Stuart, 2018; Stern, 2013). Compelling examples of parallelism and convergence demonstrate that similar phenotypes may share underlying neural, physiological, molecular, and/or genetic mechanisms, even across highly divergent taxa (e.g. Insel and Young, 2000; Manceau et al., 2010; Pankey et al., 2014; Rosenblum et al., 2010). Similar mechanisms underlying parallel evolutionary transitions suggest the genotype-phenotype map is constrained by limits on the possible ways to construct adaptive phenotypes (Losos, 2011). In this case, phenotypic evolution may rely on a limited number of mechanistic paths, repeatedly targeting

65 those mechanisms that yield the greatest phenotypic responses with the smallest pleiotropic
66 costs. Indeed, the recent view of complex traits as “omnigenic” highlights the potential for near
67 ubiquitous pleiotropy to constrain evolutionary trajectories to a very limited number of paths (Boyle
68 et al., 2017).

69 In contrast, non-shared mechanisms giving rise to shared, independently derived
70 phenotypes suggest that mechanistic flexibility may facilitate evolution by providing multiple,
71 alternative ‘solutions’ to a given adaptive problem, thereby providing alternative paths by which
72 organisms can reach adaptive peaks (Badyaev & Morrison, 2018; Drion, O’Leary, & Marder, 2015;
73 Grashow, Brookings, & Marder, 2009). Empirical studies demonstrate that different mechanistic
74 ‘solutions’ can give rise to shared phenotypes in closely related species, among populations of
75 the same species, or even among individuals of the same population (e.g. Abouheif and Wray,
76 2002; Crawford and Oleksiak, 2007; Drion et al., 2015; Grashow et al., 2009; Mandic et al., 2018).
77 Mechanistic flexibility may facilitate evolution by allowing organisms to reach the same adaptive
78 peak from alternative starting points (e.g. distinct genetic backgrounds), and/or buffering
79 organisms from pleiotropic effects via compensatory mechanisms that exploit the alternative
80 paths created by many-to-one mappings. Whether the potentially “omnigenic” nature of complex
81 traits contributes to constraints due to widespread pleiotropy or facilitates alternative paths is an
82 area ripe for exploration (Boyle et al., 2017).

83 In sum, empirical evidence for both shared and distinct mechanisms underlying parallel
84 phenotypic evolution leaves open the question of when and why either pattern dominates. Further
85 addressing this question is a focus of theoretical and empirical work, with implications for
86 evolutionary biology, conservation, and medicine (reviewed in Bolnick et al., 2018). In view of the
87 potential importance of many-to-one mappings, gene expression studies provide promising
88 starting points for surveying broadscale patterns of (non)parallelism in the mechanisms underlying
89 complex traits. Despite this promise and substantial recent interest in (non)parallel evolution
90 (Bolnick et al., 2018; Kaeuffer, Peichel, Bolnick, & Hendry, 2011; Oke et al., 2016; Paccard et al.,

2020; Stuart et al., 2017), gene expression mechanisms underlying parallel evolution have only recently begun to be explored (Ghalambor et al., 2015; Manousaki et al., 2013; Pankey et al., 2014).

Does phenotypic plasticity bias phenotypic evolution?

One feature of biological systems that may channel evolution into particular paths is environmentally induced phenotypic plasticity. First, both plastic and evolutionary processes may rely on a limited number of mechanistic paths, repeatedly targeting those mechanisms that yield the greatest phenotypic responses with the smallest pleiotropic costs. In this case, we expect plastic and evolutionary responses to share underlying mechanisms. Theory predicts that plastic and evolutionary changes will be in the same direction when plasticity in a novel environment is adaptive, increasing immediate survival and allowing time for evolutionary divergence via co-option of mechanisms involved in environmentally induced responses (Baldwin, 1896; Ghalambor, McKay, Carroll, & Reznick, 2007; Lande, 2009; West-Eberhard, 2003). In contrast, plastic and evolutionary changes will be in opposite directions when plasticity in a novel environment is non-adaptive, thereby increasing the strength of selection, or when plastic responses 'overshoot' adaptive optima and are compensated by selection (Conover, Duffy, & Hice, 2009; Ghalambor et al., 2007; Grether, 2005; Velotta & Cheviron, 2018). Importantly, plasticity may facilitate adaptation under either scenario and empirical studies document both adaptive (e.g. Fraser et al., 2014; Gleason & Burton, 2015; Li et al., 2018; Mäkinen, Papakostas, Vøllestad, Leder, & Primmer, 2016; Scoville & Pfrender, 2010; Shaw et al., 2014; Wang & Althoff, 2019) and non-adaptive (e.g. Dayan, Crawford, & Oleksiak, 2015; Ghalambor et al., 2015; W. Ho & Zhang, 2018; Pespeni et al., 2013; Schaum, Rost, Millar, & Collins, 2013) plasticity associated with adaptive evolution. Finally, plastic and evolutionary processes may rely on shared mechanistic paths because the mechanisms mediating phenotypic plasticity promote the accumulation of cryptic genetic variation that is released under new environmental conditions,

117 thereby fostering associations between plasticity and divergence (Draghi & Whitlock, 2012;
118 Espinosa-Soto, Martin, & Wagner, 2011).

119 In the present study, we combine measures of phenotypic plasticity and parallel adaptation
120 to ask whether flexibility in underlying mechanisms shapes evolutionary trajectories. While the
121 potentially important role of phenotypic plasticity in this process has been pointed out, few studies
122 have explicitly considered the role of environmentally induced plasticity in parallelism (for rare
123 examples see Oke et al., 2016; Torres-Dowdal, Handelsman, Reznick, & Ghalambor, 2012), nor
124 asked how plasticity itself evolves (West-Eberhard, 2003). By comparing transcriptional
125 mechanisms associated with adaptation within and across parallel lineages, we are able to assess
126 whether plasticity biases evolutionary outcomes, to ask whether parallel phenotypic evolution is
127 associated with shared gene expression patterns, and to speculate on the role of mechanistic
128 flexibility in adaptive evolution.

129

130 *Guppies as a model system*

131 Trinidadian guppies (*Poecilia reticulata*) have become a model system in ecology and
132 evolutionary biology due to repeated, independent adaptation of natural guppy populations to
133 distinct predation environments (Endler, 1995; Haskins, Haskins, McLaughlin, & Hewitt, 1961; D.
134 Reznick, Butler IV, & Rodd, 2001). In Trinidad, high- and low-predation population pairs from
135 different river drainages represent independent evolutionary lineages (Barson, Cable, & Van
136 Oosterhout, 2009; Fraser, Künstner, Reznick, Dreyer, & Weigel, 2015; Gilliam, Fraser, & Alkins-
137 Koo, 1993; Willing et al., 2010) in which colonization of low-predation environments has led to
138 parallel, adaptive changes in life history traits, morphology, and behavior (Endler, 1995;
139 Magurran, 2005; D. A. Reznick, Bryga, & Endler, 1990; D. Reznick et al., 2001; D. N. Reznick,
140 1997). For example, low-predation guppies shoal less tightly (Huizinga, Ghalambor, & Reznick,
141 2009; Magurran & Seghers, 1990b, 1991), escape more slowly (Ghalambor, Reznick, & Walker,
142 2004), are slower to re-commence movement following a predator encounter (Elvidge,

143 Ramnarine, & Brown, 2014; Harris, Ramnarine, Smith, & Pettersson, 2010), and perform fewer
144 predator inspections (Magurran & Seghers, 1990a, 1994) than their high-predation counterparts.
145 Building on decades of work comparing high- and low-predation populations in the wild,
146 more recent laboratory experiments have deployed breeding designs to disentangle genetic and
147 environmental influences of predation on phenotypic differences. These recent studies
148 demonstrate that a combination of genetic and environmental influences shape guppy life history
149 (Torres Dowdall et al., 2012), morphology (Fischer, Soares, Archer, Ghalambor, & Hoke, 2013;
150 Handelsman, Ruell, Torres-Dowdall, & Ghalambor, 2014; Ruell et al., 2013; Torres-Dowdal et al.,
151 2012), physiology (Fischer, Harris, Hofmann, & Hoke, 2014; Handelsman et al., 2013), and
152 behavior (Fischer, Ghalambor, & Hoke, 2016b; Huizinga et al., 2009; Torres-Dowdal et al., 2012).
153 Yet despite many years of work on whole-organism phenotypes, the mechanisms underlying
154 adaptive phenotypic differences in guppies remain largely unexplored. A single study
155 characterized brain gene expression in multiple populations from the same lineage during the
156 earliest stages of adaptation (~3 generations after colonization of low-predation environments)
157 and found a negative relationship between phenotypic plasticity and adaptive divergence
158 (Ghalambor et al., 2015).
159
160 *Transcriptional mechanisms of plasticity, evolution, and parallel adaptation*
161 In the present study, we take advantage of parallel phenotypic evolution in independent
162 lineages of Trinidadian guppies (*Poecilia reticulata*) to explore patterns of flexibility and constraint
163 in transcriptional mechanisms mediating repeated adaptation. We compare the effects of genetic
164 background (high- versus low-predation populations) and developmental environment (rearing
165 with and without predator cues) on brain gene expression patterns in two parallel, independent
166 evolutionary lineages. These lineages diverged at least 600,000 years ago, with subsequent,
167 more recent colonization of low-predation environments by high-predation ancestors within each
168 river drainage (Fajen & Breden, 1992; Fraser et al., 2015; Willing et al., 2010). We compare whole-

169 brain gene expression patterns *within* and *between* lineages based on evolutionary history with
170 and rearing with predators. We focus on gene expression in the brain because the previous gene
171 expression study in guppies used brain tissue (Ghalambor et al., 2015) and because of previous
172 evidence for behavioral plasticity in our focal populations (Fischer et al., 2016b).

173 We test four hypotheses concerning the influences of underlying mechanisms on adaptive
174 evolutionary outcomes. **Hypothesis 1** (H1), that genes with significant expression plasticity are
175 more likely to show genetic divergence in expression; **Hypothesis 2** (H2), that the direction of
176 plastic responses predicts the direction of genetic divergence; **Hypothesis 3** (H3), that gene
177 expression plasticity itself evolves; and **Hypothesis 4** (H4), that parallel phenotypic adaptation
178 across independent lineages relies on parallel gene expression changes. To test our hypotheses
179 we (H1) quantify the extent of overlap in genes exhibiting significant expression plasticity as well
180 as genetic expression divergence within each drainage, (H2) examine associations in the
181 direction of developmental and genetic expression differences within each lineage, (H3) ask
182 whether expression plasticity itself evolves, and (H4) assess whether parallel phenotypic
183 adaptation between lineages relies on similar changes in gene expression profiles.

184 A number of non-mutually exclusive, interrelated predictions follow from our hypotheses.
185 If developmental plasticity in gene expression does indeed predict genetic divergence in gene
186 expression, we expect the magnitude (H1) and direction (H2) of gene expression plasticity to be
187 associated with the magnitude and direction of expression divergence between high- and low-
188 predation populations within a river drainage (i.e. within an evolutionary lineage). If gene
189 expression plasticity itself evolves (H3), we expect the extent and/or direction of plastic responses
190 to differ between high- and low-predation populations within a lineage. Finally, if only a few gene
191 expression configurations (i.e. mechanistic paths) can give rise to shared adaptive phenotypes,
192 we expect parallel adaptation to low-predation habitats to be characterized by parallel expression
193 changes in set of genes shared between lineages (H4). In contrast, if transcriptional mechanisms
194 are flexible, we expect gene expression divergence between high- and low-predation populations

195 within each river to occur in largely non-overlapping gene sets. Taken together, our results allow
196 us to assess the flexibility of transcriptional mechanisms of adaptation across timescales – from
197 developmental plasticity, to genetic divergence within rivers, to parallel adaptation across
198 lineages.
199
200 **Material and Methods**
201 *Fish collection and rearing*
202 We used a laboratory breeding design to disentangle the influences of evolutionary history
203 with predators and developmental experience with predators on brain gene expression patterns.
204 We established lab populations of guppies from high- and low-predation populations pairs
205 collected from the Aripo and Quare river drainages in 2012 and 2014, respectively. To maintain
206 the genetic variation of the original wild-caught fish, we established 20 - 30 unique family lines for
207 each population (i.e., for each generation a single female from each family was crossed to a single
208 male from another family) (D. N. Reznick & Bryga, 1987). We used second-generation lab born
209 fish from these unique family lines in this study to minimize environmental and maternal effects.
210 At birth, we split second-generation siblings into rearing environments with (pred+) or without
211 (pred-) predator chemical cues, and they remained in these environments until the completion of
212 the experiment (as in Fischer et al., 2016). We used only mature males in this study. To maximize
213 the range of genetic variation captured among focal fish, all males in a given experimental group
214 (i.e. population and rearing environment) were from distinct families; however, our split-brood
215 design allowed us to simultaneously leverage genetic similarity among siblings and control for the
216 effects of genetic background on developmental responses. Figure 1 provides an overview of our
217 experimental design and interpretation of comparisons.
218 All guppies were individually housed in 1.5 liter tanks on a 12:12 hour light cycle at
219 Colorado State University. Fish were fed a measured food diet once daily, receiving Tetramin™
220 tropical fish flake paste and hatched *Artemia* cysts on an alternating basis. Prior to tissue

221 collection for this study, behavioral and hormone data were collected in an identical manner for
222 all fish and these results are reported elsewhere (Fischer et al., 2016b). All experimental methods
223 were approved by the Colorado State University Animal Care and Use Committee (Approval #12-
224 3818A).
225
226 *Tissue collection and processing*
227 We collected brain tissue from the Aripo and Quare lineage males described above in
228 2013 and 2015, respectively. To standardize any effects of recent experience, behavior, and
229 circadian rhythm on gene expression, we collected whole brains within 10 minutes of lights-on in
230 the morning. We collected tissue from a maximum of four fish each day, one from each
231 experimental group per day, collected in a random order. We interpret our transcriptional data as
232 baseline, in the sense that fish were minimally stimulated prior to tissue collection, and expression
233 levels should therefore reflect genetic background and developmental experience more strongly
234 than responses to immediate environmental context. Fish were anesthetized by immersion in ice
235 water followed by rapid decapitation. Whole brains were removed, flash frozen in liquid nitrogen,
236 and stored at -80°C until further processing. Tissue collection took <2 minutes per fish, rapid
237 enough to minimize changes in gene expression responses to handling and dissection.
238 We extracted total RNA from brain tissue using the Qiagen RNeasy Lipid Tissue Mini Kit
239 (Qiagen, Germany) following manufacturer guidelines. We prepared separate sequencing
240 libraries for each individual using the NEBNext Ultra RNA Library Prep Kit for Illumina (New
241 England Biolabs, Massachusetts, USA) following manufacturer instructions. Libraries were
242 sequenced on an Illumina HiSeq 2000 at the Florida State University College of Medicine
243 Translational Science Laboratory (Tallahassee, Florida) in May 2014 (Aripo dataset) and January
244 2016 (Quare dataset). For the Aripo dataset, 40 samples (N=10 per group) were combined with
245 unique barcodes into eight samples per pool and each pool was sequenced on a single lane. For
246 the Quare dataset, 60 samples (N=12-16 per group) were combined into three pools with 20

247 samples per pool and each pool was sequenced in two separate lanes. Blocks of individuals run
248 in the same week were processed and sequenced together, and experimental groups were
249 balanced across sequencing lanes.
250
251 *Transcript abundance estimation*
252 To quantify gene expression, we mapped RNA sequencing reads to the guppy genome.
253 We received 1.45 billion 100-bp paired-end reads that passed the HiSeq quality filter, averaging
254 14.5 million reads per sample. We used Rcorrector to amend Illumina sequencing errors (Song &
255 Florea, 2015), and removed adapter sequences and trimmed reads for high-quality sequence
256 using Trim Galore! (Babraham Bioinformatics, Babraham Institute). Following developer
257 recommendations, we used a quality score of 33, a stringency of 5, and a minimum read length
258 of 36bp. We aligned corrected, trimmed reads from both datasets to the guppy genome
259 (http://uswest.ensembl.org/Poecilia_reticulata; downloaded November 2019) and estimated
260 transcript abundance using STAR (Dobin et al., 2013) with default parameters. On average, 91%
261 (range: 88.8% – 94.2%) of sequences per individual mapped to the guppy genome assembly.
262
263 *Data filtering and screening*
264 Prior to analysis, we performed data filtering and quality screening of gene expression
265 data. Preliminary cluster analyses revealed retinal contamination in a subset of our Aripo dataset
266 brain samples. While opsins are expressed at low levels in the brain, the very high expression
267 levels (>10,000 copies) in three samples pointed to retinal contamination. To deal with this issue,
268 we devised a sample filtering and screening procedure to remove genes in which expression
269 differences between samples were likely dominated by retinal contamination. Briefly, we first
270 filtered genes with low expression, then we used contigs annotated as known retinal genes
271 (Rhodopsin, red/green-sensitive opsins, blue-sensitive opsins) as seed contigs to identify other
272 contamination-related transcripts based on high positive correlations of expression levels with

273 seed genes. We calculated the gene-wise sum of correlations between candidate genes and seed
274 genes and performed multiple hypothesis testing using a false discovery rate (FDR) controlling
275 procedure. The nominal level of FDR was set to α=0.2 to remove presumptive contaminant
276 contigs. Using this approach, we identified 1,559 contigs as presumptive retina-enriched genes
277 (~ 3% of all contigs in our final assembly) which we removed from both datasets in all subsequent
278 analyses (Table S1). More detailed descriptions of statistical procedures used for filtering are in
279 the Supplemental Methods.
280
281 *Differential expression analysis*
282 To test our specific hypotheses, we first needed to identify transcripts differentially
283 expressed between experimental groups, i.e. fish with differences in evolutionary history with
284 predators and/or developmental experience with predators (Figure 1). Due to differences in the
285 timing of fish rearing, sample processing, and sequencing, we did not combine Aripo and Quare
286 datasets for statistical analyses. We instead performed analysis in an identical fashion for both
287 drainages and subsequently conducted separate analyses to test hypotheses concerning
288 mechanistic parallelism across drainages (see below). Standard differential expression analysis
289 packages could not accommodate the random effects in our experimental design (i.e. family and
290 week; see below) but including these effects improved model fit and reflected our split-brood
291 experimental design, in which siblings were split into different rearing environments at birth, and
292 tissue was collected from fish in balanced groups of four in different weeks (see above). Fitting a
293 generalized linear mixed model with negative binomial link to each data set, revealed that a subset
294 of genes had statistically significant random effects due to either family, week, or both. Model
295 comparisons between the generalized linear model without random effects and the generalized
296 linear mixed model including week and family effects supported incorporating random effects, as
297 likelihood ratio tests indicated better fit of the mixed models for 391 out of 19,004 genes in the
298 Aripo dataset and 1,194 out of 19,902 genes in the Quare dataset. For consistency, we therefore

299 performed differential expression analysis using generalized linear mixed models with random
300 effects for all genes. Additional details of linear mixed model procedures are in the Supplemental
301 Methods.

302 We normalized read counts using DESeq2 in R (Love, Huber, & Anders, 2014) and
303 performed differential expression analysis using the lme4 package in R (github.com/lme4).
304 Normalized count data were modeled using a generalized linear mixed model with negative
305 binomial distribution as described above. We included population of origin, rearing environment,
306 and their interaction as fixed effects. In addition, we included family (siblings split across rearing
307 environments) and week (tissue was collected from animals in balanced blocks across multiple
308 weeks) as random effects. A Wald's test provided p-values for main effects and interaction effects
309 for each gene (Lehmann & Romano, 2005). We adjusted p-values for multiple hypothesis testing
310 using a direct approach for FDR correction (Storey, 2002) as implemented in the fdrtool package
311 in R (Strimmer, 2008). We considered transcripts differentially expressed (DE) if the adjusted p-
312 value for an effect was below 0.05.

313 To examine whether differential expression calls were influenced by transcript abundance,
314 we compared mean and median counts of DE and non-DE genes using two sample t-tests and
315 Wilcoxon rank sum tests. We also compared overall gene-wise variance between DE and non-
316 DE groups with respect to main effects of population of origin or rearing environment using Siegel-
317 Tukey tests and Kolmogorov-Smirnov tests. As sample sizes differed between our two datasets,
318 we also performed a down-sampling simulation to test for the effects of sample size on DE gene
319 number (Supplemental Methods).

320 We performed GO term enrichment analysis for all sets of DE transcripts using 'Biological
321 Processes' in the topGO package in R (Alexa & Rahnenfuhrer, 2016) and GO annotation
322 information from the guppy genome (http://uswest.ensembl.org/Poecilia_reticulata; downloaded
323 November 2019).

324

325 *Statistical tests of specific hypotheses*

326 To test Hypothesis 1, we evaluated overlap in transcripts differentially expressed between
327 populations (i.e. population DE genes) and between rearing conditions (i.e. rearing DE genes).
328 To test the hypothesis that initial plastic responses following colonization of low-predation
329 environments might drive subsequent divergence, we specifically compared genes with
330 significant ancestral plasticity (i.e. differences between high-predation siblings reared with and
331 without predators; see Glossary and Figure 1) to genes with significant genetic expression
332 divergence (high- versus low-predation fish reared in the derived, predator-free environment)
333 within each drainage. We used FDR-corrected post-hoc tests of simple effects to identify DE
334 genes in these comparisons and estimate log-fold gene expression changes.

335 To test Hypothesis 2, we compared the association between directions of plasticity and
336 divergence (concordant vs. non-concordant) in (1) those genes with *both* significant ancestral
337 plasticity and significant population differences, and (2) *all* transcripts with significant population
338 differences in expression, including those without significant expression plasticity. The second
339 comparison addressed the possibility that even subthreshold expression plasticity in response to
340 rearing environment could influence patterns of expression divergence, especially given the high
341 false negative rate in our conservative DE analyses. We used log-fold expression changes to
342 determine whether gene expression differences were in the same/concordant direction (i.e.
343 upregulated in high-predation populations and in response to rearing with predator cues, or down-
344 regulated in high-predation populations and in response to rearing with predator cues) or
345 opposite/non-concordant direction (upregulated in high-predation populations but down-regulated
346 in response to rearing with predators, or vice versa).

347 Statistical approaches to test Hypotheses 1 and 2 that compare gene-wise contrasts with
348 respect to a common experimental group (high predation fish reared without exposure to
349 predators) are biased and nontrivial to correct, (for illustration of this point see a classical example
350 in the Supplementary Methods and the discussion by Mallard, Jakšić, & Schlötterer, 2018). We

351 therefore instead evaluated whether significant effects of rearing condition in the ancestral
352 population tended to be associated with significant population effects across all genes rather than
353 evaluating individual genes. To do so, we focused on (a) the probability of significant population
354 simple effects conditional on significant rearing effects in the high-predation population; and (b)
355 the probability of significant population simple effects conditional on non-significant rearing effects
356 (Cao, Zhou, Breidt, & Peers, 2020; Hwang & Liu, 2010; Storey, 2007). We tested whether the
357 conditional probabilities of gene expression divergence between high- and low-predation
358 populations differed for genes that exhibited ancestral plasticity and those that did not (Hypothesis
359 1). We further tested whether conditional probabilities for upregulated or downregulated genes in
360 the low-predation population compared to the high-predation population differed depending on
361 the direction of plasticity (Hypothesis 2). To draw inference on these conditional probabilities
362 across all genes, we adapted a parametric bootstrap method (Efron & Tibshirani, 1993) similar to
363 the approach implemented by (W. C. Ho & Zhang, 2019). As the number of genes is large, the
364 estimated conditional probabilities are accurate and have statistical properties that make the use
365 of parametric bootstrap valid. We calculated parametric bootstrap confidence intervals for these
366 differences in conditional probabilities as detailed in the Supplemental Methods, with confidence
367 intervals that do not include zero indicating a significant association between gene expression
368 divergence and plasticity.

369 To test Hypothesis 3, we examined the subset of transcripts with significant interaction
370 effects in our differential expression analysis (see above). We grouped genes with significant
371 interaction effects into categories for the evolution of plasticity outlined by Renn and Schumer
372 (2013) based on *post hoc* differences between rearing environments within a population ($p<0.05$):
373 (1) Assimilated: plasticity in the ancestral high-predation population but a loss of plasticity in the
374 derived low-predation population; (2) Accommodated: a change in the degree, but not the
375 direction, of plasticity in the derived as compared to the ancestral population; (3) Reversed:
376 opposing directions of plasticity in high- versus low-predation populations; (4) Evolved plastic: no

377 plasticity in the ancestral high-predation population but an emergence of plasticity in the derived
378 low-predation population; and (5) Unclassified: genes that had a significant interaction effect but
379 no significant *post hoc* rearing differences and therefore could not be unambiguously classified
380 into one of the above categories. All statistical tests and data visualization were performed in R
381 (version 3.5.1; The R Foundation for Statistical Computing).

382 To test Hypothesis 4, we evaluated overlap in DE transcript identity and expression
383 direction between the Aripo and Quare lineages. We used chi-square tests of independence to
384 test for greater than chance overlap *between* drainages in those gene sets differentially expressed
385 based on rearing environment or population of origin *within* drainages. We also used chi-square
386 tests to test for biases in concordant versus non-concordant expression between drainages. We
387 performed two additional comparisons to address potential bias in our conclusions due to high
388 false negative rates in gene expression data (Rice, Schork, & Rao, 2008). First, we performed
389 the same analysis as before at two less stringent p-value cut-offs (p=0.1 and 0.2). Second, for
390 comparisons of expression direction, we made an additional, more conservative comparison by
391 including not only those genes differentially expressed in *both* drainages (i.e. the intersection of
392 DE gene lists), but also those genes differentially expressed in *either* drainage (i.e. the union of
393 DE gene lists) in the analysis. We again used log-fold expression differences to call gene
394 expression as being in the same/concordant or opposite/non-concordant direction between
395 drainages.

396

397

398 **Results**

399 *Differential gene expression and GO term enrichment*

400 In order to test our specific hypotheses, we first summarized differential expression
401 patterns in both datasets. In the Aripo drainage, 766 genes were differentially expressed (DE)
402 between high- and low-predation populations, 1,118 genes were differentially expressed between

403 pred- and pred+ fish, and 825 genes had interaction effects (Fig. 2; Table S2). DE genes were
404 enriched for GO categories including DNA integration, immune function, and morphogenesis. In
405 the Quare drainage, 3,831 genes were differentially expressed between high- and low-predation
406 populations, 791 genes were differentially expressed between pred- and pred+ fish, and 586
407 genes had interaction effects (Fig. 2; Table S3). DE genes were enriched for GO categories
408 including immune function, developmental processes, and neurogenesis. Complete results of GO
409 enrichment analyses for population of origin, rearing, and interaction effects are in the
410 Supplemental Information (Tables S4 & S5).

411 Given the marked difference in the total number of DE genes between datasets, we looked
412 for evidence that this pattern might arise from technical variation, expression bias, or differences
413 in sample size between the two datasets. While we cannot rule out some influence of technical
414 variation, we found no evidence for differences in sequence quality, read alignment statistics, or
415 variance in transcript abundance to suggest that variation in the number of DE transcripts between
416 drainages was of technical origin. Nor did we find evidence that identification of DE transcripts
417 was biased by transcript expression level: mean and median gene expression were significantly
418 lower in DE (population, rearing, and/or interaction effects) as compared to non-DE transcripts in
419 both the Aripo (Fig. S1; mean: DE=263, non-DE=344, $p<0.0001$; median: DE=65, non-DE=107,
420 $p<0.0001$), and Quare (Fig. S1; mean: DE=264, non-DE=334, $p<0.0001$; median: DE=86, non-
421 DE=101, $p<0.0001$) datasets. A down-sampling simulation provided no evidence for a reliance of
422 the greater number of population DE genes in the Quare dataset on the larger sample size of this
423 dataset (Figure S2). In sum, these tests suggest that identification of DE transcripts was not
424 influenced by relatively greater expression magnitude or variance of these transcripts, nor by
425 sample size differences.

426

427 *Expression plasticity biases the propensity for genetic expression divergence*

428 Evolutionary theory and empirical studies suggest that environmentally induced plasticity
429 may facilitate adaptation to novel environments. To test Hypothesis 1, we asked whether genes
430 with significant ancestral expression plasticity were more likely to show genetic divergence in
431 expression. We examined the extent of overlap in gene sets (1) differentially expressed between
432 high-predation fish reared with and without predators, to those (2) differentially expressed
433 between populations adapted to high- versus low-predation environments. In both drainages, the
434 DE genes with significant ancestral plasticity were more likely to show significant population
435 divergence than expected by chance (Aripo: 264 transcripts, 95% CI for differences in conditional
436 probability of significant divergence for plastic genes compared to conditional probability of
437 divergence for non-plastic genes: [0.125, 0.150]; Quare: 409 transcripts, 95% CI: [0.211,0.239]),
438 supporting an association between plastic responses and genetic divergence.
439
440 *Expression plasticity biases the direction of genetic expression divergence*
441 To test Hypothesis 2, we asked whether the directions of expression plasticity and genetic
442 expression divergence were associated. We did so for those genes with both significant ancestral
443 plasticity and population differences, as well as the larger set of all transcripts with significant
444 population differences (i.e. including those with and without significant rearing differences). We
445 performed the second, more conservative comparison because we reasoned that even
446 subthreshold expression plasticity could influence the direction of adaptive divergence. Within
447 both drainages, we found a signature of non-adaptive plasticity associated with genetic
448 expression divergence. Approximately 84% of DE genes exhibited population and rearing
449 expression changes in non-concordant directions in the Aripo drainage (95% CI for difference in
450 probability of same versus opposite expression direction: [-0.564,-0.499]), and 68% of DE genes
451 exhibiting non-concordant expression in the Quare drainage (95% CI: [-0.520,-0.456]) (Fig. 3).
452 Patterns were consistent in both drainages when we considered the larger set of all genes with
453 significant population expression differences (Aripo: 80% of genes with non-concordant

454 expression, 95% CI: [-0.811,-0.752]; Quare: 66% of genes with non-concordant expression, 95%
455 CI: [-0.803,-0.733]).
456
457 *Evolution of expression plasticity*
458 We next explored the evolution of expression plasticity itself to test Hypothesis 3. We
459 identified genes with evolved expression plasticity as those in which the direction and/or degree
460 of rearing effects depended on population of origin, i.e. those genes with significant population-
461 by-rearing interaction effects. The number of genes with significant expression plasticity that
462 evolved (i.e. significant rearing and interaction effects) was approximately a quarter to a third of
463 the number of genes with significant expression plasticity that did not evolve (i.e. significant
464 rearing effect but no interaction; 263 (24%) in Aripo and 286 (36%) in Quare) (Fig. 2).
465 To further characterize how expression plasticity evolved, we subdivided genes with
466 significant population-by-rearing interaction effects into one of five categories: assimilated,
467 accommodated, reversed, evolved plastic, or unclassified (Fig. 4A; based on Renn and Schumer,
468 2013). We found many transcripts that exhibited evolution of plasticity, with all five categories
469 represented in both datasets (Fig. 4B). Of the genes with significant interaction effects, in the
470 Aripo drainage 263 (32%) transcripts showed patterns of expression assimilation, 81 (10%)
471 transcripts showed patterns of expression accommodation, 113 (14%) transcripts exhibited
472 reversed plasticity, and 182 (22%) transcripts evolved plasticity in the derived population. In the
473 Quare drainage, 164 (28%) transcripts showed patterns of expression assimilation, 110 (19%)
474 transcripts showed patterns of expression accommodation, 128 (22%) transcripts exhibited
475 reversed plasticity, and 95 (16%) transcripts evolved plasticity in the derived population (Fig. 4B).
476
477 *Parallelism in gene expression patterns across drainages*
478 To test Hypothesis 4 that shared mechanisms mediate parallel phenotypic adaptation
479 across drainages, we asked whether differentially expressed genes *within* drainages were

480 overlapping in identity and expression direction *between* drainages. Of the genes that diverged
481 between high- and low-predation populations within a drainage (i.e., population main effect), 174
482 were overlapping between drainages (Table S6), more than expected by chance (χ^2=8.02,
483 p=0.0046). However, the direction of expression divergence among these overlapping genes was
484 not associated across the two drainages (χ^2=0.16, p=0.686): 91 genes (52%) had concordant
485 expression changes and 83 genes (48%) had non-concordant expression changes between
486 drainages (Fig. 5). To control for the influence of false negatives on our conclusions, we repeated
487 this comparison at less stringent significance adjusted p-value cut-offs. At a p<0.1, we found
488 marginally significant overlap in gene identify (347 overlapping genes; χ^2=3.38, p=0.06), but no
489 association in expression direction (χ^2=0.65, p=0.42), and at p<0.2 we found no significant overlap
490 or association in expression direction. These patterns remained unchanged when we restricted
491 the analysis to those genes with strongest evidence for adaptive divergence (see Methods and
492 Table S8).

493 Of the genes that showed significant expression plasticity (i.e. main effect of rearing), 64
494 transcripts were overlapping between drainages (Table S7), more than expected by chance
495 (χ^2=14.06, p=0.0001). Of these 43 (67%) had plastic changes in the same direction (i.e.
496 concordant) and 21 (33%) had expression changes in opposite directions (i.e. non-concordant)
497 between drainages, a stronger signature of concordant expression between river drainages than
498 expected by chance (χ^2=7.56, p=0.006) (Fig. 5). At less stringent p-value cut-offs for differential
499 expression we found no more overlap in gene identity than expected by chance (p<0.1: 108
500 overlapping genes; χ^2=0.05, p=0.81; p<0.2: 276 overlapping genes; χ^2=1.36, p=0.24).

501

502

503 **Discussion**

504 We examined transcriptomic differences associated with adaptive divergence and rearing
505 environment across repeated, independent evolutionary lineages in guppies. Within lineages, we

506 observed phenotypic plasticity in gene expression patterns as well as the evolution of gene
507 expression plasticity itself. Plastic genes were more likely to exhibit population differences in
508 expression, and these population differences were more likely to be in the opposite, rather than
509 the same, direction as ancestral expression plasticity. Comparing across lineages, DE gene sets
510 for population and rearing within each lineage were largely non-overlapping (>75% non-shared
511 identity) between lineages. Nonetheless, the small number of DE genes that did overlap between
512 drainages was more than expected by chance. Taken together, our findings suggest that a small
513 number of core genes may be repeatedly targeted during colonization of low-predation
514 environments, but that largely non-shared, alternative transcriptional solutions are associated with
515 parallel phenotypic adaptation across lineages. Combining studies of phenotypic plasticity and
516 parallel adaptation, we highlight a role for transcriptional flexibility in the parallel phenotypic
517 evolution of a species known for rapid adaptation.
518
519 *Impacts of gene expression plasticity on expression evolution*
520 A growing number of studies are using transcriptomic and proteomic surveys to address
521 the long-standing debate on the role of plasticity in evolution, with contrasting results favoring the
522 alternative hypotheses that adaptive (Fraser et al., 2014; Gleason & Burton, 2015; Li et al., 2018;
523 Mäkinen et al., 2016; Scoville & Pfrender, 2010; Shaw et al., 2014; Wang & Althoff, 2019) versus
524 non-adaptive (Dayan et al., 2015; Ghalambor et al., 2015; W. Ho & Zhang, 2018; Pespeni et al.,
525 2013; Schaum et al., 2013) plasticity facilitates adaptation. We recently proposed that non-
526 adaptive plasticity dominates during the earliest stages of rapid evolution, and that adaptive
527 plasticity may contribute to subsequent fine-tuning of phenotypes (Fischer et al., 2016a). In line
528 with this prediction and our previous findings, we report here that the strong signature of non-
529 adaptive plasticity in brain gene expression (89% of transcripts) observed in guppy populations in
530 the earliest stages of adaptation to low-predation environments (Ghalambor et al., 2015) is
531 present but weaker in long-established low-predation populations. Furthermore, in the current

532 study, this signature of non-adaptive plasticity was more apparent in the more recently diverged
533 Aripo lineage (80% of genes) as compared to the older Quare lineage (65% of genes). Our
534 findings highlight the need to explicitly consider how plasticity relates to divergence throughout
535 successive stages of adaptation in order to build a more holistic understanding of the role of
536 plasticity in adaptation.
537
538 *Evolution of plasticity in gene expression*
539 Diverse transcriptional patterns could accompany evolved differences in behavioral
540 plasticity (Renn & Schumer, 2013), but relevant data characterizing the evolution of gene
541 expression plasticity has been lacking. We found that plasticity itself evolved in approximately one
542 third of genes with significant ancestral expression plasticity in both lineages. As was observed
543 for transcriptomic evolution of gill tissue in stickleback fish (Gibbons, Metzger, Healy, & Schulte,
544 2017), plasticity evolution showed no consistent pattern, with genes gaining, losing, and switching
545 the direction of expression plasticity between ancestral and derived populations in both lineages.
546 The lack of consistency in these patterns and the dearth of studies that have characterized the
547 evolution of gene expression plasticity make these patterns difficult to interpret at present. Some
548 of the diversity is likely associated with adaptive phenotypic divergence between high- and low-
549 predation populations, in which initially plastic behavioral shifts may become fixed, eliminated, or
550 altered over time. For example, evolution in gene expression plasticity could reflect the gains,
551 losses, and switches behavioral plasticity in these populations (Fischer et al., 2016b).
552 Alternatively, compensatory and homeostatic mechanisms could promote diversity among plastic
553 responses in gene expression without altering higher-level phenotypic traits such as morphology
554 and behavior (Badyaev, 2018; Fischer, Ghalambor, & Hoke, 2016a; Renn & Schumer, 2013).
555 Finally, because fish in low-predation habitats experience relaxed selection on predator-induced
556 plasticity in conjunction with low effective populations sizes, some of the evolution of
557 transcriptional plasticity we report likely arose as a product of genetic drift, rather than selection

558 for altered plastic responses (Lynch, 2007). Documenting the evolution of expression plasticity is
559 an important first step, and additional studies are needed to understand the ubiquity and
560 evolutionary sources of these patterns.
561
562 *Transcriptomic signatures of population divergence in two independent evolutionary lineages*
563 We identified many genes differentially expressed between high- and low-predation
564 populations in each river drainage. The absolute number of population DE transcripts was smaller
565 in the Aripo drainage as compared to the Quare drainage, but the number of developmental and
566 interaction differences was greater in the Aripo drainage. Our analyses provided no evidence that
567 technical differences in expression magnitude, expression variance, or sample size influenced
568 these differences between datasets in the number of genes reaching statistical significance. We
569 therefore propose that differences in DE gene number between drainages are biological in origin,
570 as high- and low-predation populations in the Quare drainage show greater genetic divergence
571 than those in the Aripo drainage (Willing et al., 2010). Indeed, we note that the larger number of
572 genes in the Quare dataset come from a large difference in the number of genes with population,
573 but not rearing or interaction, effects. In sum, we suggest that the difference in the number of
574 differentially expressed transcripts between datasets is primarily driven by the greater degree of
575 genetic divergence between high- and low-predation populations within the Quare as compared
576 to the Aripo river drainage (Willing et al., 2010).
577 When we compared DE gene sets between drainages, we found a small, but significant,
578 number of population and rearing DE genes shared across lineages. However, the majority of DE
579 genes were non-shared across drainages for both population (Aripo: 78%; Quare: 96%), and
580 rearing (Aripo: 94%; Quare: 92%) effects, and we note that this pattern persisted at less stringent
581 p-values cutoffs. Rearing DE genes showed a significant association in expression direction
582 across lineages, with 67% of genes concordantly differentially expressed between lineages. In
583 contrast, genes associated with genetic divergence between high- and low-predation populations

584 in both drainages showed no signature of expression concordance across lineages. We suggest
585 that these patterns across drainages point to a small number of core genes that exhibit
586 predictable, plastic expression responses upon colonization of low-predation environments, but
587 that lineage-specific selection pressures, differences in genetic background, non-adaptive
588 processes (e.g. drift, inbreeding, founder effects), and alternative gene expression responses give
589 rise to largely non-overlapping, non-concordant expression differences associated with parallel
590 phenotypic adaptation.

591 A previous study in guppies performed a similar comparison of gene expression changes
592 associated with adaptation to low-predation environments (Ghalambor et al., 2015) and found a
593 strong signal of concordant differential expression in genes differentially expressed based on
594 population of origin. Whereas the present study compared long-term natural population
595 divergence across drainages, Ghalambor et al. (2015) characterized the early stages of
596 adaptation of experimentally introduced low-predations populations derived from founders from a
597 single high-predation source population within the same drainage. These contrasting findings in
598 comparisons of population pairs within the same drainage versus across genetically distinct
599 drainages highlight the impacts of standing genetic variation within the source population on
600 mechanisms of divergence (Feiner, Rago, While, & Uller, 2017; Thompson, Osmond, & Schluter,
601 2019), particularly at early stages of evolution (Barrett & Schluter, 2008): while alternative
602 transcriptional 'solutions' are possible, shared genetic background appears to bias evolutionary
603 outcomes toward shared patterns. These findings are exciting as they extend to a mechanistic
604 level the growing recognition of and interest in the non-parallel aspects of parallel adaptation,
605 even in systems considered classical examples of parallel/convergent evolution (Bolnick et al.,
606 2018; Manousaki et al., 2013; Paccard et al., 2020; Stuart et al., 2017).

607 Both adaptive and non-adaptive processes may contribute to the combination of shared
608 and distinct transcriptional mechanisms we find associated with parallel, adaptive life-history,
609 morphological, and behavioral phenotypes across lineages in guppies. First, as described above,

differences in standing genetic variation likely influence which mechanisms are available to selection in response to common environmental conditions in different drainages (Barrett & Schluter, 2008; Thompson et al., 2019). Second, low-predation populations are typically established by a small number of individuals (Barson et al., 2009; Fraser et al., 2015; Willing et al., 2010), making them susceptible to the unpredictable, non-adaptive influences of founder effects, genetic drift, and/or inbreeding on gene expression divergence; although we note that work in three-spine stickleback suggests deviations from parallel evolution tend to result from selection rather than non-adaptive processes (Kaeuffer et al., 2011). Third, the large number of significantly evolved genes that did not overlap between drainages may also represent adaptive responses to drainage- or site-specific environmental factors other than predation (Fitzpatrick, Torres-Dowdall, Reznick, Ghalambor, & Chris Funk, 2014; Zandonà et al., 2011). Finally, compensatory or homeostatic gene expression responses may arise in response to any of the above factors, leading to alternative transcriptional configurations associated with similar higher-level phenotypes.

In sum, we suggest that genetic similarity among ancestral populations may channel low-predation populations within the same drainage toward shared transcriptional solutions (as in Ghalambor et al. 2015), while differences in standing genetic variation, drainage-specific environmental conditions, founder effects, and alternative compensatory changes can result in distinct mechanistic paths to arrive at shared organism-level phenotypes. We cannot definitely distinguish causal from non-adaptive and compensatory gene expression differences under these scenarios – indeed, it is likely a combination of these factors that contribute to distinct transcriptional patterns associated with parallel adaptation. Nonetheless, alternative transcriptional patterns suggest that mechanistic flexibility and ‘many-to-one’ mapping of gene expression to organism level phenotypes may facilitate adaptation.

Conclusions

We assessed the extent to which adaptation to common environments targets predictable changes in transcript abundance across independent evolutionary events. Within lineages, genes with greater expression plasticity were more likely to diverge in expression between populations, but in the direction opposite of developmental changes. Despite a small number of differentially expressed genes shared across drainages, parallel adaptation to low-predation environments in independent lineages was associated with divergence in largely non-overlapping transcripts. While identification of shared genes is generally used as the starting point for work exploring mechanisms of parallel adaptation – and our work here indeed suggests that a small number of core genes may indeed be critical during the earliest stages of adaptation – we propose that parallel evolutionary transitions are not limited to a small set of possible transcriptional mechanisms in guppies. Instead, our results highlight the potential for alternative transcriptional solutions associated with parallel, adaptive trait evolution even within a single species. Transcriptional network versatility, in which diverse alterative network configurations can produce common network outputs and behavioral phenotypes, may allow underlying networks to simultaneously accommodate the influences of selection, drift, and genetic background and thereby facilitate evolution in a species known for rapid adaptation to novel environments.

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662

663 **Author contributions**: EKF and KLH conceived of the study; EKF collected samples and
664 performed molecular work; EKF and KAH performed gene expression mapping, transcript
665 abundance estimation, and preliminary differential expression analyses; YS and WZ devised
666 and performed statistical analyses with input from EKF and KLH; EKF and KLH wrote the
667 manuscript with contributions from all authors. All authors gave final approval for publication.

668

669 **Data Accessibility**: Raw sequencing reads are available through the NCBI SRA repository
670 (PRJNA601479). R code for statistical analyses is available on GitHub.

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Glossary

convergent evolution	The evolution of similar phenotypes in multiple independent populations, in response to similar selection pressures, but from different initial conditions.
parallel evolution	The evolution of similar phenotypes in multiple independent populations, in response to similar selection pressures, and from similar initial conditions.
genetic divergence	Differences between populations adapted to distinct environments. Here specifically, genetic differences in gene expression between high- and low-predation fish. These differences are identified through a common garden breeding design.
phenotypic plasticity	The potential for multiple phenotypes to arise from a single genotype, e.g. in response to environmental changes.
ancestral plasticity	The phenotypic plasticity present in individuals/populations adapted to ancestral environmental conditions when exposed to environmental conditions reflecting the derived environment. Here specifically, the plastic response of high-predation fish to rearing in predator free environments. This plasticity may be adaptive or non-adaptive (see below).
adaptive plasticity	Phenotypic plasticity that brings a trait closer to the optimum in the novel environment as compared to the trait value in the ancestral environment. Here specifically, gene expression plasticity in high-predation fish reared without predators that mirrors the direction of expression divergence between high- and low-predation adapted populations.
non-adaptive plasticity	Phenotypic plasticity that moves a trait further away from the optimum in the novel environment as compared to the trait value in ancestral environment. Here specifically, gene expression plasticity in high-predation fish reared without predators that is opposite the direction of expression divergence between high- and low-predation adapted populations.
concordant expression differences	When gene expression differences among parallel comparisons are in the same direction, i.e. either both up- or down-regulated across comparisons. Here specifically, when gene expression differences between low-predation populations and high-predation ancestors are either upregulated or downregulated in distinct drainages.
non-concordant expression differences	When gene expression differences among parallel comparisons are in opposite directions, i.e. up-regulated in one comparison and down-regulated in the other, or vice versa. Here specifically, when gene expression differences between high- and low-predation population in distinct drainages are in opposite directions.

Figures

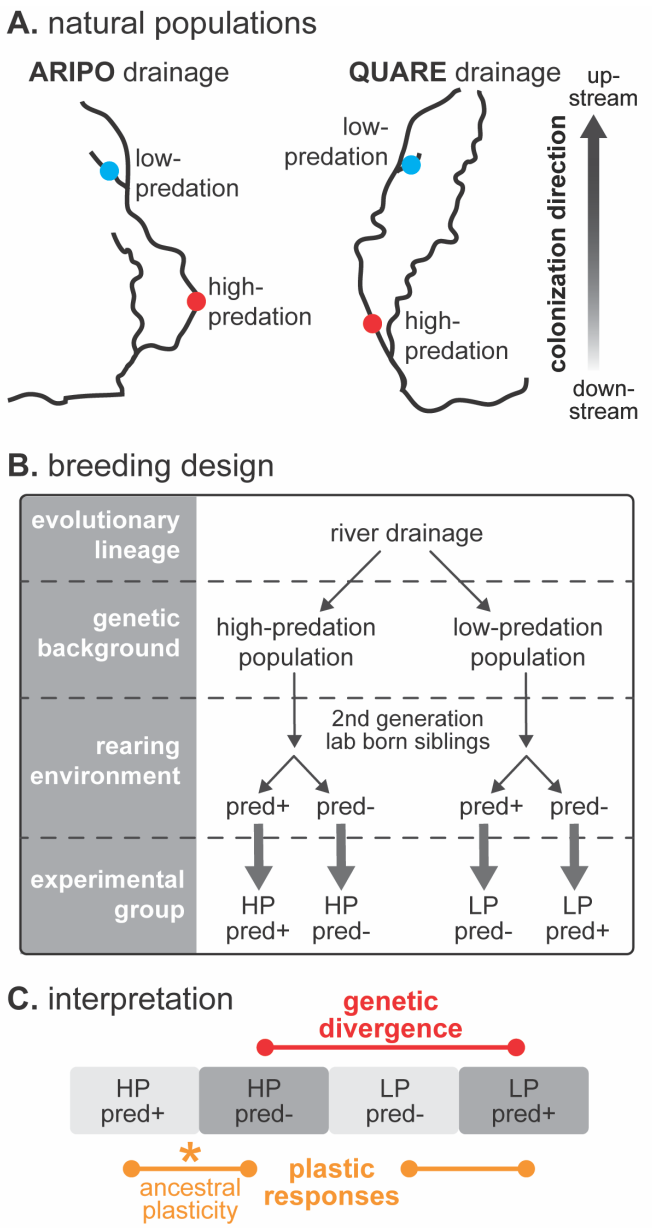

Figure 1. Overview of experimental design and interpretation. (A) Schematic shows Aripo and Quare river drainages in the Northern Range Mountains of Trinidad. We sampled population pairs from these two drainages, which represent distinct evolutionary lineages. (B) Wild-caught fish from each high- and low-predation population pair were reared to the second generation in the lab to control for maternal and environmental effects. At birth, second generation siblings from each population were split into rearing environments with (pred+) and without (pred-) predation cues. (C) This rearing design allows us to discern lineage effects (contrast between drainages), genetic divergence (contrast between different populations in the same environment), phenotypic plasticity (contrast between genetically similar fish in different rearing condition), and their interactions.

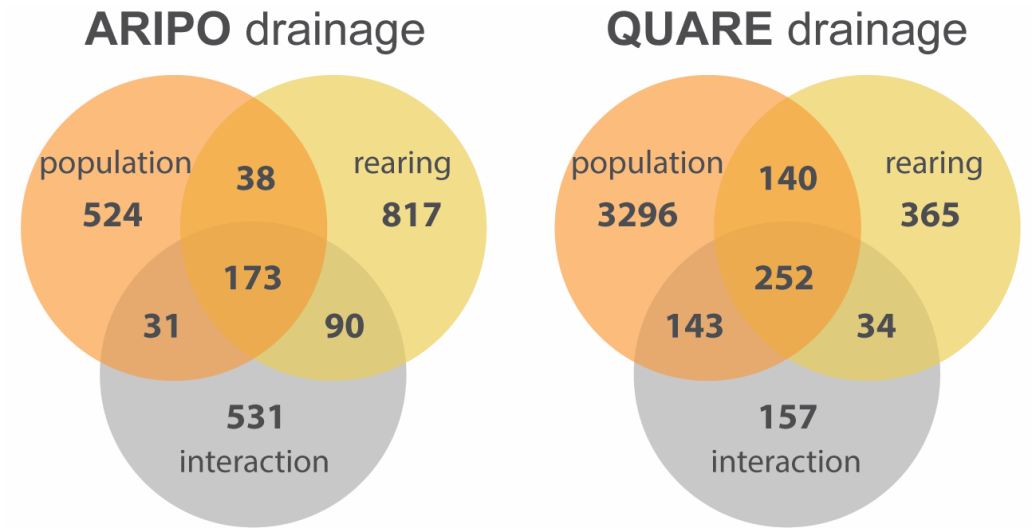

Figure 2. Summary of differential expression analyses. We identified many transcripts in both the Aripo (left) and Quare (right) drainages whose expression levels differed based on evolutionary history of predation (population of origin; orange), developmental experience of predation (rearing; yellow), and their interaction (grey).

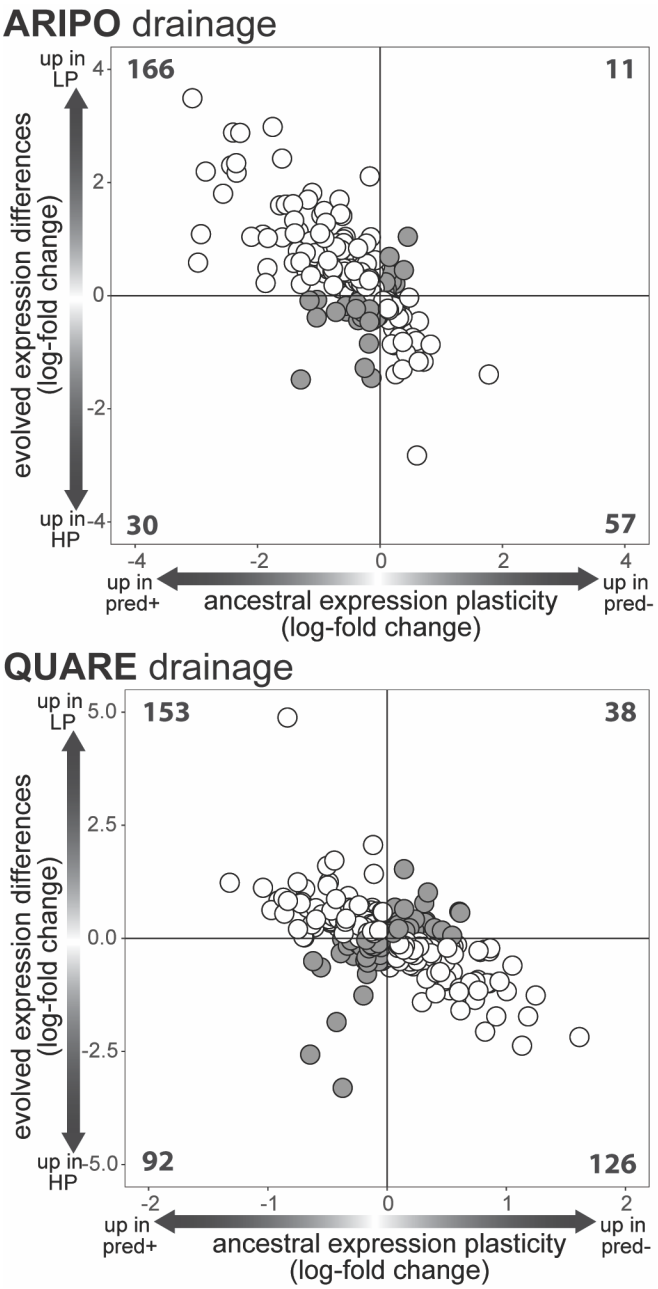

Figure 3. Relationship between ancestral plasticity and evolved expression divergence. Genes exhibiting significant ancestral expression plasticity (i.e. different between high-predation fish reared with and without predator cues) were more likely to also differ in expression between high- and low-predation populations reared without predator cue exposure in both the Aripo (top) and Quare (bottom) lineages. Each circle represents expression differences in a single transcript with statistically significant ancestral plasticity in the high-predation population as well as significant expression differences between high- and low-predation populations. Plastic expression changes were more likely to be in the opposite (white circles) than in the same (grey circles) direction as genetic expression divergence. The number of transcripts in each quadrant is indicated on the graphs. Note that axes differ between plots in order to best visualize variation within each dataset.

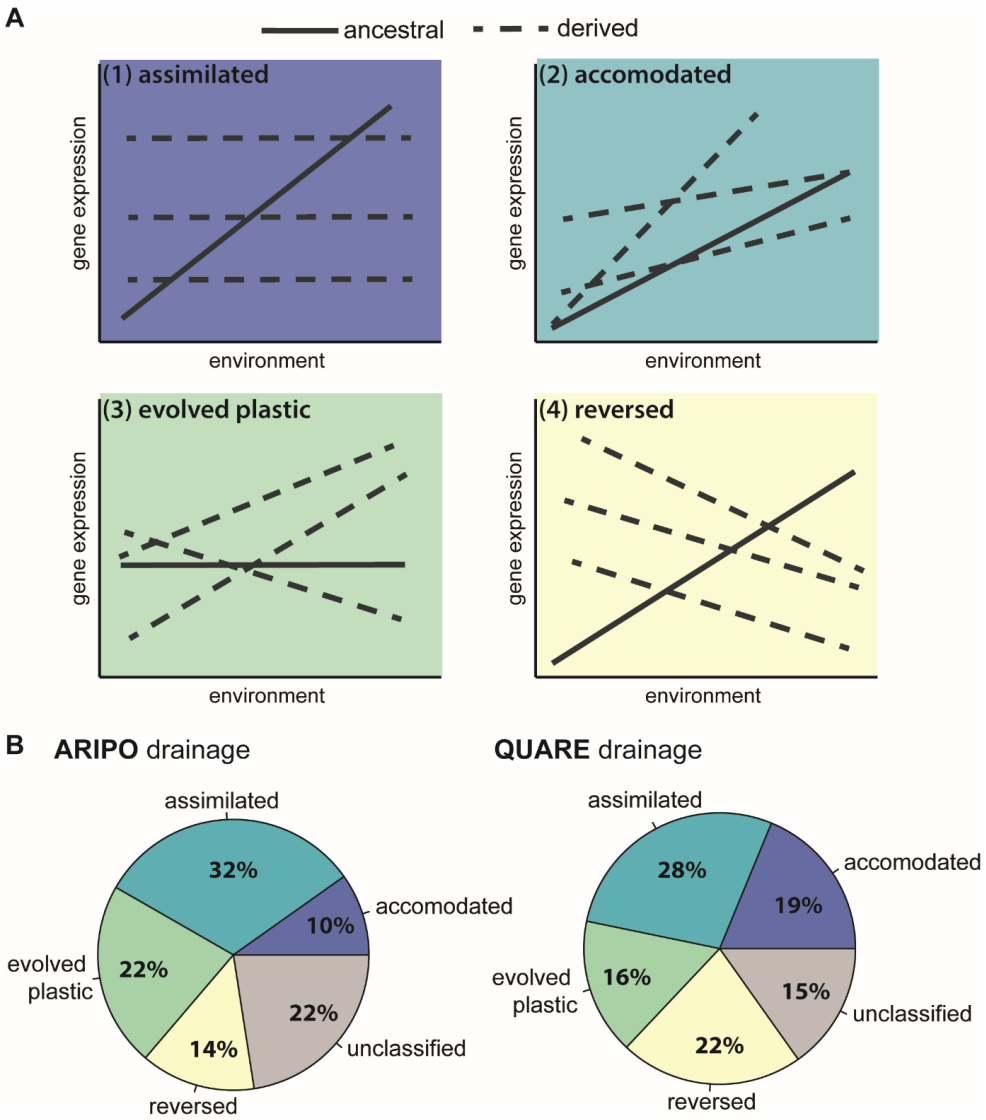

Figure 4. Evolution of transcript expression plasticity. (A) We grouped transcripts with significant interaction effects into one of four categories based on patterns of evolution in expression plasticity: (1) Assimilated: plasticity in the ancestral high-predation population but a loss of plasticity in the derived low-predation population; (2) Accommodated: a change in the degree, but no the direction, of plasticity in the derived as compared to the ancestral population; (3) Evolved plastic: no plasticity in the ancestral high-predation population but an emergence of plasticity in the derived low-predation population; (4) Reversed: opposing directions of plasticity in high- versus low-predation populations. We categorized remaining transcripts that had a significant main interaction effect, but no significant *post hoc* rearing differences as unclassified. Adapted from Renn & Schumer (2013). (B) All categories were represented in both Aripo and Quare datasets.

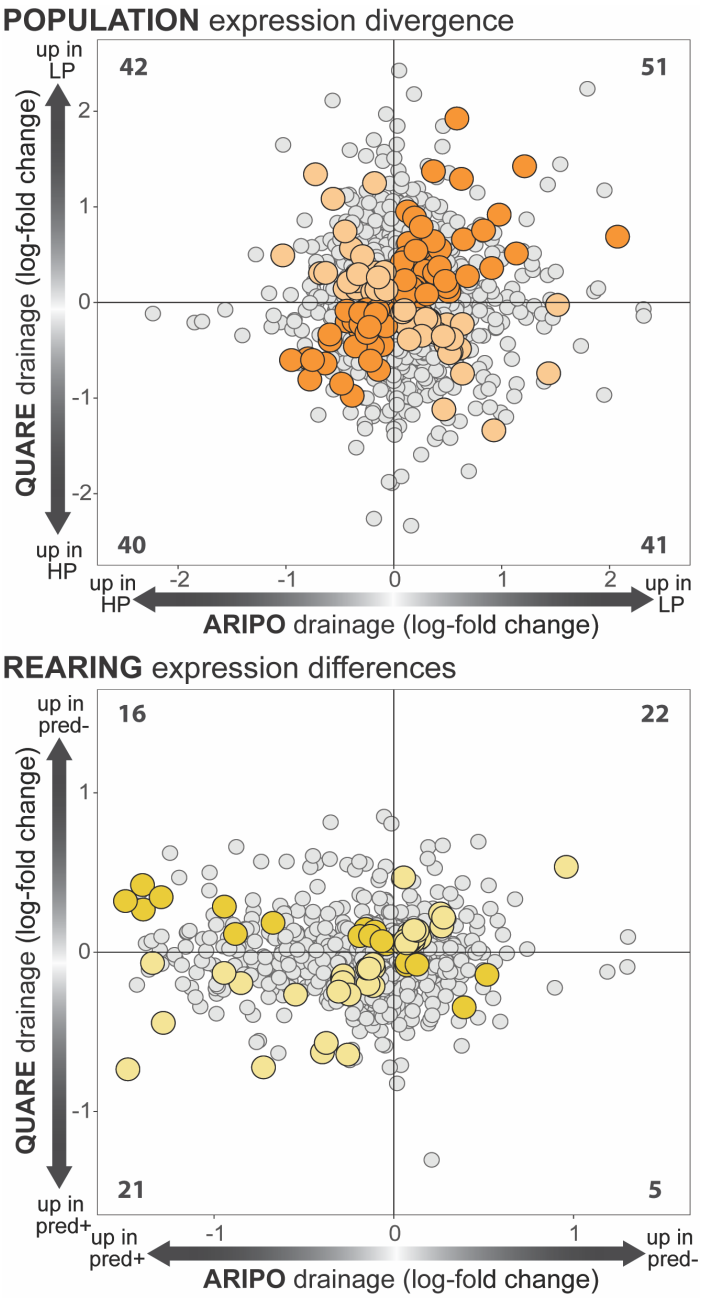

Figure 5. Overlap in population and rearing expression differences across evolutionary lineages. Of the transcripts differentially expressed based on population of origin (top), 124 were overlapping between drainages. Of these, 66 were concordantly differentially expressed (i.e. expression divergence in the same direction in both drainages; dark orange circles) and 58 were not (i.e. expression divergence in opposite directions between drainages; light orange circles), no different than expected by chance. Of the transcripts differentially expressed based on rearing environment (bottom), 35 were overlapping in the two drainages. Of these, 19 were concordantly differentially expressed (dark yellow circles) and six were not (light yellow circles), more non-concordance than expected by chance. The number of differentially expressed transcripts in each quadrant is indicated on the graphs and transcripts differentially expressed in one but not both drainages are shown in grey.

Supplemental Information

Table S1. Transcripts filtered from the analysis following identification as retinal contaminants. Provided as Excel file.

Table S2. Transcripts significantly differentially expressed between high- and low-predation populations in the Aripo drainage. Provided as Excel file.

Table S3. Transcripts significantly differentially expressed between high- and low-predation populations in the Quare drainage. Provided as Excel file.

Table S4. Results of GO enrichment analyses for population, rearing, and interaction effects in the Aripo drainage. Provided as Excel file.

Table S5. Results of GO enrichment analyses for population, rearing, and interaction effects in the Quare drainage. Provided as Excel file.

Table S6. Population DE transcripts overlapping between Aripo and Quare drainages. 185 transcripts were DE in both drainages, of these 89 had expression differences in the same direction in both datasets (concordant differential expression). Provided as Excel file.

Table S7. Rearing DE transcripts overlapping between Aripo and Quare drainages. 16 transcripts were DE in both drainages, of these 7 had expression differences in the same direction in both drainages (i.e. concordant differential expression). Provided as Excel file.

Table S8. Pst estimates for the Aripo and Quare drainages.

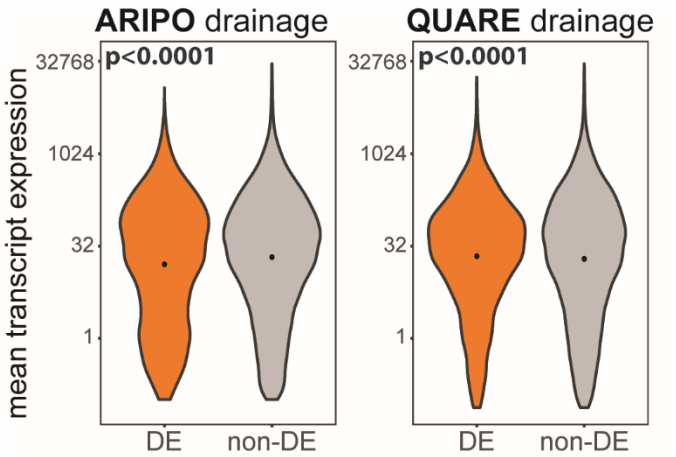

Figure S1. Differences in means but not variance of differentially expressed (DE) and non-differentially expressed (non-DE) transcripts. DE transcripts had significantly higher means in the Aripo drainage and lower means in the Quare drainage as compared to non-DE transcripts. Due to the large range of expression values, mean differences are plotted on a log scale (y-axis).

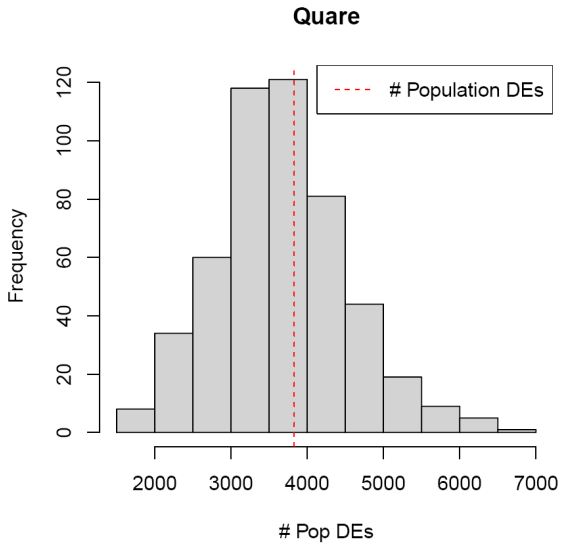

Figure S2. Outcome of down-sampling simulations**.** The Quare dataset was randomly down-sampled to generate a sample size equivalent to that of the Aripo dataset (40 samples, 10 per group). The histogram shows the distribution of the number of population DE genes obtained from 500 random down-samples. The number of DE genes in the full dataset (red dashed line) is not extreme compared to the distribution of the down-sampled datasets, indicating that sample size is not a critical factor for the difference in DE gene number between the two datasets.