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Counter-gradient variation in gene expression between fish populations facilitates colonization of low-dissolved oxygen environments

Janay A. Fox | David A. G. A. Hunt | Andrew P. Hendry | Lauren J. Chapman | Rowan D. H. Barrett

Department of Biology, McGill University, Montreal, Quebec, Canada

Correspondence
Janay A. Fox and Rowan D. H. Barrett, Department of Biology, McGill University, Stewart Biology Building, 1205 Av du Docteur Penfield, Montreal, QC, Canada.
Email: janay.fox@mail.mcgill.ca and rowan.barrett@mcgill.ca

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Abstract

The role of phenotypic plasticity during colonization remains unclear due to the shifting importance of plasticity across timescales. In the early stages of colonization, plasticity can facilitate persistence in a novel environment; but over evolutionary time, processes such as genetic assimilation may reduce variation in plastic traits such that species with a longer evolutionary history in an environment can show lower levels of plasticity than recent invaders. Therefore, comparing species in the early stages of colonization to long-established species provides a powerful approach for uncovering the role of phenotypic plasticity during different stages of colonization. We compared gene expression between low-dissolved oxygen (DO) and high-DO populations of two cyprinid fish: *Enteromius apleurogramma*, a species that has undergone a recent range expansion, and *E. neumayeri*, a long-established native species in the same region. We sampled tissue either immediately after capture from the field or after a 2-week acclimation under high-DO conditions, allowing us to test for both evolved and plastic differences in low-DO vs high-DO populations of each species. We found that most genes showing candidate-evolved differences in gene expression did not overlap with those showing plastic differences in gene expression. However, in the genes that did overlap, there was counter-gradient variation such that plastic and evolved gene expression responses were in opposite directions in both species. Additionally, *E. apleurogramma* had higher levels of plasticity and evolved divergence in gene expression between field populations. We suggest that the higher level of plasticity and counter-gradient variation may have allowed rapid genetic adaptation in *E. apleurogramma* and facilitated colonization. This study shows how counter-gradient variation may impact the colonization of divergent oxygen environments.

KEYWORDS
adaptation, cyprinid, dissolved oxygen, fish, gene expression, plasticity

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1 | INTRODUCTION

Populations are increasingly faced with drastic shifts in their environment due to human activity and climate change (Chen et al., 2011; O'Hara et al., 2021; Yan et al., 2021). These environmental shifts can result in existing phenotypes not being well suited for current conditions, meaning that organisms must either move to a more suitable habitat and/or shift their phenotypes to avoid extirpation (Parmesan & Yohe, 2003). Phenotypes can shift to new optima through adaptive genetic change, termed 'evolutionary rescue' (Bell, 2013, 2017; Carlson et al., 2014); however, populations may be unable to persist long enough for evolutionary rescue to occur (Bell, 2013). Phenotypic plasticity, broadly defined as the ability of a single genotype to produce different phenotypes depending on the environment (West-Eberhard, 2003), allows for rapid phenotypic change in response to environmental conditions. Plasticity has been suggested to play a major role in the colonization of new environments (Bilandžija et al., 2020; Walter et al., 2022; Wang & Althoff, 2019; Yeh & Price, 2004), range expansions (Doudová-Kochánková et al., 2012; Otaki et al., 2010; Zarco-Perello et al., 2022), responses to climate change (Charmantier et al., 2008; Franks et al., 2014; Potts et al., 2021), and invasive species' ability to invade (Jardeleza et al., 2022; Liao et al., 2016; Pichancourt & Klinken, 2012).

Understanding how phenotypic plasticity affects population persistence during colonization and range expansion is important for predicting species responses to environmental change. However, the role that phenotypic plasticity plays during these challenges remains unclear due to inconsistent results across studies (reviewed in Hendry, 2016). For example, two meta-analyses published in the same year that investigated levels of plasticity expressed in invasive versus non-invasive plants found conflicting results—one study indicated that invasive plants did not have higher levels of plasticity than non-invasive plants (Palacio-López & Gianoli, 2011), while the other found that invasive plants had significantly higher levels of plasticity (Davidson et al., 2011). When considering how plasticity will help species persist under climate change, some studies have found that higher plasticity led to increased persistence (Henn et al., 2018; Urban et al., 2014; Vedder et al., 2013), whereas other studies have suggested that plasticity will have a limited impact (Gill et al., 2014; Gunderson & Stillman, 2015; Kellermann et al., 2020). These inconsistent results suggest that the impact of plasticity is trait and context-dependent. This could be due to the different ways that adaptive versus maladaptive plasticity alters species responses to environmental shifts. If plasticity is adaptive, it can allow organisms to persist in novel or changing environments through 'plastic rescue' (Chevin et al., 2010; Kovach-Orr & Fussmann, 2013; Lande, 2009; Snell-Rood et al., 2018). Adaptive plastic phenotypic shifts can then be followed by additive genetic change in the same direction, termed 'genetic assimilation' (Schlichting & Wund, 2014; West-Eberhard, 2003). If the plasticity is maladaptive, plastic shifts move populations further from the optimum phenotype, which could hinder survival in a new environment. Genetic change in the opposite direction of the plasticity, termed 'genetic compensation', is then required to push phenotypes closer to the optimum (Grether, 2005). This leads to 'counter-gradient variation' where individuals from different environments display higher trait similarity in the field than when acclimated in a common environment (Conover & Schultz, 1995).

Another potential reason for these inconsistencies may lie in the shifting importance of plasticity across timescales. Both genetic assimilation and genetic compensation are subsets of 'genetic accommodation', whereby genetic responses can reduce plasticity either by reinforcing the adaptive plastic phenotype such that it no longer needs to be environmentally induced or by reversing the maladaptive plastic phenotype so that it is no longer expressed (Grether, 2005; Waddington, 1942; West-Eberhard, 2003). Thus, one could predict that species that are new to an environment would show higher levels of plasticity than those that have been in the environment for a longer evolutionary timescale and have had time for genetic accommodation to take effect. This shifting importance of plasticity through time becomes an issue when comparing native species to invading species that have already become well-established in the novel environment, as is done in many studies due to difficulties in capturing initial range expansions or colonization events. It would be more informative to compare levels of plasticity between species that differ in their experience with an environment (i.e., newly invading vs. native species).

This study takes advantage of a system in which fish communities have undergone recent range shifts in response to changing environmental conditions to compare plasticity across two different time scales—very recent range-expanding (RE) versus long-established populations. Long-term monitoring of the Mpanga River drainage in Kibale National Park, Uganda has captured the range expansion of the cyprinid *Enteromius apleurogramma* northwards into the Rwembaita Swamp System (RSS), which includes a low-dissolved oxygen (DO) swamp and high-DO tributary streams. Monitoring of the RSS since 1990 indicated that the system hosted only two native fishes until 2012, the cyprinid *Enteromius neumayeri* and the air-breathing catfish *Clarias liocephalus*, both of which occur in low-DO and high-DO environments. *E. apleurogramma* was first recorded in the RSS in 2015 but has since spread throughout the entire swamp and associated streams (Hunt et al., 2023). It is one of three native fish species known to have expanded their range northward in the Mpanga River system, the others being the cyprinodontid *Platypanchax modestus* (appeared in 2012) and the cichlid *Pseudocrenilabrus multicolor* (appeared in 2022). *E. apleurogramma* inhabits both low- and high-DO areas in its historical habitat. Despite seasonal fluctuations in DO (Chapman et al., 1999), there is a strong phenotypic divergence between low-DO (swamp) and high-DO (stream) sites in *E. neumayeri*, with swamp-dwelling populations characterized by greater tolerance to hypoxia (Chapman, 2007; Olowo & Chapman, 1996), larger gills (Chapman et al., 1999; Langerhans et al., 2007), higher haematocrit (Chapman, 2007; Martinez et al., 2004), higher liver LDH

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activities, and higher glycolytic capacity (Chapman, 2015; Martínez et al., 2011). Populations do exchange some migrants between high-DO and low-DO habitats (Chapman et al., 1999; Harniman et al., 2013); however, a combination of long-term acclimation (Martínez et al., 2011) and genetic studies (Chapman et al., 1999; Harniman et al., 2013) suggest that there is divergent selection between oxygen regimes potentially leading to local adaptation even over small spatial scales. *E. neumayeri* and *E. apleurogramma* inhabit very similar habitats, are phylogenetically closely related (Ndeda, 2018), and display similar patterns of divergence across DO gradients in their native range (Hunt et al., 2023). Therefore, this study system allows us to compare levels of plasticity between two similar species that have different time scales of experience with the habitat: one experiencing a recent range shift (began 2 years prior to sampling) and another that has a much longer evolutionary history within the area.

This range expansion of *E. apleurogramma* was likely enabled by a recent increase in temperature in the RSS that made it more similar in temperature to the original habitat of *E. apleurogramma* (Hunt et al., 2023). It is expected that the colonizing *E. apleurogramma* individuals originated from high-DO populations based on the most direct route; however, it is possible that some individuals have previous experience with low-DO environments. Hypoxia, defined as DO levels under 2–3 mg O^2/L (Vaquer-Sunyer & Duarte, 2008), is common in the heavily vegetated papyrus swamp of the RSS due to lower flow and higher rates of decomposition. DO levels can be especially limiting in aquatic environments and impose strong selective pressures on fish species (Chapman, 2015). Accordingly, variation in DO levels can shape species ranges and result in many different behavioural and physiological adaptations (Nikinmaa & Rees, 2005; Richards, 2009, 2011). Due to the strong pressure that DO levels exert, it is likely that phenotypic plasticity in traits underlying hypoxia tolerance facilitated the colonization of *E. apleurogramma* (Crispo & Chapman, 2010).

Gene expression connects genotypes to phenotypes; therefore, plasticity in gene expression can serve as a link between environmental change and adaptive phenotypic plasticity (Rivera et al., 2021; Schlichting & Wund, 2014). Gene expression plasticity has been found to allow species to cope with variable environments, including hypoxia (Gracey et al., 2001; Nikinmaa & Rees, 2005; Storz et al., 2010), and to facilitate the colonization of new environments (Bittner et al., 2021; Morris et al., 2014). Hypoxia-induced plasticity in gene expression can occur very rapidly—goby fish (*Gillichthys mirabilis*) exposed to hypoxia showed shifts in gene expression within 8 h that were maintained for at least 6 days (Gracey et al., 2001). While all genes likely display a level of plasticity in their expression, the magnitude and direction of gene expression plasticity can be compared across populations and different environments to reveal potential differences in levels of phenotypic plasticity. Additionally, some differences in gene expression have a heritable basis that selection can act on (Crawford & Oleksiak, 2007; Whitehead & Crawford, 2006). Therefore, gene expression can be involved in both plastic and evolutionary divergence.

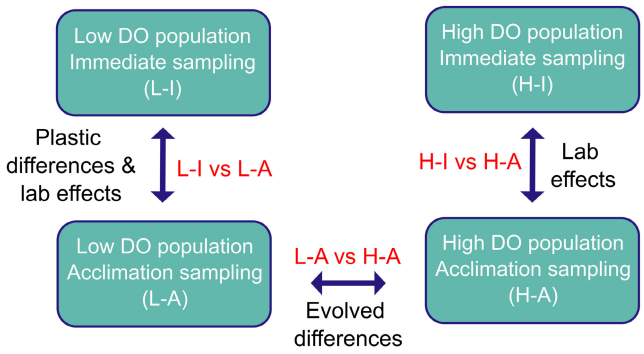

FIGURE 1 Pairwise comparisons made within each species for differential gene expression analysis. DO, dissolved oxygen.

In this experiment, we compared gene expression between *E. apleurgramma*, representing a RE species, and *E. neumayeri*, representing a native (N) species with evolutionary history in the area, caught from low-DO and high-DO habitats within the RSS. We sampled tissue either immediately after capture of the fish from the field or after a 2-week acclimation period at high-DO in small ponds. This allowed us to test for both candidate-evolved and short-term plastic differences in low-DO vs high-DO populations of each species by making three comparisons within each species (Figure 1). We cannot differentiate between evolved differences and changes that may reflect developmental plasticity or phenotypic change requiring a longer acclimation period. Therefore, differences we detect that are not induced by 2-week acclimation represent candidate-evolved differences. We hypothesized that plasticity in gene expression underlying hypoxia tolerance facilitated the colonization of *E. apleurogramma* into divergent oxygen environments within the RSS and that plasticity in *E. neumayeri* has over time been replaced by fixed differences. We predicted that gene expression would differ between colonizing and native populations such that in low-DO versus high-DO comparisons, colonizers exhibit primarily plastic gene expression, whereas native populations exhibit lower plasticity but more evolved divergence due to inherited differences in gene expression. Throughout our analysis, we assumed that candidate-evolved divergence between habitats reflects adaptive change. Therefore, plasticity was considered adaptive if it showed gene expression that occurs in the same direction as evolved divergence. For example, if both the plastic and candidate-evolved differences show the upregulation of a particular gene in low-DO relative to high-DO environments, this would be considered adaptive plasticity. In contrast, maladaptive plasticity would show gene expression plasticity and evolved divergence occurring in opposite directions. We additionally tested for genetic signatures of local adaptation and whole genome differentiation between low- and high-DO populations of both species. Since previous studies on *E. neumayeri* have shown that there is likely divergent selection between the two different habitats (Chapman et al., 1999; Harniman et al., 2013), we expected to find signatures of local adaptation between DO populations with there being more local adaptation in *E. neumayeri* than *E. apleurogramma* due to increased time in the area.

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2 | METHODS

2.1 | Ethics statement

Permission to carry out this work came from the Uganda National Council for Science and Technology, the Uganda Wildlife Authority, and McGill University Animal Care (AUP 5029).

2.2 | Study site

This study was conducted within the RSS (00.58875° N 030.37222° E) in Kibale National Park, Uganda. In this papyrus (*Cyperus papyrus*) swamp, low water flow and mixing combined with high input of organic matter and levels of shade result in low-DO levels, averaging 0.99 mg/L between 1993 and 2019 (for DO data, see Chapman et al., 2022). However, the swamp has associated streams and river tributaries where increased flow and turbulence lead to much higher average DO levels (~6 mg/L). Between 1994 and 2016, average local air temperatures have increased 1.45°C and concordantly average water temperatures have increased by 1.41°C (Lauren Chapman, unpublished data). It is possible that this shift in temperature has facilitated the expansion of *E. apleurogramma* into the swamp as historical populations reside in locations approximately 200 m lower in elevation that would have a predicted average temperature that is 1.3°C higher than the RSS (Hunt et al., 2023), although actual temperature records for the lower site are not available.

2.3 | Fish collection and acclimation trials

Similarly sized adult *E. neumayeri* (average standard length (SL): 5.97 cm ± 1.05 cm) and *E. apleurogramma* (average SL: 4.23 cm ± 0.28) were collected from a swamp and stream pair separated by ~200 m. Collections were done on June 6, 2017 using barrel minnow traps with a mesh size of 6.35 mm and throat openings of 25.4 mm baited with bread. Fish were randomly divided into two categories: those to be immediately sacrificed and those to be acclimated for 2 weeks. Fish selected for immediate sacrifice were euthanized using clove oil within 10 minutes of being pulled from the trap; gills were then extracted as quickly as possible and placed into RNAlater (Qiagen, Hilden, Germany). Gill tissue was chosen due to the central role it plays in respiration and its known plasticity in response to different DO levels (Sollid & Nilsson, 2006). Samples were initially stored at ambient temperature (~30°C) for 4–8 h before being returned to the field station (Makerere University Biological Field Station, MUBFS) where they were stored at 4°C. Fish selected to be acclimated were returned to the field station in small containers of well-oxygenated water. Fish were marked with a subdermal dye mark just below the dorsal fin, with combinations of colour and side of body indicating population and species. Fish were held for 14 days at ambient temperature in two open-air ponds (~1 m diameter by 50 cm depth) equipped with air pumps to ensure full oxygenation of the water. Approximately 10 fish of each of the two species were held in each pond for a total of 20 fish per pond: five from each species from the hypoxic swamp and five from each species from the normoxic stream. Pools were monitored daily for temperature and DO (Table S1), and for fish morbidity and mortality. Fish in the pools were fed ad libitum, and water changes were performed every 3 days. Acclimated fish were sacrificed after 14 days in a manner identical to immediately sacrificed fish: euthanized by clove oil and the gills immediately extracted and placed in RNAlater. Samples were held at 4°C for 30 days before being transported at ambient temperature over a period of 36 h to McGill University, where they were stored at −20°C until extraction. Sex is cryptic in these species, therefore, there are no sex data for these samples.

TABLE 1 Number of *E. neumayeri* (EN) and *E. apleurogramma* (EA) fish retained for analysis for each sampling category and species.

	Population	
Sampling category	Low DO	High DO
Immediate	EN:8, EA:10	EN: 9, EA: 9
Acclimation	EN: 7, EA: 11	EN: 8, EA: 7

2.4 | RNA extraction and sequencing

DNA and RNA were extracted using AllPrep DNA/RNA Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. We measured RNA quality using the Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, United States) and quantity using the Quant-it RiboGreen RNA Assay Kit (Invitrogen, Waltham, United States). Out of 80 samples, 70 were deemed of sufficient quantity and quality for sequencing (Table 1). Samples were sent to the McGill Genome Center (Montréal, Canada) for library preparation and sequencing. Libraries were prepared using the NEB Ultra II Directional RNA Library Prep Kit (New England BioLabs, Ipswich, United States), and all samples were run on one lane of the NovaSeq6000 S4 v1.5 (Illumina, San Diego, United States).

2.5 | Read quality control

We used Rcorrector v1.0.4 (Song & Florea, 2015) to remove erroneous k-mers using default settings and then TrimGalore! v0.6.6 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to trim low quality bases (phred <5) and adaptor contamination. Next, we generated quality reports on our samples using FastQC (Andrews, 2019) and found that there were many overrepresented sequences that corresponded to rRNA when searched using BLAST (Altschul et al., 1990). To counteract this, we constructed a rRNA database using the SSUParc and LSUParc v138.1 files in the silva database (accessed Dec 2022; www.arb-silva.de) and then mapped the reads to the database using bowtie2 v2.3.4.3 (Langmead & Salzberg, 2012) with the –nofw flag for dUTP-based libraries and

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the –very-sensitive-local preset option. Only read pairs for which neither read mapped to the database were retained for further analysis. Again, FastQC was run, and read quality was checked. One *E. neumayeri* (low-DO, immediate) sample was removed due to poor sample quality.

2.6 | Trinity de novo assembly

As there is no reference genome available for either species, we performed de novo assembly for each species separately using Trinity v2.15.0 (Grabherr et al., 2011) with –SS_lib_type set to RF for dUTP-based libraries and default settings. To assess the quality of our trinity assemblies we looked at several assessment metrics. First, we used bowtie2 v2.3.4.3 (Langmead & Salzberg, 2012) to align reads from each individual to its corresponding assembly and examined the RNA-seq read representation of the assembly. Next, we computed 'gene' contig Nx length statistics where at least x% of the assembled transcript nucleotides are found in contigs of at least Nx length for x = 10–50 along with counts of transcripts and 'genes' and median contig length using a custom perl script in the Trinity toolbox (TrinityStats.pl). Then, we used BUSCO v5.2.2 (Manni et al., 2021) with the vertebrata_odb10 BUSCO set (accessed Feb 2023) using the transcriptome setting to estimate the completeness and redundancy of the assembly. To functionally annotate the assemblies, we used TransDecoder v 5.7.0 to predict coding regions (Haas, BJ. https://github.com/TransDecoder/TransDecoder). Then, we used Trinotate v4.0.0 (Bryant et al., 2017) to compare predicted coding regions and entire transcripts to established protein databases, Swiss-Prot (Bairoch & Apweiler, 1997) and PFam (Punta et al., 2012) (both accessed May 2023). We also used Infernal v1.1.4 (Nawrocki & Eddy, 2013) to search the noncoding RNA database Rfam v14.9 (accessed May 2023). Using Trinotate, we annotated coding regions for signal peptides with Signal P v6.0 (Teufel et al., 2022), transmembrane helices with tmHMM v2 (Krogh et al., 2001) and domain content with Eggnog-mapper v2 (Cantalapiedra et al., 2021).

2.7 | Quantification and differential gene expression analysis

We used *Salmon* v1.10.1 (Patro et al., 2017) to quantify transcripts and generate gene counts using the Trinity gene transcript map generated for each species. All further analyses were performed in R v4.2.2 (R Core Team, 2022). Differential gene expression was analysed using *edgeR* v3.40.2 (Robinson et al., 2010). Out of all possible contrasts, we chose a subset of three planned contrasts a priori to test our hypotheses and reduce type-I errors. Within each species, we ran the following comparisons: (1) Low-DO population, immediate sampling vs, low-DO population, sampling after acclimation (plastic differences and laboratory effects; L-I vs L-A); (2) High-DO population, immediate sampling vs high-DO population, sampling after acclimation (laboratory effects; H-I vs H-A); and (3) Low-DO population, sampling after acclimation vs high-DO population, sampling after acclimation (candidate-evolved differences; L-A vs H-A) (Figure 1). We filtered out lowly expressed genes using a count-per-million (CPM) threshold of one, corresponding to a count of six reads in the sample with the smallest number of reads, and requiring a gene to be past this threshold in at least seven individuals—representing the number of individuals in the smallest sampling group. To determine whether holding pond should be included as a covariate in the analysis, we ran a principal component analysis (PCA) on expression across all genes and assessed the first six principal components (PCs) for an effect of pond. When analysing the PCA for any impact of pond on sample clustering, we found that samples did not cluster by pond, suggesting no or little impact (Figure S1). To further confirm the absence of an effect of pond on gene expression, we ran ANOVAs on the first six PCs and found no significant effect of pond ($p > 0.05$ for all tests). We therefore did not include pond as a covariate in our analysis. Genes were considered differentially expressed at a false discovery rate cut-off of 0.01 and a minimum four-fold difference in expression.

2.8 | Cluster analyses

Further statistical analysis was performed on trimmed mean of M values (TMM) normalized, log2-transformed and median-centred gene expression values. To evaluate trends in total gene expression and differential gene expression, we ran PCA on all genes and on all differentially expressed genes (DEGs). We assessed the PCAs for effects of population (low- and high-DO) and sample type (immediate vs. acclimation). We also ran hierarchical clustering with Euclidean distance and Ward's linkage on samples using *cluster* v2.1.4 (Maechler et al., 2022).

To identify and visualize expression patterns across genes, we used *MFuzz* v2.60.0 (Kumar & Futschik, 2007) to perform soft (fuzzy c-means) clustering on our DEGs. This package groups genes with similar expression patterns together and assigns each gene a membership value to the cluster it is assigned to, representing how closely its expression aligns with the rest of the cluster. First, we estimated the optimal fuzzifier parameter using the *mestimate* function and then used *Dmin* and *cselection* to investigate the potential optimal number of clusters. For both species, the suggested number of clusters was 2 with the minimum centroid distance rapidly decreasing after 16 clusters. However, it is advised to visually review the data before choosing the number of optimal clusters as these tools may not always accurately identify all patterns, so we performed repeated clustering for a range of cluster numbers (c = 2–20) and visually assessed expression patterns to determine the number of clusters at which no uniquely shaped expression patterns were collapsed. For *E. neumayeri*, at the suggested number of clusters (c = 2), many unique expression profiles were collapsed, which then became separate at c = 9; but at c > 9 redundant expression patterns became apparent. Therefore, we selected c = 9. Following the same

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reasoning, we selected c = 7 for *E. apleurogramma*. After selecting the final cluster number, we visualized expression across these clusters, requiring a minimum membership value of 0.7 for all genes. Lastly, we constructed a heatmap that displayed the clustering results for samples and DEGs with relative gene expression using *pheatmap* v1.0.12 (https://CRAN.R-project.org/package=pheatmap) and visually identified clusters of interest that contained genes with expression patterns showing differential expression between H-I samples and L-I samples that are no longer differentially expressed between the H-A and L-A samples as these genes are likely to be involved in plastic responses to DO.

2.9 | Comparing plastic to evolutionary changes in gene expression within and between species

We identified three sets of genes: (1) Candidate-evolved changes (hereafter evolved): identified in the H-A versus L-A comparison; (2) Plastic changes: identified as DEGs in the L-I versus L-A comparison (laboratory effects and plasticity) that are not present in the H-I vs H-A comparison (laboratory effects); and (3) Shared changes: identified by finding the overlap between the evolved DEGs and plastic DEGs. We compared log2-fold change (FC) in the shared DEGs using Pearson's correlation to determine whether these DEGs showed shifts in gene expression in the same or opposite direction. If a gene was upregulated or downregulated in both the L-A samples relative to the H-A samples and the H-A samples relative to the L-I samples, it was said to be in the same direction. We compared the observed correlation to a distribution produced through permutation by randomly sampling the number of shared genes (31 for *E. neumayeri* and 269 for *E. apleurogramma*) from all genes retained in the DEG analysis 10,000 times and recalculating Pearson's correlation. We also used a chi-squared test with Yates correction to determine whether there was a higher or lower proportion of evolutionary divergence DEGs overlapping with the plastic genes than expected. Within species, we ran a chi-squared test with Yates' correction to test for differences in the proportion of significant DEGs for evolved and plastic DEG gene sets. To compare the plastic responses and evolved divergence between species, we ran Mann–Whitney *U*-tests on the average magnitude of log2-FC in DEGs from the L-A vs L-I and the H-A. vs L-A comparisons between species. We used the *rstatix* package to calculate the effect sizes of the comparisons (Kassambara, 2023).

2.10 | Population genetic analysis

To assess genetic differentiation between populations, we used the Trinity toolbox to convert our de novo assemblies into SuperTranscripts for each species (Davidson et al., 2017), thus providing a genome-like reference that allows for the identification of sequence variants. For each individual, we mapped all reads to the SuperTranscripts using *STAR* v.2.7.11b (Dobin et al., 2013) and identified duplicated reads using *Picard* v2.26.3 (https://github.com/broadinstitute/picard). We merged bam files for all individuals of a species and then used FreeBayes v1.3.6 (Garrison & Marth, 2012) to detect single-nucleotide proteins (SNPs). Only SNPs with a genotype and site quality of >30 were retained. *BCFtools* v1.16 (Danecek et al., 2021) was used to filter out SNPs that had missing data, were multiallelic sites, deviated from Hardy–Weinberg equilibrium ($q < 0.05$), or had a minor allele frequency less than 5%. *BCFtools* was also used to calculate linkage disequilibrium in 10,000 bp windows. We retained only one SNP from any tightly linked pairs ($r^2 > 0.8$). With this filtered SNP set we used the *Hierfstat* R package (Goudet, 2005) to calculate Weir and Cockerham's F_{st} between DO populations.

SNPs putatively subject to natural selection were detected using *pcadapt* v4.4.0 (Privé et al., 2020). We started with $K = 25$ PCs and then identified the number of useful PCs following the package guidelines. We retained 5 PCs for *E. apleurogramma* and 8 PCs for *E. neumayeri*. We visually assessed PCA plots and determined which PCs separated samples by population. Outlier SNPs that were associated with these PCs were identified as potential SNPs under selection between the two DO populations, and we identified a list of genes containing outlier SNPs. Lastly, we used a chi-squared test with Yates correction to determine whether there was a difference in the proportion of outlier SNPs between the species.

2.11 | Gene Ontology enrichment analysis

We conducted Gene Ontology (GO) enrichment analysis on the gene clusters of interest identified in the cluster analysis using the package *Goseq* v3.17 (Young et al., 2010) which performs GO enrichment analysis while correcting for gene length bias. All three GO branches (Cellular Components, Biological Processes and Molecular Functions) were used to test for enrichment using the Wallenius approximation while restricting to a background list of the genes that were retained for the differential expression analysis after filtering for minimum expression. Then, we corrected *p*-values for multiple testing by converting to *q*-values using the package *qvalue* v2.32.0 (Storey et al., 2023) and considered terms as significantly enriched at a false discovery rate of $q < 0.05$. To conduct a GO enrichment analysis on genes containing outlier SNPs, we used *topGO* v2.56.0 (Adrian Alexa, 2017). The background list of genes consisted of all genes containing a SNP after filtering and we used Fisher's exact test with the default weight algorithm that corrects for GO topology. Since we used a method that accounts for GO topology we did not correct *p*-values for multiple testing as per the algorithm guidelines (Alexa et al., 2006). GO terms were considered significantly enriched at $p < 0.05$. To further analyse and plot enriched GO terms, we used REVIGO (http://revigo.irb.hr/) on all significant GO terms in the biological process category using default settings (whole UniProt database, medium list size and SimRel similarity measure). REVIGO removes redundant GO terms and performs SimRel clustering to plot the similarity of given GO terms in semantic space (Supek et al., 2011).

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3 | RESULTS

3.1 | Sequence count overview, Trinity assemblies and differential gene expression analysis

E. neumayeri (native: N) samples had a sequencing depth range of 11.6 million to 39.4 million trimmed paired end (PE) reads (average 20,929,949 ± 5,964,781) and *E. apleurogramma* (RE) samples had a range of 12.4 million to 71.6 million reads (average 33,856,401 ± 14,498,298) (Tables S2 and S3). The Trinity assembly generated for *E. neumayeri* contained 1,094,565 transcript contigs grouped into 611,156 'genes' (median contig length: 374, N50 of 1009) (Table S4). The *E. apleurogramma* Trinity assembly had 899,947 transcript contigs grouped into 546,319 'genes' (median contig length: 406, N50 of 1197) (Table S4). BUSCO reports generated for each assembly indicated near complete gene sequence information for 91.6% of genes for *E. neumayeri* and 92.7% of genes for *E. apleurogramma* (Table 2). An average of 97% and 98.4% of reads per sample aligned back to the *E. neumayeri* and *E. apleurogramma* assemblies, respectively, with most of these reads mapped as proper pairs (Tables S2 and S3). Using Trinotate to annotate the *E. neumayeri* assembly, we found 185,053 transcripts matching 35,511 unique Swiss-Prot proteins, 11,869 of which matched at least 80% of the protein's length. For *E. apleurogramma*, we found 181,074 transcripts matching 34,996 unique Swiss-Prot proteins with 12,086 of which matched at least 80% of the protein's length. After filtering, 33,427 and 71,534 Trinity 'genes' were retained for differential gene expression analysis for *E. apleurogramma* and *E. neumayeri*, respectively. We identified a total of 1015 DEGs for *E. neumayeri* (N) and 1085 DEGs for *E. apleurogramma* (RE) (see Figure S2 for full breakdown and additional attachments for full results).

To test whether the difference in the number of retained genes between species could have had an impact on our ability to detect DEGs, we ran additional filtering of lowly expressed genes that reduced gene sets to 32,460 genes for *E. apleurogramma* and 57,385 genes for *E. neumayeri*, thus reducing the differential in number of genes detected in each species. After re-running the DEG analysis, this additional filtering did not change the number of DEGs detected. We also re-ran the differential gene expression analysis for *E. neumayeri* by randomly sub-setting 30,000 genes from the total gene set to see whether there are any impacts of reducing the gene set to roughly the same number of genes as *E. apleurogramma*. We found that for every comparison, this subset of genes resulted in the same amount or fewer DEGs being detected. Furthermore, 100% of the DEGs detected from the subset overlapped with the DEGs detected in the original analysis, indicating that reducing the number of genes did not result in additional genes being detected. Therefore, we do not expect that the difference in the number of retained genes impacted our results.

TABLE 2 BUSCO reports for each species.

Species	Summary in BUSCO annotation
E. neumayeri	C: 91.6% [S: 18.5%, D73.1%], F: 4.8%, [M: 3.6%, *n*: 3354]
E. apleurogramma	C: 92.7% [S: 15.9%, D76.8%], F: 4.7%, [M: 2.6%, *n*: 3354]

Abbreviations: C, complete; D, complete and duplicated; F, fragmented; M, missing; *n*, number of BUSCOs searched; S, complete and single copy.

3.2 | Cluster analyses

For the PCA on all genes in *E. neumayeri* (N), there was divergence between the immediate samples of each population (H-I and L-I) and between the sample types (immediate vs. acclimation). In contrast, we observed overlap between the acclimation samples from both populations (H-A and L-A) (Figure 2a). In *E. apleurogramma* (RE), the PCA on all genes showed high levels of overlap between the immediate samples from each population, whereas the acclimation samples from each population showed divergence along PC1, with the L-A samples diverging the most from the immediate samples (Figure 2b). In the PCA on DEGs, there was clear clustering by population and sample type for both species (Figure 2c,d). *E. neumayeri* (N) showed a slight overlap between the acclimation samples (Figure 2c), while *E. apleurogramma* (RE) showed separation between those samples and instead slight overlap between the immediate samples (Figure 2d). The hierarchical clustering on samples showed H-I and L-I samples clustering together for both species, however, for *E. apleurogramma* (RE) this cluster is then nested within the H-A samples and the L-A samples are the least like the rest (Figure 3a,b). For *E. neumayeri* (N), the H-A and L-A samples form a separate cluster (Figure 3a). Fuzzy cluster analysis on the DEGs using soft clustering identified nine clusters for *E. neumayeri* and seven clusters for *E. apleurogramma* (Figures S3 and S4). Of these clusters, Cluster 5 was determined to be of interest and potentially involved in plastic responses to DO levels in *E. apleurogramma* (Figure 3b) and Clusters 5 and 6 were of interest for *E. neumayeri* (Figure 3a).

3.3 | Comparison between plastic and evolved gene expression

We identified 344 plastic, 63 candidate evolved, and 31 shared DEGs for *E. neumayeri* (Figure 4a). *E. apleurogramma* (RE) had 556 plastic, 320 candidate evolved, and 269 shared DEGs (Figure 4b). Log2-FC was highly negatively correlated between shared DEGs in both species (*E. neumayeri*: Pearson's correlation = −0.838; *E. apleurogramma*: Pearson's correlation = −0.792) (Figure 5a,b). We compared this result to a permutation test where we randomly sampled the number of DEGs retained in the analysis out of all genes 10,000 times and found that the observed correlation was stronger than expected by chance for both species ($p = 0.0458$ for *E. neumayeri* and $p < 0.0001$ for *E. apleurogramma*) (Figure 5a,b; Figure S5). There were no shared DEGs that showed changes in the same direction. In addition, genes

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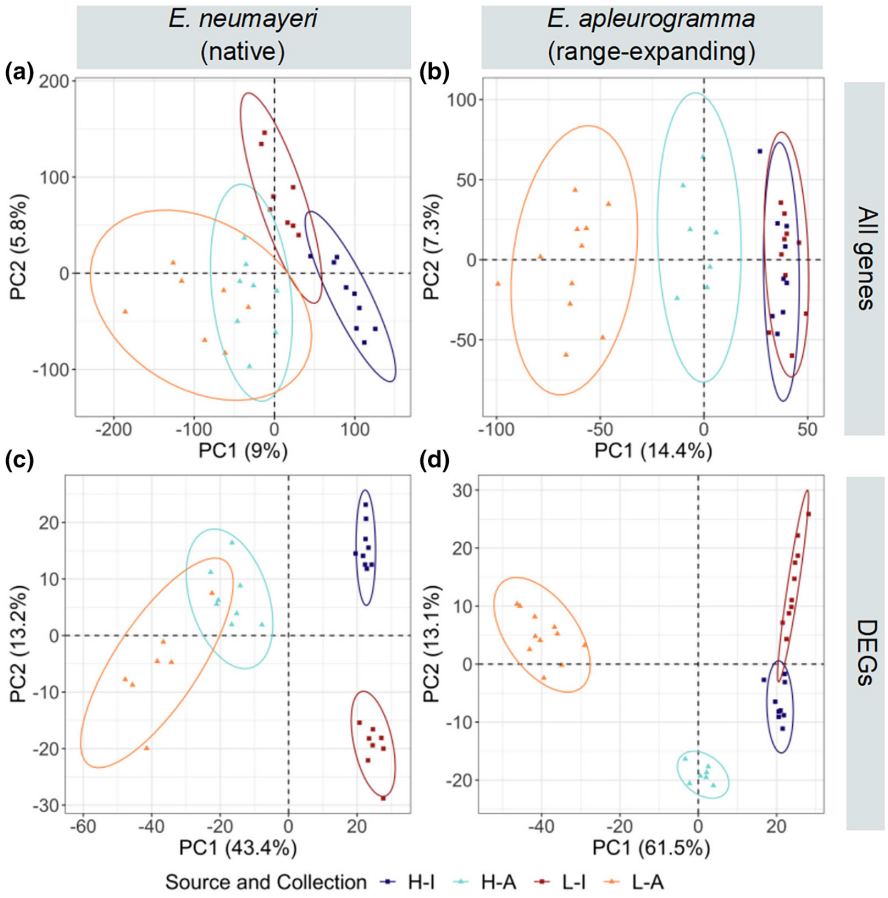

FIGURE 2 Principal component analysis (PCA) on all expression (a and b) and all differentially expressed genes (DEGs) (c and d) in *E. neumayeri* (native) (a and c) and *E. apleurogramma* (range-expanding) (b and d). Gene expression values were trimmed mean of M values (TMM) normalized, log2 transformed and median centred prior to analysis. *L*, low-DO population; H, high-DO population; I, immediately sampled, A, sampled after acclimation.

showing evolutionary divergence overlapped with those showing plastic divergence at a higher rate (EN: 9.01%, EA: 48.38%) than they did within all genes (EN: 0.12%, EA: 1.05%) for both species (EN: $X^2 = 1537.6$, df = 1, $p < 0.0001$; EA: $X^2 = 6560$, df = 1, $p < 0.0001$).

Both species showed a higher proportion of significant plastic DEGs than evolved DEGs (EN: $X^2 = 230.5$, df = 1, $p < 0.0001$; EA: $X^2 = 58.5$, df = 1, $p < 0.0001$). *E. apleurogramma* (RE) had a higher median and larger interquartile range (IQR) of magnitude log2-FC than *E. neumayeri* (N) in plastic DEGs (EA: median = 4.05, IQR = 7.14; EN: median = 2.52, IQR = 1.11). In evolved DEGs, *E. apleurogramma* (RE) had a higher median but a smaller IQR of magnitude log2 FC than *E. neumayeri* (N) (*E. apleurogramma*: median = 7.30, IQR = 2.48; *E. neumayeri*: median = 5.31, IQR = 3.91). These differences were significant in the Mann–Whitney *U*-tests ($p < 0.0001$ for both comparisons; Figure 6). However, the effect size for the comparison between the plastic DEGs was smaller ($r = 0.28$) than that of the evolved DEGs ($r = 0.318$).

3.4 | Signatures of local adaptation

After filtering, we retained 118,685 out of 2,502,400 SNPS from the *E. apleurogramma* (RE) and 227,702 out of 3,821,249 SNPs from *E. neumayeri* (N). Using these SNPs, we found that both species had very low genome-wide F_{st} between DO populations (EN = 0.001, EA = 0.002). However, we identified a significant number of outlier SNPs that could be under natural selection for both species, with significantly more outlier SNPs identified for *E. neumayeri* (N) (1434 SNPs) than for *E. apleurogramma* (RE) (330 SNPs) ($X^2 = 189.8$, df = 1, $p < 0.0001$).

3.5 | Gene Ontology enrichment analysis and REVIGO

For *E. neumayeri* (N), we conducted GO enrichment analysis and then REVIGO on gene expression Clusters 5 and 6. Cluster 5 contained genes with decreased expression in the L-I samples compared with the rest of the samples (Figure 4a). This cluster showed significant GO terms related to immune responses and regulation of the immune system (Figure S6a). Cluster 6 contained genes that were upregulated in L-I samples (Figure 4a), and this cluster contained significant GO terms related to cellular responses to hypoxia, protein hydroxylation and metabolic processes (Figure S6b). *E. apleurogramma* (RE) had GO enrichment analysis and REVIGO run on gene expression Cluster 5 which contained genes that were upregulated in the L-I samples (Figure 4b) and showed GO terms related to responses to hypoxia and nitric oxide, protein hydroxylation and post-synaptic processes (Figure S6c; Tables S5 and S6 for all significant GO terms).

GO enrichment analysis was also done on genes that contained the identified outlier SNPs and there was an enrichment for many

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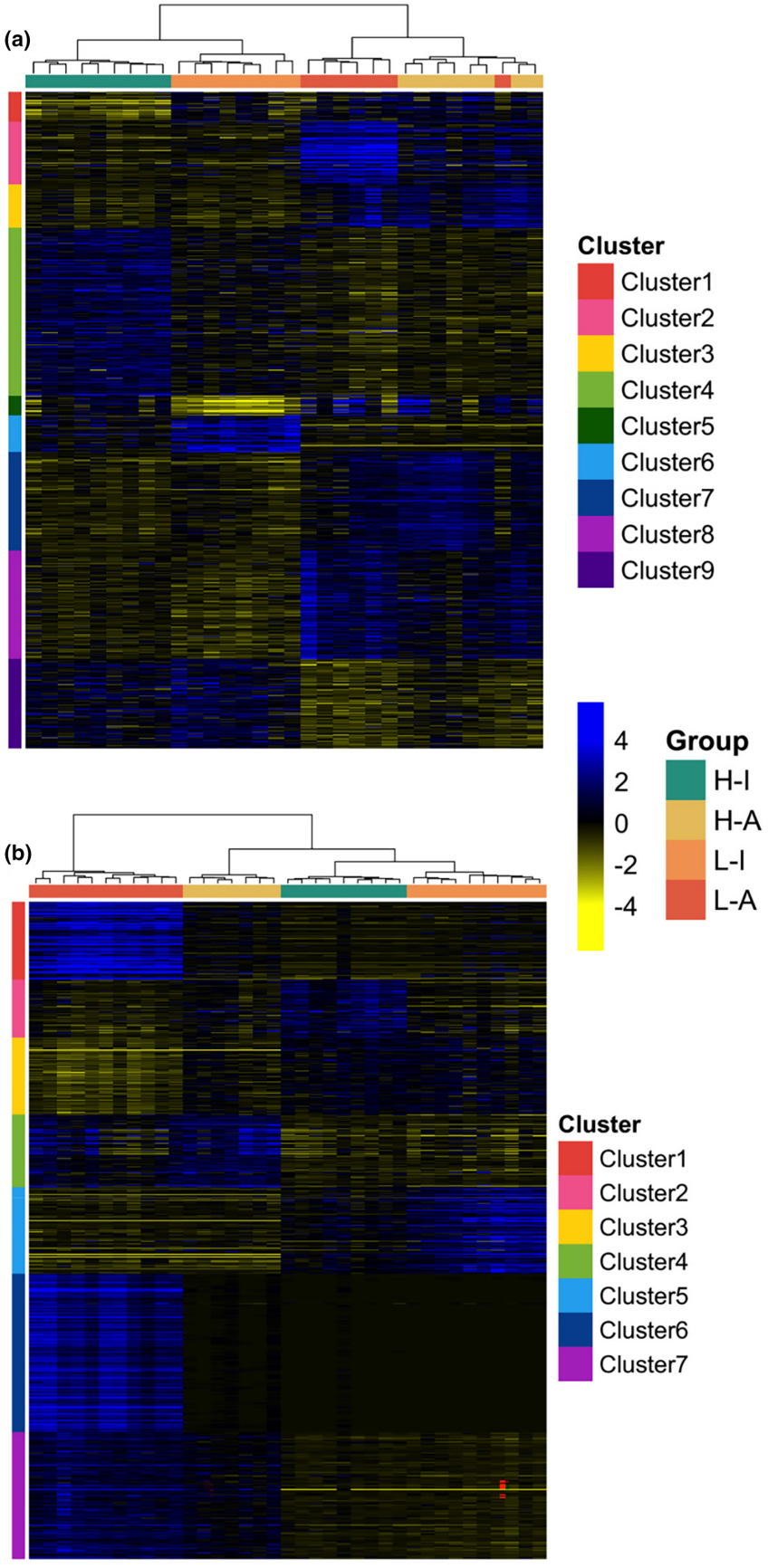

FIGURE 3 Heatmaps of differentially expressed genes (DEGs) with cluster analysis for (a) *E. neumayeri* (native) and (b) *E. apleurogramma* (range-expanding). Gene expression values are trimmed mean of M values (TMM) normalized, log2 transformed and median centred. Hierarchical clustering with Euclidean distance and Ward's linkage was run on samples and results are shown along the top of the heatmap. Soft (fuzzy c-means) clustering was run on DEGs to identify expression patterns with clustering results shown along the left side of the heatmap. Clusters of interest that contained genes with expression patterns showing differential expression between H-I samples and L-I samples that are no longer differentially expressed between the L-A and H-A samples were clusters 5 and 6 for *E. neumayeri* and cluster 5 for *E. apleurogramma*. L = low-DO population, H = high-DO population, I = immediately sampled, A = sampled after acclimation.

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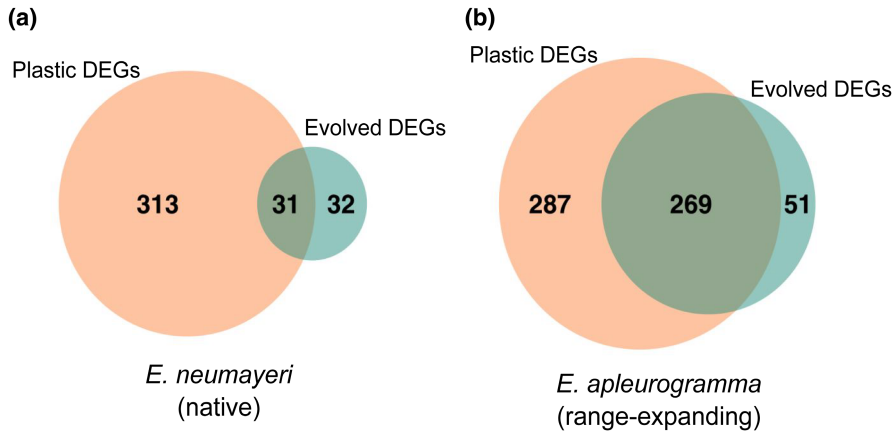

FIGURE 4 Venn diagram of plastic and candidate-evolved differentially expressed genes (DEGs) and the overlap between the two for (a) *E. neumayeri* (native) and (b) *E. apleurogramma* (range-expanding). Evolved DEGs were identified through the high dissolved oxygen (H-DO) population, acclimation sampling vs low-dissolved oxygen population (L-DO), acclimation sampling comparison. Plastic DEGs were identified by finding the DEGs from the L-DO, immediate sampling versus L-DO, acclimation sampling comparison and then excluding DEGs that were also found in the H-DO, immediate sampling vs H-DO, acclimation sampling comparison.

genes with putative functions in hypoxia response. For both species, outlier SNPs were found in genes involved in regulating gene expression, DNA replication, various metabolic processes and heart function. Heart function and metabolic processes are known to play a role in hypoxia adaptations (discussed further below). *E. apleurogramma* additionally showed outlier SNPs located in genes involved in similar functions as the gene clusters identified above, with roles in the regulation of reactive oxygen species, immune function and protein modifications (Figure S7). *E. neumayeri* showed outlier SNPs located in genes involved in ion homeostasis and muscle development, two processes known to be impacted by hypoxia (Figure S8) (Tables S7 and S8 for all significant GO terms). Putative functions are discussed in more detail below.

4 | DISCUSSION

We compared gene expression plasticity in response to DO levels between two non-model fish species that have experienced different timescales of exposure to a naturally varying environment: one range-expanding (RE) species (*E. apleurogramma*) and one native (N) species (*E. neumayeri*). We identified gene clusters involved in plastic responses to DO levels with significant GO terms, many of which match findings found in mammalian study systems. Using our gene expression data, we also identified SNPs and found many SNPs potentially under selection that were located in genes involved in responses to hypoxia, suggesting there is local adaptation between the two DO populations. Sampling fish from low-DO and high-DO field populations both immediately after capture and after a high-DO acclimation trial allowed us to disentangle candidate evolutionary differences from short-term plastic differences in each species. Across our analyses, we found results that point to the importance of maladaptive plasticity in promoting divergence between high- and low-DO populations through counter-gradient variation for both species. However, our results suggest that the counter-gradient variation may be stronger in the recently colonizing species and could be facilitating colonization by promoting local adaptation between low-DO and high-DO populations.

4.1 | Samples clustered differently by population origin and DO exposure for each species

Cluster analyses showed higher than expected levels of similarity between the field populations in both species. In the PCA on all genes, there is complete overlap of the high-DO and low-DO immediate samples for *E. apleurogramma* (RE), while in *E. neumayeri* (N), there is slight overlap between the immediate samples but much more overlap between the acclimation samples. The PCA on DEGs indicates a similar pattern, with more overlap between the immediate samples of each population for *E. apleurogramma* (RE) than *E. neumayeri* (N). Similar results were found in the hierarchical analysis of all DEGs, the immediate samples clustered most closely together in both species. It is unknown which DO environment the colonizing *E. apleurogramma* individuals originated from, but it is expected that they primarily come from the high-DO population because this population has a much more direct route for migration to the RSS. Additionally, the field populations of *E. apleurogramma* (RE) have had much less time to diverge from each other than the field populations of *E. neumayeri* (N). Therefore, we would expect to see closer clustering between the immediate samples in *E. apleurogramma* (RE) than *E. neumayeri* (N). However, gene expression could alternatively reflect how close an individual is to the adaptive peak in its environment, with gene expression plasticity instead reflecting a lack of adaptation to counteract the negative effects of a stressful DO environment (Ghalambor et al., 2007). Under this scenario, it would instead be predicted that since *E. neumayeri* (N) has had more time to locally adapt to each habitat,

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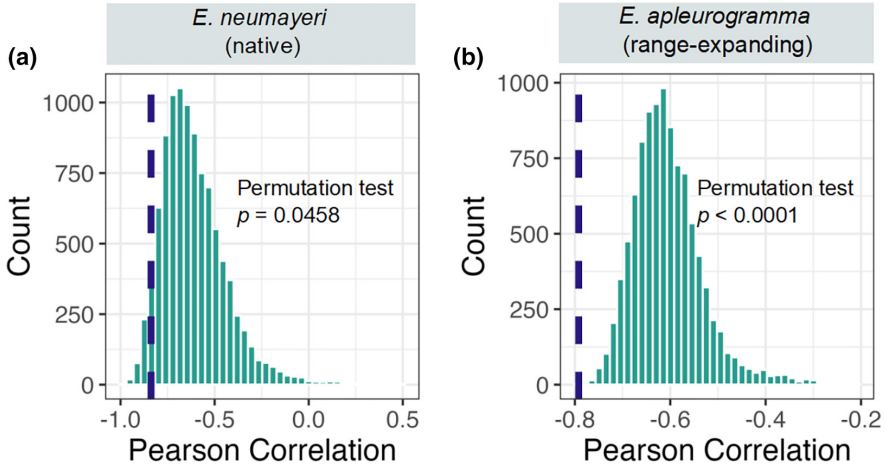

FIGURE 5 Correlation between log2 fold change in the candidate evolutionary divergence and plastic shifts in (a) *E. neumayeri* and (b) *E. apleurogramma*. We ran permutation tests where we randomly sampled the number of DEGs that overlap out of all genes 10,000 times for each species and then recalculated Pearson's correlation. The distribution of correlations is displayed along with the observed correlation indicated by the dashed line.

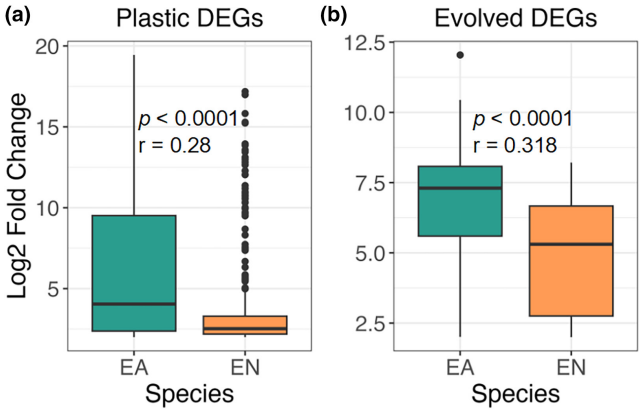

FIGURE 6 Difference in average magnitude log2 fold change between species for (a) Plastic changes or (b) candidate evolutionary changes. Candidate-evolved differentially expressed genes (DEGs) were identified through the high dissolved oxygen (H-DO) population, acclimation sampling versus low-dissolved oxygen population (L-DO), acclimation sampling comparison. Plastic DEGs were identified by finding the DEGs from the L-DO, immediate sampling vs L-DO, acclimation sampling comparison and then excluding DEGs that were also found in the H-DO, immediate sampling vs H-DO, acclimation sampling comparison. Significance was tested using Mann–Whitney *U*-tests. EA = *E. apleurogramma* (range-expanding), EN = *E. neumayeri* (native).

immediate samples would cluster more closely together than *E. apleurogramma* (RE) fish that have had less time to adapt and could be experiencing a greater environmental challenge.

4.2 | Counter-gradient variation in DEGs that overlap between plastic and candidate-evolved changes

We identified candidate-evolved DEGs (H-A vs. the L-A) and plastic DEGs (L-I vs. L-A minus H-I vs. H-A). By comparing these sets of DEGs, we found that the majority did not overlap, suggesting that plasticity and evolved divergence occurs mostly in different subsets of genes. This finding is consistent with other studies. For example, in killifish (*Fundulus heteroclitus*) that experience varying temperatures there was very little overlap between plasticity DEGs and adaptation DEGs (Dayan et al., 2015). This result could suggest that plasticity and evolved divergence act on different mechanisms. Alternatively, it could suggest that plasticity impedes adaptive divergence since the genes that experience plasticity do not diverge between populations. One way to determine whether plasticity is impeding evolutionary divergence is to compare the proportion of evolutionary divergence DEGs in all genes to the proportion in plastic genes. We found that there was a significantly higher proportion of evolutionary divergence genes within the plastic gene set than within all genes for both species, suggesting that plasticity does not impede genetic divergence. The subset of genes that did overlap, demonstrating both plastic and evolved differences, were highly negatively correlated in both species. This correlation was stronger than expected by chance as tested in a permutation analysis. Additionally, the fold change of expression was found to operate in the opposite direction for all shared DEGs. Adaptive differences that occur in the opposite direction as the plastic differences are suggestive of counter-gradient variation (Conover & Schultz, 1995).

Counter-gradient variation can evolve through genetic compensation, a subset of genetic accommodation, where a plastic change in phenotype reduces fitness in a new environment but selection subsequently acts to shift the phenotype back to the ancestral state without reducing phenotypic plasticity (Grether, 2005). As a result, genetic compensation may lead to populations from different environments displaying higher trait similarity in the field than when acclimated in a common environment. In the cluster analysis, the RE *E. apleurogramma* showed more similarity between populations from different DO habitats when they were sampled immediately in the field than after they had been acclimated to normoxia. This pattern could be due to genetic compensation acting to reduce phenotypic variation between the populations. However, this pattern was not observed in the native *E. neumayeri*, which may suggest that this species is experiencing less counter-gradient variation. This could be due to *E. neumayeri* (N) expressing less maladaptive plasticity than *E. apleurogramma* (RE). Indeed, *E. neumayeri* (N) showed fewer counter-gradient genes than *E. apleurogramma* (RE). However, it is unclear whether *E. neumayeri* (N) has always possessed fewer

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counter-gradient genes or whether there might have been similar levels of counter-gradient variation during initial stages of colonization which were then reduced over time. In guppies (*Poecilia reticulata*), between two lineages that have shown parallel evolution in response to high predation, the lineage that was more recently diverged showed a stronger signature of nonadaptive plasticity than the older lineage (Fischer et al., 2021). This suggests that studies must consider how plasticity impacts divergence across all stages of colonization to fully understand its role.

Previous studies have found conflicting results for counter-gradient variation in gene expression in fish. The previously referenced study on *F. heteroclitus* found patterns of counter-gradient variation in gene expression in response to thermal environments (Dayan et al., 2015). Another study on guppies adapting to predator-free environments found that 89% of transcripts showed shifts in gene expression that were in the opposite direction of evolved changes and concluded that maladaptive plasticity potentiates the rapid evolution of brain gene expression during the early stages of adaptation (Ghalambor et al., 2015). However, this study was criticized for making conclusions based on gene expression data only and collecting no data on organismal plasticity directly (van Gestel & Weissing, 2018). It is important to consider that gene expression is only one measurement of plasticity, and the complexity of regulation mechanisms make it possible for divergent changes in gene expression to lead to convergent phenotypes. Indeed, another study investigating gene expression directly from the proteome in populations of European grayling (*Thymallus thymallus*) instead found that plastic and evolved changes were in the same direction (Mäkinen et al., 2016). Furthermore, a follow up study on guppies again showed nonadaptive plasticity but also suggested that alternative transcriptional configurations could be associated with shared phenotypes across distinct evolutionary lineages (Fischer et al., 2021). Therefore, our results are consistent with counter-gradient variation; however, further studies are needed to fully elucidate the role of maladaptive plasticity in this system. For example, it is also possible that plasticity is acting on stress responses that are beneficial in the short term but costly in the long term and are, therefore, reduced over evolutionary time.

4.3 | Higher plasticity and evolutionary divergence in range-expanding *E. apleurogramma*

One way to assess the relative levels of plastic and evolved divergence is to compare the proportion of genes that are significantly differentially expressed for each type of change. We found that there were a significantly higher proportion of plastic DEGs than candidate-evolved DEGs for both species. This result is expected for *E. apleurogramma* (RE) which has had less time for populations in low- and high-DO habitats to show evolved divergence; however, it is surprising for *E. neumayeri* (N), which is expected to be under divergent selection between low- and high-DO sites. A level of DO-induced plasticity is likely maintained in *E. neumayeri* (N) despite evolved divergence across DO habitats since DO fluctuates seasonally (Chapman et al., 1999) and the proximity of the sites (~200 m) means that some individuals likely cross DO boundaries. If individuals are experiencing frequent shifts in DO, due to seasonal fluctuations and/or travel between DO habitats, it could instead be expected that plasticity would be favoured and therefore increase over evolutionary time. These different predictions could be more thoroughly tested by carrying out a common garden in hypoxia, which would allow further disentangling of the plastic vs evolved divergence in these two species.

Support for plasticity playing a more important role in *E. apleurogramma* (RE) than *E. neumayeri* (N) comes from comparisons of log2-fold change (FC), which show that *E. apleurogramma* had a higher median magnitude of log2-FC for plastic DEGs. Interestingly, the range of magnitude log2-FC for the plastic DEGs for *E. apleurogramma* (RE) was much larger than the range for *E. neumayeri* (N). Although *E. apleurogramma* has only a slightly larger magnitude of plastic change than *E. neumayeri*, it possesses some genes that show very strong plastic responses. Further work could be done to identify genes that show larger amounts of plasticity than others. If these highly plastic genes are adaptive, they could be responsible for facilitating *E. apleurogramma*'s colonization of the RSS by enabling the species to persist in the low-DO environments and thereby allowing for genetic assimilation (Crispo, 2007; Schlichting & Wund, 2014). However, the counter-gradient variation we observed indicates that these plastic genes could also be maladaptive. Maladaptive plasticity has been hypothesized to aid adaptive divergence in some cases by increasing the strength of selection (Ghalambor et al., 2007). This could facilitate colonization by increasing the speed of adaptation. Experimental range shifts of the seed beetle, *Callosobruchus macuulatus*, into cooler and more variable conditions showed that heat and cold tolerance rapidly evolved, however, this adaptation was associated with maladaptive plasticity in the novel conditions which resulted in a pattern of counter-gradient variation (Leonard & Lancaster, 2020). Beetles that colonized only colder but not more variable environments expressed only adaptive plasticity and no evolved response. The RSS has temporal and spatial variation in DO levels (Chapman et al., 1999) that could be promoting rapid adaptation through maladaptive plasticity and counter-gradient variation. Multiple studies have found evidence that swamp populations of *E. neumayeri* (N) could be maladapted to their environment. One study found swamp populations have lower fecundity, reproductive investment and condition (Baltazar, 2015) while another showed no growth advantage for swamp fish over stream fish in a swamp environment (Martínez et al., 2011). While these results could be due to transgenerational epigenetic negative effects of living in a swamp, our results suggest these patterns could also be due to maladaptive plasticity in gene expression.

Additionally, we found that *E. apleurogramma* (RE) had a higher median magnitude of log2-FC for candidate-evolved DEGs relative to *E. neumayeri* (N). This is surprising given that *E. neumayeri* (N) has a longer evolutionary history in the RSS and was expected to be more divergent between high- and low-DO populations than

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E. apleurogramma (RE), which is new to the RSS. This finding could suggest that there are more migrants exchanged between populations of *E. neumayeri* than previously hypothesized. One mark–recapture study on *E. neumayeri* found that 7% of individuals dispersed from their location of capture with some individuals travelling across DO environments (Chapman et al., 1999). Indeed, we found low genetic differentiation between DO populations of both species, suggesting high gene flow between the habitats (discussed in more detail below). This finding could also give further support to the hypothesis that the observed counter-gradient variation could be contributing to the development of rapid divergence between the two populations of *E. apleurogramma* (RE).

In contrast, *E. neumayeri* (N) shows a smaller variation in plasticity between genes and a lower magnitude log2-FC for plasticity and evolutionary divergence. While plasticity can allow populations to persist in an environment long enough for selection to occur, it is hypothesized that costs of plasticity lead to decreasing levels of plasticity over time as evolutionary changes begin to take effect (Crispo, 2007). The smaller range of plasticity seen in *E. neumayeri* could suggest that the level of plasticity has been reduced by selection. The lower magnitude of plasticity could alternatively suggest that the counter-gradient variation was less strong in this species, which could explain the smaller evolutionary divergence between the two populations. In tree sparrows (*Passer montanus*), the amount of genetic divergence between populations experiencing varying oxygen environments due to altitude depends on the magnitude of counter-gradient variation (She et al., 2023). As previously mentioned, cluster results suggest that *E. apleurogramma* (RE) may be experiencing stronger counter-gradient variation than *E. neumayeri*, which could result in more divergence between populations.

4.4 | Genetic signatures of local adaptation in native species

We identified SNPs from our gene expression data and used these SNPs to assess whole genome genetic differentiation and identify signatures of local adaptation. We found that genetic differentiation between DO populations of both species was low, suggesting high gene flow between these habitats. It is not surprising that our results indicate high gene flow given the close distance between our two sample sites. Additionally, a mark–recapture study done on *E. neumayeri* showed that there was migration of individuals across DO habitats, supporting our finding of high gene flow (Chapman et al., 1999). However, other genetic results have suggested low gene flow and divergent selection between DO environments in *E. neumayeri* (Chapman et al., 1999; Harniman et al., 2013). These studies used only two loci each, while in our study we calculated whole genome differentiation which is likely to include some sites that are differentiated and many that are not. Additionally, there is reason to expect that widespread purifying selection in mRNA can decrease estimates of genetic differentiation relative to estimates generated using microsatellites or other genetic data (Hershberg & Petrov, 2008; Smith et al., 2013). One study that directly compared genetic differentiation calculated using mRNA SNPs to microsatellites found that estimates using mRNA SNPs were approximately 40% lower (Thorstensen et al., 2021).

Due to previous studies showing phenotypic divergence between habitats (Chapman, 2007; Chapman et al., 1999; Martinez et al., 2004; Olowo & Chapman, 1996), we expected to find signs of divergent selection and local adaptation. Despite low genetic differentiation between populations, we identified many outlier SNPs that could be under natural selection and involved in local adaptation. Increasingly, studies are finding that local adaptation can occur over ecological timescales and in the absence of population isolation (Butlin et al., 2014; Kinnison & Hendry, 2001; Papadopulos et al., 2014). Important phenotypic divergence can even be found among populations that do not show differences in neutral genetic polymorphisms (Karhu et al., 1996; Rheindt et al., 2011; Sæther et al., 2007). Therefore, our outlier SNPs, along with previously discussed results showing phenotypic divergence, suggest local adaptation between DO populations despite high gene flow. Our GO analysis showed that outlier SNPs were involved in processes that are likely tied to DO adaptation (discussed in more detail below), further suggesting there is local adaptation between populations. Additionally, we found significantly more outlier SNPs in the native species than the RE species, which supports our prediction that the native species would be more locally adapted due to a longer evolutionary history in this habitat.

4.5 | Gene clustering and identification of genes related to hypoxia responses

Using soft clustering, we identified two clusters of interest for *E. neumayeri* and one cluster of interest for *E. apleurogramma* that had expression profiles, suggesting involvement in plastic responses to DO levels. The GO analysis of these clusters of interest identified genes involved in responses to hypoxia for both species that are upregulated in the low-DO, immediate samples. In mammals, research on hypoxia has identified the hypoxia-inducible factor (HIF) that regulates gene expression cascades in response to lower oxygen levels (Nikinmaa & Rees, 2005). HIF-mediated gene expression is oxygen sensitive due in part to the degradation of the HIF-α subunit that is mediated by an oxygen-dependent degradation (ODD) domain. In this domain, specific proline residues are hydroxylated and then degraded under normoxic conditions. Under hypoxic conditions, hydroxylation does not occur and HIF-α accumulates and then binds to promoter or enhancer regions of hypoxia-inducible genes. Interestingly, there was upregulation of genes involved in protein hydroxylation or proline hydroxylation and in genes related to 4-hydroxyproline metabolic processes in both species. This may be evidence that these species utilize different oxygen-dependent steps in HIF gene expression pathways. Alternatively, these shifts in expression could represent mechanisms to reduce the impact of hypoxia. Previous studies have

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found that HIF pathways are less activated in human populations that are adapted to high elevation compared with populations at sea level (Storz, 2021). Therefore, it is possible that the fish show adaptation to low-DO that allows for the suppression of HIF pathways. Other gene groups known to be involved in HIF gene expression cascades were found to be significantly enriched as well. In *E. apleurogramma*, genes related to the regulation of CAMKK-AMPK signalling cascade were upregulated under low-DO, which has been previously found to be upregulated in gill tissue under hypoxic stress (Ren et al., 2022). There was also an increase in expression of genes related to responses to nitric oxide, which mediates vasodilation to help deliver more oxygen to tissues (Ho et al., 2012).

In *E. neumayeri*, we also identified a cluster containing genes that were downregulated in L-I samples relative to the samples under normoxic conditions. Most of the significant GO terms in this cluster were related to immune and defence responses. Downregulation of immune-related genes under hypoxia stress has also been found in zebrafish (*Danio rerio*) (van der Meer et al., 2005), tilapia (*Oreochromis niloticus*) (Li et al., 2017), and large yellow croaker (*Larimichthys crocea*) (Mu et al., 2020) in various tissues including in gill tissue, which could be especially detrimental to the health of fish experiencing hypoxic conditions due to gill tissue being a primary barrier to pathogens.

We also ran GO analysis on genes that contained outlier SNPS and again found many genes likely involved in hypoxia adaptations. Genes involved in protein modifications and metabolic processes were implicated in both species and genes involved in immune function, in *E. apleurogramma*. It is unknown what functional impact these SNPs have but they could be tied to DO adaptations in similar ways as the previously discussed shifts in gene expression and may represent local adaptation between DO populations. Additionally, outlier SNPs were also found in genes involved in heart function in both species. Low-DO is known to have significant impacts on heart function, with fish exposed to hypoxia often developing bradycardia (Furimsky et al., 2003; Gehrke & Fielder, 1987; Rantin et al., 1993). These outlier SNPs could therefore be involved in key adaptations that adjust heart function to survive in a hypoxic environment, as has been shown in many other fish species (Stecyk, 2017). Other outlier SNPs in *E. apleurogramma* were involved in the regulation of reactive oxygen species, which are known to accumulate under hypoxia and could play a role in regulating HIFs (Kietzmann & Görlach, 2005). Several *E. neumayeri* outlier SNPs are involved in muscle development, which has also been shown to be affected by hypoxia (Gracey et al., 2001; Martínez et al., 2011).

4.6 | Future directions

This study adds to the growing evidence that counter-gradient variation in gene expression plays a role in the early stages of colonization; however, there are several important issues that should be addressed in future research. One limitation is that we are assuming that the gene expression patterns displayed between the H-A vs the L-A comparison represent heritable differences between the two populations because they persist after the acclimation trial to a common DO environment. However, it is possible that this comparison also includes irreversible developmental plasticity. Future studies could disentangle levels of developmental plasticity from evolved divergence by raising multiple generations under acclimation trials. Additionally, work on developing analytical frameworks to quantify co-gradient and counter-gradient variation suggests that the best experimental design to decipher between the two is a reciprocal transplant design where individuals are exposed to both environments (Albecker et al., 2022). Low-DO acclimations are logistically difficult to run at field stations, however, to confirm whether there is indeed counter-gradient variation in these species, a future study should run the acclimation study in both low-DO and high-DO and try to apply these new analytical techniques. As previously mentioned, measuring plasticity at the phenotypic level would also further distinguish counter-gradient from co-gradient variation by determining which changes in gene expression result in divergent phenotypes and what the adaptive consequences are. Another limitation is that the acclimation used in this study represents a shift in only one environmental parameter, whereas the low-DO and high-DO environments likely vary in many biotic and abiotic conditions that could covary with DO. While this study focuses on plastic and adaptive responses to DO, adaptation to these different environments likely requires plasticity or local adaptation in a suite of traits that may not be directly impacted by DO. Future research could study multiple overlapping environmental parameters to obtain a more comprehensive understanding of the relationship between adaptive divergence and plasticity.

4.7 | Conclusion

In this study, we described gene expression responses to hypoxia in two fish species and compared plastic to candidate-evolved changes. We found that plastic changes mostly occur in different genes from evolutionary divergence and uncovered evidence suggesting counter-gradient variation in plasticity and evolved divergence in both a recently RE and long-established species. This counter-gradient variation might be due to maladaptive plasticity that is being genetically compensated for. We suggest that plasticity may not need to be adaptive to facilitate colonization of novel environments; maladaptive plasticity could also aid colonization by increasing the strength of selection and promoting rapid adaptive genetic divergence. This study provides insight into how phenotypic plasticity and genetic divergence interact to shape populations diverging across varying environments.

AUTHOR CONTRIBUTIONS

Janay Fox carried out laboratory work, analysed data, and wrote the manuscript. David Hunt carried out fieldwork and gave feedback

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on the manuscript. Andrew Hendry, Lauren Chapman, and Rowan Barrett conceptualized the experimental design, provided funding, and gave feedback on the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors of this paper do not report any conflicts of interest.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI Sequence Read Archive (SRA) repository, accession no.: PRJNA1041828. Full GO results and tables with gene counts, log2FC, and FDR-corrected *p*-values are available in the supplemental materials.

ORCID

Janay A. Fox https://orcid.org/0000-0002-7895-2357
David A. G. A. Hunt https://orcid.org/0000-0002-7771-8569
Andrew P. Hendry https://orcid.org/0000-0002-4807-6667
Lauren J. Chapman https://orcid.org/0000-0002-2802-5889
Rowan D. H. Barrett https://orcid.org/0000-0003-3044-2531

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