FLEXIBILITY IN GENE COEXPRESSION AT DEVELOPMENTAL AND EVOLUTIONARY TIMESCALES

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ABSTRACT

The explosion of next-generation sequencing technologies has allowed researchers to move from studying single genes, to thousands of genes, and thereby to also consider the relationships within gene networks. Like others, we are interested in understanding how developmental and evolutionary forces shape the expression of individual genes, as well as the interactions among genes. To this end, we characterized the effects of genetic background and developmental environment on brain gene coexpression in two parallel, independent evolutionary lineages of Trinidadian guppies (*Poecilia reticulata*). We asked whether connectivity patterns among genes differed based on genetic background and rearing environment, and whether a gene's connectivity predicted its propensity for expression divergence. In pursuing these questions, we confronted the central challenge that standard approaches fail to control the Type I error and/or have low power in the presence of high dimensionality (i.e., large number of genes) and small sample size, as in many gene expression studies. Using our data as a case study, we detail central challenges, discuss sample size guidelines, and provide rigorous statistical approaches for exploring coexpression differences with small sample sizes. Using these approaches, we find evidence that coexpression relationships differ based on both genetic background and rearing environment. We report greater expression divergence in less connected genes and suggest this pattern may arise and be reinforced by selection.

INTRODUCTION

Genes neither act nor evolve in isolation. Rather, genes are members of physically and functionally interacting networks. The nature of these interactions influences the degree to which changes in gene sequence and gene expression influence higher-level phenotypes, and therefore the extent to which sequence and expression changes are constrained at developmental and evolutionary timescales. On the one hand, genes with many interaction partners (i.e., those that occupy central 'hub' positions in a network) may be targets of developmental switches and evolutionary selection because they most strongly influence network output and higher-level phenotypes (Chateigner *et al.*, 2020; Friedman *et al.*, 2020). Alternatively, expression changes in highly connected genes may be constrained by pleiotropic effects imposed by their many connections (Jeong *et al.*, 2001; Hahn & Kern, 2005), and developmental and evolutionary changes may therefore be more prevalent in peripheral genes that have lower connectivity and presumably lower pleiotropic loads (Kim *et al.*, 2007; Mähler *et al.*, 2017). These ideas are of long-standing interest but have historically been difficult to test because physical interaction networks were well-characterized in only very few species (e.g. protein networks in yeast (Jeong *et al.*, 2001; Hahn *et al.*, 2004; Jovelin & Phillips, 2009)) and simultaneously surveying expression in large numbers of genes was challenging, if not impossible. The proliferation of next generation sequencing technologies, and specifically RNA-sequencing (hereafter RNAseq), has removed these constraints from a technical perspective. Yet, analytical and statistical methods lag behind our ability to generate big data and – despite falling costs – the number of biological replicates remains small in many gene expression studies (Fischer et al. 2021). These factors impact network and gene coexpression analyses in particular.

Although network and coexpression analyses of RNAseq datasets remain less common than gene-wise differential expression analyses, studies in this area provide intriguing – albeit conflicting – results. Recent studies have shown that centrality in coexpression networks is negatively correlated with divergence in gene expression (Warnefors & Kaessmann, 2013; Mähler

63 *et al.*, 2017; Kuo *et al.*, 2023) as well as gene sequence evolution (Josephs *et al.*, 2017; Masalia
64 *et al.*, 2017; Harnqvist, 2021). Conversely, genes in peripheral positions show greater magnitude
65 expression divergence (Mähler *et al.*, 2017) and signatures of positive selection (Kim *et al.*, 2007).
66 Collectively, these findings suggest evolutionary constraints imposed on genes with high network
67 centrality and evolutionary flexibility in genes at the network periphery. Yet this conclusion is in
68 opposition with evidence for a bias toward changes in the expression of and selection on genes
69 with high centrality (Koubkova-Yu *et al.*, 2018; Chateigner *et al.*, 2020; Friedman *et al.*, 2020;
70 Rennison & Peichel, 2022). Evidence for changes in central genes include examples for positive
71 selection on genes with more interaction partners in human protein networks (Luisi *et al.*, 2015)
72 and high centrality in coexpression networks of genes associated with lung cancer (Wachi *et al.*,
73 2005). Together, these latter examples are more consistent with a contrasting hypothesis that
74 central genes better predict phenotypic variation and are therefore targets of selection.
75 The contradictory evidence for how gene centrality influences gene expression and
76 sequence evolvability begs the question of what leads to these opposing patterns. One eventuality
77 is that the above alternatives are not mutually exclusive, but complementary. Indeed, an extensive
78 survey of stress responses in *Arabadopsis thaliana* found expression differences associated with
79 drought stress in peripheral genes but cold stress in central genes (Des Marais *et al.*, 2017).
80 Similarly, some evidence suggests that changes in gene sequence versus gene expression may
81 be favored for physiological versus morphological traits (Warnefors & Kaessmann, 2013) and that
82 selection for sequence versus expression changes could act more strongly at central versus
83 peripheral network positions. The authors of (Hämälä *et al.*, 2020) suggest a 'goldilocks'
84 phenomenon, in which intermediate levels of pleiotropy facilitate evolution while excessive
85 pleiotropy inhibits evolution and insufficient pleiotropy makes selection inefficient (Hämälä *et al.*,
86 2020). If these seemingly contradictory observations – across taxa as well as within species – are
87 indeed driven by interactions within gene regulatory networks, then disentangling how interactions

among genes shape development and evolution requires understanding at what timescales and to what extent interactions among genes themselves change.

If the relationships among genes are flexible, then the degree of pleiotropy and its presumed consequences are not fixed. Understanding how changes in coexpression influence phenotypes is of keen interest for both basic and biomedical research (Gysi & Nowick, 2020; Stanford *et al.*, 2020). Evolutionary biologists are interested in whether and how changes in coexpression influence species' propensity for adaptation to novel and changing environments and whether changes in coexpression patterns can explain distinct genetic mechanisms underlying convergent phenotypes (Hu *et al.*, 2016; Koubkova-Yu *et al.*, 2018; Yu *et al.*, 2020b). Behavioral biologists are increasingly asking how 'rewiring' of coexpression networks drives behavioral changes in health and disease. For example, (Bloch *et al.*, 2021) found changes in coexpression associated with mating behavior in guppies, and meta-analyses implicated changes in gene coexpression patterns associated with behavioral disorders in humans (Gaiteri *et al.*, 2014). Changes in coexpression patterns are also of broader biomedical interest as they have been documented as a feature of diverse cancers (Wachi *et al.*, 2005; Anglani *et al.*, 2014).

The above studies highlight both the interest in and the potential of coexpression analyses. This demand is being met by a growing collection of tools for (differential) gene coexpression analysis (Wang *et al.*, 2017; Chowdhury *et al.*, 2020; Tommasini & Fogel, 2023). Yet while these software packages make advanced network analyses accessible, they do not eliminate the statistical limitations of these approaches. These limitations arise primarily from the combination of small sample sizes and high-dimensional data (tens of thousands of genes) emblematic of transcriptomic studies. While sample sizes have increased as sequencing costs have decreased, per group sample sizes commonly remain less than ten. Pooling samples across experimental groups or multiple studies can bring the total experimental sample size into the range recommended for network analyses (e.g., N=20 by (Langfelder & Horvath, 2008; Ballouz *et al.*, 2015)). However, when explicitly asking if coexpression relationships differ between two or among

a few experimental groups, small per group samples remain a problem. In brief, inference from coexpression analyses is problematic without sufficient sample sizes and power, but also when samples are pooled across experimental groups that can differ in coexpression structure. This leaves researchers trapped between an experimental rock and hard place.

Like others, we are interested in using transcriptomic analyses to understand the biological basis of complex phenotypes, and specifically in exploring changes in individual genes as well as the interactions among genes. To this end, we characterized the effects of genetic background (high-predation versus low-predation populations) and developmental environment (rearing with and without predator chemical cues) on brain gene coexpression in two parallel, independent evolutionary lineages of Trinidadian guppies (*Poecilia reticulata*). In Trinidad, downstream, high-predation fish have repeatedly and independently colonized upstream, low-predation environments (Gilliam *et al.*, 1993; Barson *et al.*, 2009; Willing *et al.*, 2010; Fraser *et al.*, 2015), leading to parallel adaptive changes in life-history, morphology, and behavior (Reznick *et al.*, 1990, 2001; Endler, 1995; Reznick, 1997; Magurran, 2005). In other words, each river drainage represents a naturally replicated experiment demonstrating parallel phenotypic adaptation. Recent studies have used laboratory breeding designs to disentangle genetic from environmental influences, demonstrating that both evolutionary history with predators and developmental experience with predators shape life history (Torres Dowdall *et al.*, 2012), morphology (Torres-Dowdal *et al.*, 2012; Fischer *et al.*, 2013; Ruell *et al.*, 2013; Handelsman *et al.*, 2014), physiology (Handelsman *et al.*, 2013; Fischer *et al.*, 2014), and behavior (Huizinga *et al.*, 2009; Torres-Dowdall *et al.*, 2012; Fischer *et al.*, 2016b). Using this breeding (Figure 1), we asked (1) whether connectivity patterns among genes differed among groups, and (2) whether connectivity influenced a gene's propensity for expression divergence.

We previously demonstrated genetic and developmental differences in brain gene expression (Fischer *et al.*, 2021). Here, we were interested in testing the hypothesis that – in addition to expression changes in single genes – the relationships among genes are targets of

developmental and evolutionary processes. In pursuing these questions, we confronted the statistical challenges that standard approaches may fail to control Type I error and/or have low power when dimensionality is high (i.e., large number of genes) and sample size is small. From a biological perspective, we find evidence for flexibility in coexpression relationships based on both genetic background and rearing environment, suggesting that changes in the interactions among genes are associated with phenotypic divergence at developmental and evolutionary timescales. From a technical perspective, we present a case study for those interested in (differential) coexpression with small per group sample sizes. We discuss key challenges, set clear sample size guidelines to control Type I error while maintaining power, and provide rigorous statistical approaches for exploring coexpression differences even with small sample sizes that can be readily implemented for similar coexpression analyses in other studies.

RESULTS

Preliminary analyses

Guppies used in this study were second generation lab-born fish from unique family lines established from wild-caught high-predation (HP) and low-predation (LP) populations in the Aripo and Quare river drainages in the Northern Range mountains of Trinidad. At birth, we split second-generation siblings into rearing environments with (pred+) or without (pred-) predator chemical cues, and they remained in these environments until the completion of the experiment (Figure 1) (as in (Fischer *et al.*, 2016b)). In brief, each drainage therefore consists of a 2x2 factorial design that distinguishes genetic from developmental effects of predation. Pair-wise comparisons of biological relevant are: (1) HP pred- vs LP pred-, an experiment comparing populations reared in an environment lacking predator cues to identify genetic differences between populations; (2) HP pred+ vs HP pred-, to identify environmentally induced changes mimicking the situation in which high-predation fish colonize low-predation environments, i.e., “ancestral plasticity”; (3) LP pred-

vs LP pred+, to identify environmentally induced changes comparable to the situation in which low-predation fish are washed downstream and a measure of whether ancestral plasticity is maintained in the derived population; (4) HP pred+ vs LP pred+, to identify genetic differences when fish are raised with environmental cues of predation. We also compared the same experimental groups across drainages (e.g. HP pred+ in Aripo drainage vs HP pred+ in Quare drainage) to understand differences associated with parallel, independent evolutionary lineages.

Our initial approach was to characterize and explore gene coexpression using the popular Weighted Gene Correlation Network Analysis (WGCNA) package in R (Langfelder & Horvath, 2008). We calculated module preservation scores using the methods implements in WGCNA which combine a number of difference preservation statistics to calculate a summary preservation score (Langfelder *et al.*, 2011). We found that, in both drainages, ~50% of gene coexpression modules were not preserved across experimental groups (Supplemental Materials A). In other words, we identified substantial differences in network structure between groups, suggesting that the common practice of reconstructing coexpression networks by pooling samples across groups may not be valid. In brief, our preliminary analyses using WGCNA underscored the need for a statistical method to discern network differences across groups when dealing with small group sizes (N=10-12 in our case). We sought to address these issues through the alternative statistical approaches detailed in the Methods and Supplemental Materials B. We present results from the most promising approaches below.

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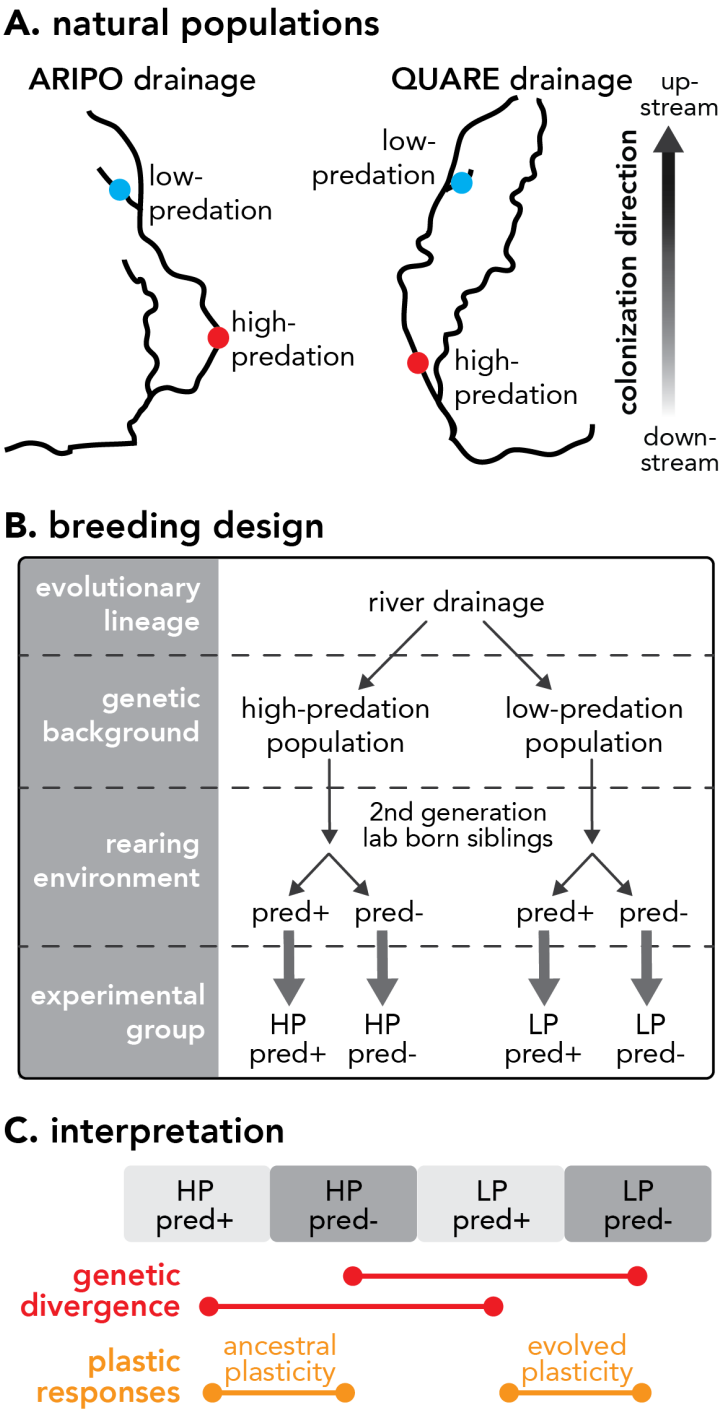

Figure 1. Conceptual overview and interpretation. (A) Natural populations in different river drainages represent distinct evolutionary lineages. (B) Overview of laboratory breeding design disentangling genetic and environmental effects. (C) Interpretation of comparisons of interest between experimental groups resulting from 2x2 breeding design shown in (B). HP = high-predation, LP = low-predation, pred+ = reared with predator chemical cues, pred- = reared without predator chemical cues. Modified from Fischer et al. 2021.

Changes in coexpression networks based on genetics and environment

To overcome problems associated with very small per group sample sizes, yet high-dimensional data, we used random projection-based tests to compare covariance structures between experimental groups. We considered the set of all genes (DE and non-DE) that passed filtering criteria (Aripo: 13,446; Quare: 14,379). We found significant differences in covariance structures between high-predation and low-predation fish reared with predators (HP pred+ vs LP pred+) in both drainages (Figure 2; Table 1). Analysis of the Quare dataset found a marginally significant difference between high-predation fish reared with and without predators (HP pred+ vs HP pred). We also compared the covariance structures between the same treatment groups across drainages. Here, we found significant differences in all comparisons (Table 2). In short, when considering the collection of all genes, we found evidence for changes in gene coexpression based on evolutionary lineage (drainage), genetic background (population), and rearing environment.

Table 1. Approximated p-values from random projection tests comparing covariance structure for all genes (DE and non-DE) between treatment groups.

	ARIPO drainage	QUARE drainage
HP pred+ vs HP pred-	1.0000	0.0639
HP pred+ vs LP pred+	0.0149	<0.0001
HP pred- vs LP pred -	1.0000	0.9568
LP pred+ vs LP pred-	0.1258	0.9943

Table 2. Approximated p-values from random projection tests comparing covariance structure across drainages.

ARIPO vs QUARE	p-value
HP pred-	<0.0001
HP pred+	0.0001
LP pred -	0.0001
LP pred+	0.0107

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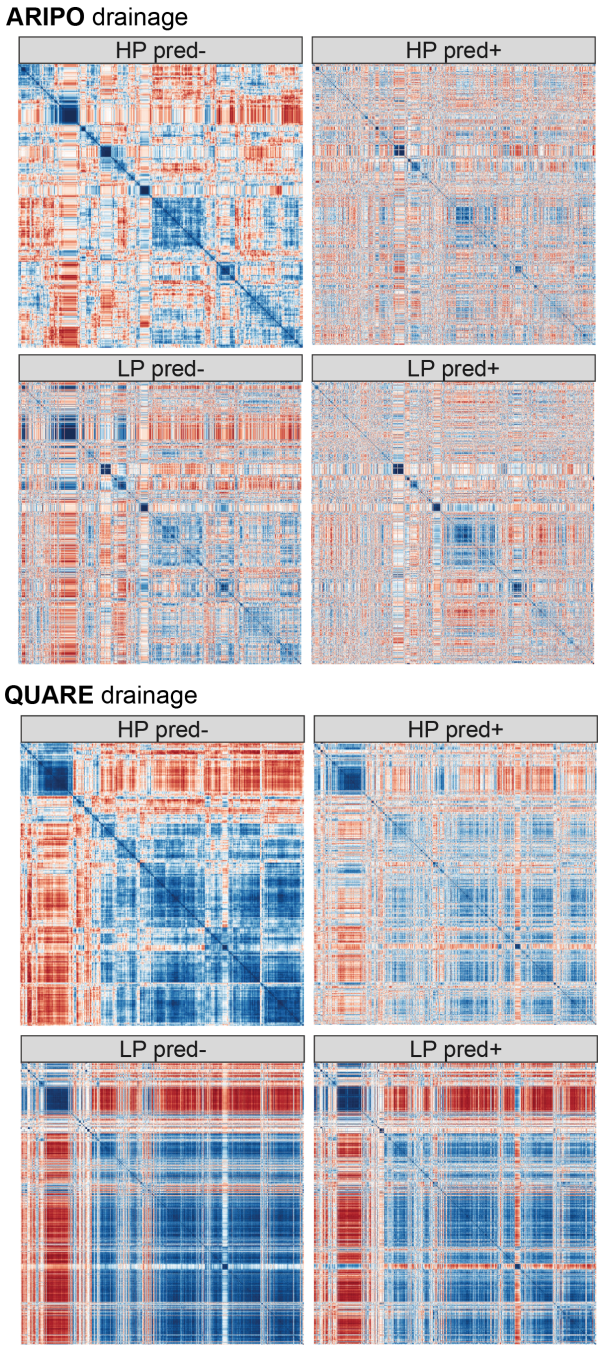


Figure 2. Visualization of coexpression differences between experimental groups and evolutionary lineages. Correlation heatmaps provide a visual approximation of statistical differences based on genetic background (high- vs low-predation), rearing environment (with (pred+) or without (pred-) predators), and evolutionary lineage (Aripo and Quare drainage). Gene order is determined by hierarchical clustering of the high-predation pred- group, meaning that the same position in two heatmaps represents the correlation of identical pairs of genes. For ease of visualization and computation, only the 1,000 most variable genes are shown.

Coexpression networks and differential expression

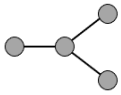
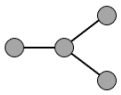
In addition to changes in coexpression among genes, we were interested in how interactions among genes might interface with a gene's propensity to be differentially expressed at developmental and evolutionary timescales. To explore this question, we compared coexpression networks between differentially expressed (DE) and non-differentially expressed (non-DE) genes. We performed these comparisons separately for each treatment group, given group-level differences in covariance structures (see above) and DE status being inherently related to differences in gene expression among treatment groups.

The comparison involved two steps: (i) reconstruction of the coexpression network using gene-wise correlations, and (ii) comparing two networks of different sizes. To achieve (i), we first tested whether the correlation between each pair of genes was zero, while controlling the FDR using the method from (Cai & Liu, 2016), as detailed in Supplemental Section 4.3. Using this method, an edge is drawn between any two genes (nodes) with nonzero correlation, forming the coexpression network. To assess the constructed network's sensitivity to different FDR levels, we compared network summary plots at multiple FDR cutoffs (α = 0.01, 0.05, 0.1). If two networks are distinct, their summary plots will differ (Maugis *et al.*, 2017). The network summary plots (Figure S6) suggest that the correlation-based coexpression network is relatively insensitive to different FDR levels. Therefore, we used a coexpression network with α = 0.05 for all subsequent analyses.

A major concern in comparing DE versus non-DE networks, which has been largely overlooked in literature, is that the different collections of genes in these two sets (e.g., DE genes are by definition a small subset of all genes) making the two corresponding networks have different numbers of unmatchable nodes [60]. To address this, we adopted the network comparison test proposed by (Shao *et al.*, 2022), which accommodates networks of different sizes by analyzing their network moments for specific motifs using the difference of two subgraph densities adjusted for their edge densities. Specifically, we used the v-shape (subgraphs with

three nodes and two edges), triangle (subgraphs with three nodes and three edges), and 3-star (subgraphs with four nodes and three edges) (see visualizations in Figure S9, Table 3) to compare the correlation-based coexpression networks. In network sciences, these subgraphs reflect connectivity and clusterability [60, 61, 62]. Apart from HP pred+ and HP pred- in the Aripo dataset, we observe that all non-DE networks had higher subgraph densities than their DE network counterparts (Figure S7 & S8; Table S8). Following this overall trend, we compared the different subgraph types described above. The Aripo dataset had fewer overall differences, especially for the 3-star subgraph type (Table S9). In the Quare dataset, DE versus non-DE networks differed in nearly all groups for all subgraph types (Table S9). To further compare subgraph densities in DE and non-DE networks, we conducted the same test with one-sided alternatives that DE gene network subgraphs are more or less connected than non-DE gene subgraphs. The results show that in the Aripo drainage, non-DE networks generally have higher sparsity-adjusted v-shape densities, while in the Quare drainage, the DE network in HPP has higher adjusted v-shape and triangle densities, but DE networks in LPP and LPNP have lower adjusted triangle and 3-star densities than their NDE counterparts (Table 3).

Table 3. Network comparisons of DE vs non-DE genes within treatment groups and across datasets. Comparisons of sparsity-adjusted subgraph densities tested the alternatives that DE gene networks had smaller or larger subgraph density than non-DE networks. P-values from one-sided alternative tests are reported for the v-shape, triangle, and 3-star subgraph types.

	ARIPO drainage			QUARE drainage		
	v-shape	triangle	3-star	v-shape	triangle	3-star
						
DE < non-DE						
HP pred-	0.0051	0.8611	0.6149	<0.0001	0.9523	1.0000
HP pred+	0.0676	0.2330	0.5027	<0.0001	<0.0001	0.0665
LP pred -	0.0056	0.1700	0.2650	1.0000	1.0000	1.0000
LP pred+	<0.0001	0.0152	0.0749	0.4844	1.0000	1.0000
DE > non-DE						
HP pred-	0.9948	0.1389	0.3742	1.0000	0.0482	<0.0001
HP pred+	0.9358	0.7676	0.5013	1.0000	1.0000	0.9347
LP pred -	0.9938	0.8277	0.7304	<0.0001	<0.0001	<0.0001
LP pred+	1.0000	0.9850	0.9252	0.5122	<0.0001	<0.0001

DISCUSSION

Our goal in this study was to understand how genetic background and rearing environment shape relationships among genes. We previously characterized expression changes at the level of individual genes (Fischer *et al.*, 2021), and here we were interested in exploring changes in coexpression patterns among genes. Our findings suggest that coexpression patterns are flexible at evolutionary and developmental timescales. Exciting from a biological perspective, exploring these questions presents statistical challenges, in particular for RNAseq datasets characterized by high-dimensionality and small sample sizes. We discuss the implications of our work from both angles.

Gene expression studies remain plagued by small per-group samples sizes and high dimensionality. Network construction is far from trivial, if not problematic, under these conditions, especially when network structure – and not just network expression level – differs among experimental groups. In our own study, we had an overall sample size of N=98 individuals, well

above the recommendation of N=30 for network construction. However, this total sample size includes samples from two drainages and four experimental groups, and – based on our analyses here and preliminary analyses using the WGCNA package – we found evidence that network structure differs between experimental groups and even more strongly between drainages. These differences are of key biological interest as they suggest that expression relationships among genes (i.e., network structure) are subject to developmental plasticity and evolutionary change. However, if network structure differs across experimental groups, then networks must be constructed separately for each experimental group to avoid construction of 'average' networks that can obscure differences of (biological) interest and lead to biased conclusions (Zhao *et al.*, 2014; Shojaie, 2021; Li *et al.*, 2022; Sai Li & Li, 2023). To take an extreme example, if two genes have opposing correlations of the same magnitude in two groups, the average correlation across groups will be zero. Thus, it is the per-group sample size that is most important for network construction and comparison when gene coexpression patterns are of interest.

While our per-group sample size of N=10-15 is relatively large for an RNAseq study, it is below the recommended threshold for network construction, such as the minimum sample of N=20 suggested for RNAseq analyses by (Ballouz *et al.*, 2015). As we illustrate in the Supplemental Materials, these sample sizes are surprisingly inadequate for recently developed high-dimensional statistical tests thought to be robust against high-dimensionality, to control Type I error, and to maintain power. Indeed, from our simulation experiments, most common methods require N>50 to retain the generally accepted nominal significance levels of 0.05 and satisfactory power exceeding 0.8. Importantly, the potential misinterpretations resulting from these shortcomings are not systematic (i.e., directionally biased) and therefore difficult to predict. As a growing number of studies consider how interactions among genes shape phenotypic differences across timescales, we present our work as a case study to increase awareness of these limitations, present complementary statistical approaches to those commonly used, and in hopes that others will consider these issues in experimental design and analysis.

We first investigated whether gene coexpression patterns differed based on genetic background and rearing environment. Using pairwise expression covariance tests, we identified differences in coexpression patterns between fish reared with and without predators, fish adapted to high- versus low-predation environments, and fish from distinct evolutionary lineages. Differences in coexpression structures in both drainages were most pronounced between high- and low-predation fish reared with predators (HP pred+ vs LP pred+). This comparison represents the ancestral population adapted to life with predators (HP pred+) versus the derived low-predation population adapted to predator and suddenly re-exposed to predator cues (e.g., as when fish are washed downstream; LP pred+). Fish adapted to a low-predation life are expected to be poorly equipped deal with the sudden stressors of predation. Indeed, we previously found HP pred+ fish to be behaviorally least variable and LP pred+ fish to be behaviorally most variable (Fischer *et al.*, 2016b). In light of findings here, we suggest that these unpredictable behavioral patterns could arise from disruptions in gene coexpression networks in the brain.

In addition to differences within drainages, we found more evidence for coexpression differences in the Quare as compared to the Aripo drainage, and that differences were ubiquitous when comparing between the two drainages. We suggest these patterns arise – at least in part – from the extent of genetic divergence between populations: high- and low-predation populations in the Quare drainage show greater genetic (Willing *et al.*, 2010) and gene expression (Fischer *et al.*, 2021) divergence than those in the Aripo drainage, and the two drainages represent distinct evolutionary lineages (Willing *et al.*, 2010). The importance of genetic background in shaping evolutionary trajectories is highlighted by our previous work demonstrating distinct underlying mechanisms associated with parallel phenotypic adaptation in guppies from distinct evolutionary lineages (Fischer *et al.*, 2016a, 2021). Similar mechanistic flexibility has also been demonstrated in other systems (Cordero *et al.*, 2018; Jacobs *et al.*, 2020), including those known for parallel phenotypic evolution (e.g. (Laporte *et al.*, 2015; Hanson *et al.*, 2017; Bolnick *et al.*, 2018)). Our findings here extend these observations from the expression of individual genes to coexpression

313 patterns among genes, suggesting that alternative gene expression network configurations can
314 give rise to shared organism-level phenotypes.

315 What are the potential consequences of changes in gene coexpression relationships at
316 developmental and evolutionary timescales? If pleiotropic interactions among genes constrain
317 their propensity for change, then flexibility in these relationships could reduce pleiotropic load
318 (e.g., (Wang *et al.*, 2010; Pavlicev & Wagner, 2012; Pavličev & Cheverud, 2015). In other words,
319 genes may be more able to change in expression and drive phenotypic change if their interactions
320 with other genes can be altered to minimize off-target effects. Conversely, flexibility in
321 coexpression relationships might improve the ability of underlying gene expression networks to
322 buffer higher-level phenotypes through homeostatic change (e.g., (Fischer *et al.*, 2016a; Badyaev,
323 2018; Hoke *et al.*, 2019)). Importantly, either scenario implies that the relationships among genes
324 may themselves be targets of selection. Alternatively, changes in gene coexpression could
325 represent transcriptional noise if these changes do not amount to selectable differences at the
326 network and/or organismal level. While this last scenario is less interesting from an adaptationist
327 perspective, such 'neutral' changes may nonetheless have consequences for evolutionary
328 trajectories, for example by giving rise to cryptic variation that is revealed under novel
329 environmental conditions (West-Eberhard, 2003; McGuigan & Sgrò, 2009; Paaby & Rockman,
330 2014). In brief, all three alternatives highlight that coexpression relationships can change at
331 developmental and evolutionary timescales with consequences in the short and long term.

332 To begin to address the above alternatives, we asked whether connectivity influenced a
333 gene's propensity for expression divergence. This question has been considered at various levels,
334 including in protein-protein interactions (Kim *et al.*, 2007; Luisi *et al.*, 2015), gene expression
335 networks (Oldham *et al.*, 2006; Bloch *et al.*, 2021), gene sequence changes (Josephs *et al.*, 2017;
336 Makinen *et al.*, 2018), and the relationship between expression and sequence divergence
337 (Harrison *et al.*, 2012; Warnefors & Kaessmann, 2013; Chateigner *et al.*, 2020). Overall, we found
338 that networks of non-DE genes were denser than networks of DE genes. This suggests that DE

339 genes are less well-connected than their non-DE counterparts, supporting the idea that differential
340 expression may be facilitated by lower connectivity and decrease pleiotropy. Comparing across
341 lineages, we observed more widespread differences in network connectivity in the Quare as
342 compared to the Aripo drainage. As high- and low-predation populations in the Quare drainage
343 are more genetically diverged than those in the Aripo (Willing *et al.*, 2010), this observations
344 suggests that greater genetic divergence may increase network sparseness, suggesting that
345 selection can reshape network structure. Taken together, our findings support greater expression
346 divergence in less connected genes and suggest this pattern could facilitate changes in network
347 topology if there is feedback between greater magnitude expression change and decreased
348 network connectivity.
349
350 *Conclusions*
351 Understanding how underlying genetic architecture shapes the maintenance and evolution of
352 complex traits is a fundamental goal of biological research. Over the past two decades, the
353 explosion of next-generation sequencing technologies has allowed us to move beyond the genetic
354 scale – considering one or a few genes or loci – to genomic scales – considering thousands to
355 tens of thousands of genes or loci. Among the key advances afforded by these approaches, are
356 the ease of conducing broadscale, exploratory studies; the opportunity to characterize underlying
357 mechanisms in non-model species; and the ability to consider genes in the context of their
358 interactions. As a growing number of studies consider how interactions among genes shape
359 phenotypic differences across timescales, we provide a case study to increase awareness of
360 limitations and provide suggestions for analysis. Our findings provide intriguing evidence of
361 extensive coexpression flexibility at multiple timescales in a species known for rapid adaptation.
362 The generality of these phenomena and their consequences for adaptation will be revealed by
363 more studies with larger sample sizes and new statistical approaches. Understanding whether
364 and how relationships among genes change at developmental and evolutionary timescales has

365 consequences for our understanding of how underlying mechanisms shape flexibility and
366 robustness in higher order phenotypes, how animals adapt to novel and changing environments,
367 and how behavior and physiology are regulated in health and disease.
368
369
370 **METHODS**
371 *Fish collection and rearing*
372 Samples here are the same as those described in (Fischer *et al.*, 2021). Briefly, guppies used in
373 this study were second generation lab-born fish from unique family lines established from wild-
374 caught high-predation (HP) and low-predation (LP) populations in the Aripo and Quare river
375 drainages in the Northern Range mountains of Trinidad. At birth, we split second-generation
376 siblings into rearing environments with (pred+) or without (pred-) predator chemical cues, and
377 they remained in these environments until the completion of the experiment (Figure 1) (as in
378 (Fischer *et al.*, 2016b)). Guppies were individually housed in 12:12 hour light cycle and fed a
379 measured food diet once daily. All experimental methods were approved by the Colorado State
380 University Animal Care and Use Committee (Approval #12-3818A).
381
382 *Tissue collection and processing*
383 We collected brain tissue from mature males in the groups described above 10 minutes after
384 lights on in the morning. We extracted RNA from whole brains using the Qiagen RNeasy Lipid
385 Tissue Mini Kit (Qiagen, Germany) and constructed a sequencing library for each individual using
386 the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Massachusetts,
387 USA). For the Aripo dataset, 40 samples (N=10 per group) were pooled with unique barcodes
388 into eight samples per sequencing library and each library was sequenced on a single lane. For
389 the Quare dataset, 60 samples (N=12-16 per group) were pooled into three sequencing libraries
390 with 20 samples per pool and each library was sequenced in two separate lanes. Libraries were

sequenced as 100bp paired-end reads on an Illumina HiSeq 2000 at the Florida State University College of Medicine Translational Science Laboratory (Tallahassee, Florida) in May 2014 (Aripo dataset) and January 2016 (Quare dataset).

Differential expression analysis

We reported results of differential expression analyses in another study (Fischer *et al.*, 2021), and we use the resulting differential expression status (DE versus non-DE) as an important criterion in analyses performed here (see below). Briefly, we normalized read counts using DESeq2 (Love *et al.*, 2014) and performed differential expression analysis using the lme4 package in R (github.com/lme4). We used generalized linear mixed models with a negative binomial link to accommodate our experimental design and data type, including family and sampling week as random effects. Population of origin (HP / LP), rearing environment (pred+ / pred-), and their interaction were included as fixed effects. We used the residuals in the covariance and correlation analyses described below to adjust for fixed and random effects included in model fitting. To compare differentially expressed and non-differentially expressed genes, we adjusted p-values for multiple hypothesis testing using a direct approach for FDR control (Storey, 2002) as implemented in the fdrtool package in R (Strimmer, 2008). We considered transcripts differentially expressed (DE) if the adjusted p-value was <0.05, and all other genes non differentially expressed (non-DE).

Challenges from small sample, high dimensional data

In wanting to understand differences in covariance structure and changes in network architecture between our experimental groups, we faced two fundamental challenges. First, controlling Type I error becomes difficult when comparing large networks or covariance structures with limited sample sizes, often leading to spurious discoveries and unreliable results. Also, detecting true effects for separating complicated networks or covariance structures in ultra-high dimensional

datasets is difficult due to low statistical power; a common phenomenon in coexpression analysis of RNAseq studies. While there are commonly accepted methods for two sample covariance tests, they either fail to control the Type I error rate with extremely small sample size (e.g., N<25 per group) or have substantially low power (see Supplemental Materials for simulations) (Li & Chen, 2012; Cai *et al.*, 2013; Chang *et al.*, 2017; Yu *et al.*, 2020a). Second, comparison of multiple networks is nontrivial, particularly when the networks are of different sizes or have unmatching nodes (Tang *et al.*, 2017; Agterberg *et al.*, 2020; Qi *et al.*, 2024). For gene coexpression analysis these issues apply, for example, when sample sizes vary between groups and are fairly small due to the limitation of experiment constraints, or when comparing subsets of genes of interest that vary in size (e.g., comparing non-DE vs DE gene sets, or differently sized coexpression modules) (Agterberg *et al.*, 2020; Alyakin *et al.*, 2024; Jin *et al.*, 2024; Qi *et al.*, 2024).

To highlight these challenges, we first present simulation studies using existing high-dimensional methods designed for valid inference on comparing large covariance structures with controlled Type I error rate and reasonable power (Li & Chen, 2012; Cai *et al.*, 2013; Chang *et al.*, 2017; Yu *et al.*, 2020a), before presenting our method and examining the real data set (see below). We summarize the outcomes of the simulation studies and exploratory comparisons here and refer the interested reader to additional details provided in the Supplemental Information.

We found that Type I error rates for existing tests were uncontrolled for small sample sizes of N<50 per group, even when the number of genes was relatively small (250 genes, orders of magnitude smaller than what is typical for RNAseq analysis) (Figure S1). In addition to uncontrolled Type I error, the existing methods were substantially underpowered for small sample sizes. Specifically, the empirical power was overall low (<0.25) for sample sizes N<30 per group (Figure S2). These issues plagued our dataset, which is representative of most RNAseq studies exploring connections between gene expression and behavior (per group samples N<10, ~20,000+ genes). Importantly, these issues are not resolved by subsampling the data to include a smaller number of genes (Figure S3), an approached commonly deployed by network analysis

443 packages (e.g., filtering for the 5,000-8,000 most variable genes in WGCNA or the approach of
444 (Qiu *et al.*, 2021)).
445
446 *Our new high-dimensional covariance comparison*
447 From the above simulations, it is clear that existing approaches to compare large covariance
448 structures fail even when using only a small subset of genes. To overcome these issues, we
449 employed the random projection (Wu & Li, 2020) to develop a new two-sample covariance
450 comparison method (Supplemental Information). This new test can control Type I error (Figure
451 S4) and have satisfactory power (Figure S5), even with very small per group sample sizes and a
452 large number of genes.
453 We conduct our newly developed random projection-based tests on residual from the
454 generalized linear mixed model described above and in (Fischer *et al.*, 2021). We focused on the
455 pairwise comparisons of biological interest (Figure 1c). Within each drainage, we compared (1)
456 HP pred- vs LP pred-, an experiment comparing populations reared in an environment lacking
457 predator cues to identify genetic differences between populations; (2) HP pred+ vs HP pred-, to
458 identify environmentally induced changes mimicking the situation in which high-predation fish
459 colonize low-predation environments, i.e., “ancestral plasticity”; (3) LP pred- vs LP pred+, to
460 identify environmentally induced changes comparable to the situation in which low-predation fish
461 are washed downstream and a measure of whether ancestral plasticity is maintained in the
462 derived population; (4) HP pred+ vs LP pred+, to identify genetic differences when fish are raised
463 with environmental cues of predation. We also compared the same experimental groups across
464 drainages (e.g. HP pred+ in Aripo drainage vs HP pred+ in Quare drainage) to understand
465 differences associated with parallel, independent evolutionary lineages. For both within and
466 between drainage comparison, we considered the four comparisons jointly to control family-wise
467 error rate.
468

Correlation network comparisons

We used correlation network analyses to examine whether DE versus non-DE genes differed in their relationships with other genes. A challenge with this analysis was the lack of consensus to define networks, in addition to the fact that derived networks will usually have very different sizes and unmatched nodes (i.e., non-DE genes far outnumber DE genes and a single gene is inherently only in one category). We constructed adjacency matrices using the correlations of residuals from the aforementioned generalized linear mixed model. From these adjacency matrices, we identified overall network structure and the frequency of specific subgraphs (i.e., small motifs or subnetworks with two to seven nodes and one to six edges) for each experimental group within each drainage. We then compared network pairs of interest to examine their structural differences, using a network summary plot and two-sample network tests based on relative frequencies of different subgraphs adjusted for the sparsity of networks (additional details in Supplemental Information) (Maugis *et al.*, 2017; Shao *et al.*, 2022).

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DATA ACCESSIBILITY

Raw sequencing reads are available through the NCBI SRA repository (PRJNA601479). R code for statistical analyses will be made available on GitHub (https://github.com/EnigmaSong/GeneFlexibilityStudy) upon publication.

AUTHOR CONTRIBUTIONS

EKF and KLH conceived of the study; EKF collected samples and performed molecular work, gene expression mapping, transcript abundance estimation, and preliminary differential expression analyses; YS and WZ devised and performed statistical analyses with input from EKF and KLH; EKF and YS performed data visualization; EKF wrote the manuscript with contributions from all authors.

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