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Are we there yet? Inter- and intraspecific approaches to evaluating phenotypic optima in a range expanding East African fish, *Enteromius apleurogramma* (Cyprinidae)

DAVID A.G.A HUNT*, ANDREW P. HENDRY and LAUREN J. CHAPMAN

Department of Biology and Redpath Museum, Stewart Biology Building, McGill University, 1205 Av du Docteur Penfield, Montreal, QC H3A 1B1, Canada

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We explored how range expansion of freshwater fishes coincident with climate warming is affected by, and then in turn affects, responses to a second environmental gradient; dissolved oxygen. Traits related to hypoxia tolerance, specifically various metrics of gill size and geometric morphometric proxies of gill size were quantified for a range-expanding cyprinid fish (*Enteromius apleurogramma*) in both its historical and novel ranges in the Mpanga River drainage of Uganda, East Africa. We found that *E. apleurogramma* followed patterns previously established in the congener *E. neumayeri*. Gill filament length and some other metrics were strongly divergent in long-established populations of both *E. apleurogramma* and *E. neumayeri*, with larger gills in hypoxic populations compared to normoxic ones. Range-expanding populations were intermediate to the two long-established populations, but divergent between themselves. Other gill traits such as filament number were weakly or not divergent. Furthermore, we show that grosser morphological traits such as opercular area can be successfully used as a proxy for gill size, both by direct measurement as well as using geometric morphometric techniques. Finally, we show that both parapatric conspecific populations and sympatric heterospecific populations can be used as reference points to approximate the 'target' of adaptation to hypoxic conditions.

ADDITIONAL KEYWORDS: cyprinid – *Enteromius* – geometric morphometrics – gill traits – interspecific comparison – intraspecific comparison – rapid evolution – range expansion.

INTRODUCTION

The only constant in biological systems is change: whether because the underlying environment is changing, such as due to climate change, or because the organisms present in an environment are changing, as with invasive species. Crucially, populations rarely have the chance to fully adapt to new environments before the environment changes again (Brady *et al.*, 2019). Although historical accounts of evolution conceptualized it as a glacially slow process, more recent work has focused on both the potential and actual rapidity of evolution (Carroll *et al.*, 2007; Sanderson *et al.*, 2022). This 'contemporary evolution' is thought to be fuelled by the large reserve of genetic variation that many (but not all) populations have at their disposal (Hendry *et al.*, 2018; Bonnet *et al.*, 2022). Further, rapid phenotypic change can originate from phenotypic plasticity, although this plasticity can be limited in its capacity to result in change, with the long-term trend in a system generally being a mosaic of 'fast' and 'slow' plastic and evolutionary dynamics (Gilchrist *et al.*, 2001; Gilchrist & Huey, 2004). However, a variety of constraints, for instance insufficient time in the case of invasive or range-expanding species; a rapidly changing environment; or Red Queen (*sensu* Lively *et al.*, 1990) dynamics, means that the phenotypic result will almost always be short of optimal trait values, even when occurring at a rapid pace (Brady *et al.*, 2019). The resulting imperfectly adapted populations can be used to further the understanding of the pace and mechanisms of adaption to changing environments.

A common case of this change is with species that are introduced into new ranges or that rapidly shift their existing ranges due to natural or anthropogenic forces. In such cases, they might have to drastically

*Corresponding author. E-mail: david.aga.hunt@gmail.com

alter their traits to thrive or even survive under the new biotic or abiotic conditions (Szűcs *et al.*, 2017; Lustenhouwer *et al.*, 2018). Furthermore, even if the general environment is analogous to other portions of their range, specific circumstances will almost certainly differ; responses will need to account for these novel conditions. For instance, species in their historical range might show diverse local adaptation to a matrix of different habitats (e.g. local predators and prey and/or abiotic extremes); yet the individuals encountering the novel conditions will almost necessarily be derived from only a subset of the potential source populations (Lewis *et al.*, 2016). Therefore, as the new populations establish and grow, they must often respond essentially *de novo* to local conditions, even if those conditions are closely analogous to their home range. In addition, the unique conditions of a range expansion will often come with their own set of trade-offs. For instance, selection under range expansion often favours fast dispersal and rapid reproduction (Burton *et al.*, 2010; Phillips *et al.*, 2010), forces that might not be as strong or even present at all in the historical ranges. In short, even when expanding into apparently similar habitats, substantial phenotypic change, including divergence between populations in the similar habitats, can occur and should even be expected. However, the speed at which this change can occur is still unclear, or even how linear such change will be; initially rapid change can be followed by a period of further slow adaptation.

In novel environments, the force of selection can be quite strong (Endler, 1986; Siepielski *et al.*, 2011, 2013; Hendry, 2017), which in combination with phenotypic plasticity presumably drives rapid phenotypic change (Sanderson *et al.*, 2022). However, the precise speed with which phenotypic traits converge on their corresponding optima in a given set of conditions is still uncertain. Indeed, rates of phenotypic change can be constrained by the availability of standing genetic variation, capacity for plasticity, population size and gene flow (Lenormand, 2002). Furthermore, the paths of traits through fitness landscapes can be circuitous (Arnold *et al.*, 2001), and even non-circuitous and unconstrained change can take a substantial period of time to reach a final optimum (Clegg *et al.*, 2008; Hunt *et al.*, 2008). The problem here lies in predicting direction and rates of change, but it is rare that the 'target' of adaption is apparent, since the new optimum for a trait can be difficult to identify or infer. Thus, it is often difficult to conclusively answer fundamental questions about adaptation in new environments except in retrospect: are populations following circuitous or direct trajectories? Are they 10% of the way to their 'target', or 90%? Yet despite the difficulty, or perhaps even because of it, these questions are fundamentally important for answering pertinent and timely topics regarding evolution and conservation, such as the invasiveness and impact of organisms spreading and adapting to new environments.

With those (substantial) caveats, one way to estimate the new optimum for a population colonizing a new environment is through comparisons to similar populations of conspecifics or closely related species in similar circumstances. Put another way, assuming that parallel or convergent evolution (*sensu* Bolnick *et al.*, 2018) is strong for similar gene pools in similar environments, the 'target' can be approximated by looking at similar populations in similar environments (Gilchrist *et al.*, 2004; Hunt *et al.*, 2008; Marques *et al.*, 2016). Of course, the underlying circumstances and capacity for adaptation of even putatively identical populations will almost certainly differ, even if just due to the role of chance, sometimes leading to noteworthy non-parallel evolution (Stuart *et al.*, 2014; Oke *et al.*, 2017), or even 'mosaics' of parallel and non-parallel adaptations within not just populations but even within the genome of individual organisms (Manousaki *et al.*, 2013). Although we must keep these complications in mind, the phenotypes of long adapted populations in particular environments can be considered to at least represent a reasonable approximation for locally optimal 'target' phenotypes for populations recently colonizing an analogous environment (Brady *et al.*, 2019). In other words, although it is unlikely long-adapted (i.e. long-established) populations are 'perfectly' adapted (Mustonen & Lässig, 2009), they can serve as a 'reference point' to approximate the magnitude of evolutionary change required to arrive at a new optimum. We will apply this approach to a serendipitous natural 'experiment' revealed by our long-term monitoring of fish communities in Uganda, East Africa.

Study system

Our work centres on the strong evolutionary pressure that variation in dissolved oxygen (DO) places on fishes. The availability of DO is a key evolutionary pressure in fishes because (1) all fish require sufficient DO for survival, and (2) DO can be extremely limiting. Even under ideal saturated conditions, water holds compared to air approximately 30 times less available oxygen per unit volume and diffuses four orders of magnitude slower (Nikinmaa, 2001). In addition, oxygen concentration is much more variable in aquatic environments than in terrestrial systems with even normally well-oxygenated sites occasionally suffering from DO depletion (Chapman, 2015). Hypoxia (defined as DO < 2 mg O_2 L^{-1}) (Vaquer-Sunyer & Duarte, 2008) can form in spatial or temporal locations with reduced inflow, low light and/or high biochemical oxygen demand, such as in heavily vegetated swamps, the profundal waters of lakes during stratification

periods or during seasonal drought. Gradients in environmental DO thus impose extremely strong selective pressures, often leading to divergence in phenotypic traits of fishes related to oxygen update and/or delivery (reviewed in Chapman, 2007, 2015).

In East and Central Africa, hypoxia is very widespread in wetlands dominated by the emergent sedge papyrus (*Cyperus papyrus*) (Chapman & Liem, 1995). The papyrus stems often average 3–4 m in height, and the terminal umbels form a canopy that creates cool, dark conditions and limits mixing, precipitating very low DO conditions. Fish that live in both hypoxic swamps and well-oxygenated tributary streams show strong adaptive divergence between the high and low DO populations in key traits related to DO uptake. For instance, in the cyprinid *Enteromius neumayeri*, populations from hypoxic swamps have larger gill filaments (Chapman *et al.*, 1999) and larger gills and heads (Schaack & Chapman, 2003; Langerhans *et al.*, 2007), as well as constant changes in certain biochemical traits such as lactate dehydrogenase (LDH) levels and inducible changes in other biochemical traits such as phosphofructokinase (PFK) levels (Martínez *et al.*, 2011). All of these traits are known in the literature to be correlated with the ability to survive under hypoxic conditions (Chapman, 2015). However, these adaptations to low DO are not without cost. For example, *E. neumayeri* with large gills have reduced feeding performance when compared to smaller-gilled conspecifics (Schaack & Chapman, 2003).

In this paper, we seek to understand how a range-expanding species (*Enteromius apleurogramma*—see Material and Methods for details) is shifting its phenotype toward a new optimum necessitated by the low DO conditions in its new range. This new optimum can be approximated by two reference points: the phenotypes of a sympatric congener (*Enteromius neumayeri*) historically present in the new range across the DO gradients between hypoxic swamps and associated normoxic streams, as well as the phenotypes of parapatric *E. apleurogramma* in its historical range where it occupies analogous DO gradients across swamps and associated streams. We do not think them fully isolated, i.e. allopatric, since the range is presumptively continuous and only partially isolated by distance alone, even if this classification is only roughly useful at best (Butlin *et al.*, 2008). This new co-habitation provides an opportunity to examine how rapidly a colonizing species might be approaching its phenotypic optimum in a novel location. Given the geography of the system, the putative source for the invading *E. apleurogramma* is a downstream normoxic (river) population and so we expect that local adaptation to the hypoxic conditions will need to proceed anew.

This opportunity leads to four distinct questions. First (Q1), previous work in *E. neumayeri* (Langerhans *et al.*, 2007) has established that the variation in specific gill structures tightly linked to oxygen uptake, such as gill filament length and hemibranch area, is well correlated with variation in environmental DO; this work can be replicated in *E. apleurogramma* in both its historical range as well as its expanding range, and then compared to *E. neumayeri* populations sympatric to the range expanding *E. apleurogramma* populations to see if patterns are similar across species and range shifts. Second (Q2), the same previous work in *E. neumayeri* also showed the link between those relatively directly functional traits and more coarse morphological traits evaluated using the much more rapid method of geometric morphometrics. We ask if a similar pattern hold true in *E. apleurogramma*. Third (Q3), do those coarser traits show expected patterns of divergence across DO gradients. Finally (Q4), we can ask how well an interspecific (but sympatric) reference point predicts trait divergence when compared to an intraspecific (but parapatric) reference point.

MATERIAL AND METHODS

Description of study species

Enteromius neumayeri (Fischer, 1884) and *Enteromius apleurogramma* (Boulenger, 1911) are members of the family Cyprinidae (subfamily Barbinae) native to freshwater streams in tropical East Africa. Until recently, both species were classified within the genus *Barbus*, but have since been reclassified (Yang *et al.*, 2015; Skelton, 2016; Hayes & Armbruster, 2017). Both are widely distributed within East Africa (Greenwood, 1962; Skelton *et al.*, 1991), and both species are found within stream and swamp habitats within their respective ranges, where they are known to be tolerant of hypoxic conditions (Olowo & Chapman, 1996; Chapman *et al.*, 1999; Rosenberger & Chapman, 2000; Langerhans *et al.*, 2007). *Enteromius apleurogramma* reach approximately 6 cm standard length; *E. neumayeri* reach a larger maximum size of 10 cm standard length. Detailed biometrics for fish in the present study can be found in Supporting Information, Tables S3–S4. *Enteromius* spp. generally feed upon small insect larvae, aquatic plants and detritus (Corbet, 1961; Chapman, 2003).

The most prominent species identification criteria are the overall darker coloration of *E. apleurogramma* and the lines or elongated dots of darker pigmentation along the lateral line of *E. neumayeri* (Fig. 1). Additional identification criteria include: one fewer pair of barbels in *E. apleurogramma* and intensely red and black coloured fins in *E. apleurogramma* (Greenwood, 1966).

Figure 1. Top: *Enteromius apleurogramma* (Boulenger, 1911) and bottom: *Enteromius neumayeri* (Fischer, 1884). Background grids are 17 mm on each side.

Description of study site

All specimens were collected in or near Kibale National Park, Uganda. The park is located in western Uganda (Fig. 2) and is mostly at 1400+ m above sea level in the foothills of the Rwenzori Mountains that form part of the border between Uganda and the Democratic Republic of the Congo. The park is mostly covered by moist mature evergreen forests, portions of which were logged approximately five decades ago. Smaller portions of the park are comprised of wetlands, open grasslands, former pine and cypress plantations, and intermediate zones between the above (Duclos *et al.*, 2013). The park is drained by two watersheds, the Mpanga and the Dura rivers, both of which ultimately drain to Lake George south of the park. There are two wet and two dry seasons, with May to August and December to February generally being drier than other portions of the year.

Our main study sites were the Rwembaita Swamp system (00.58875 °N, 030.37222 °E), a papyrus swamp (~4.6 km in length and ~50–200 m wide) within the park boundaries, as well as the Bwera Swamp system (00.36510 °N, 030.58256 °E), another large papyrus swamp (~23 km long and ~50–1500 m wide) located outside the park boundaries to the south. Both are dominated by *Cyperus papyrus* (papyrus) reaching 4+ m in height. In these swamps, the low water flow and mixing as well as shade from *C. papyrus* combined with high organic matter input and attendant decay result in low DO (~1 mg/L or less). Both swamps have associated streams and rivers, which have much greater (~6 mg/L) average DO levels due to flow-induced oxygenation.

While records for the Bwera Swamp system are sporadic at best, in the Rwembaita Swamp system (Fig. 2) DO levels have been recorded monthly or bi-monthly since the early 1990s. The Rwembaita Swamp is fed by four major streams and drains into the Njuguta River, a tributary of the Mpanga River system (Fig. 2). Chapman *et al.* (2022) reported DO at a site in the middle of the swamp averaging 0.99 mg/L over a 26-year period (1993 to 2019), whereas the inflowing tributary Mikana Stream has averaged 5.8 mg/L near its confluence with the swamp.

Across this system, the small cyprinid fish *Enteromius neumayeri* shows strong patterns of divergence between low-DO swamp habitats and high-DO tributaries. These well-adapted populations generally exchange few migrants between hypoxic and normoxic populations that are separated by only short distances (Baltazar, 2015). This results in genetic structure to the system, with low gene flow between areas of differing DO regimes (Chapman *et al.*, 1999; Harniman *et al.*, 2013). This isolation allows for highly

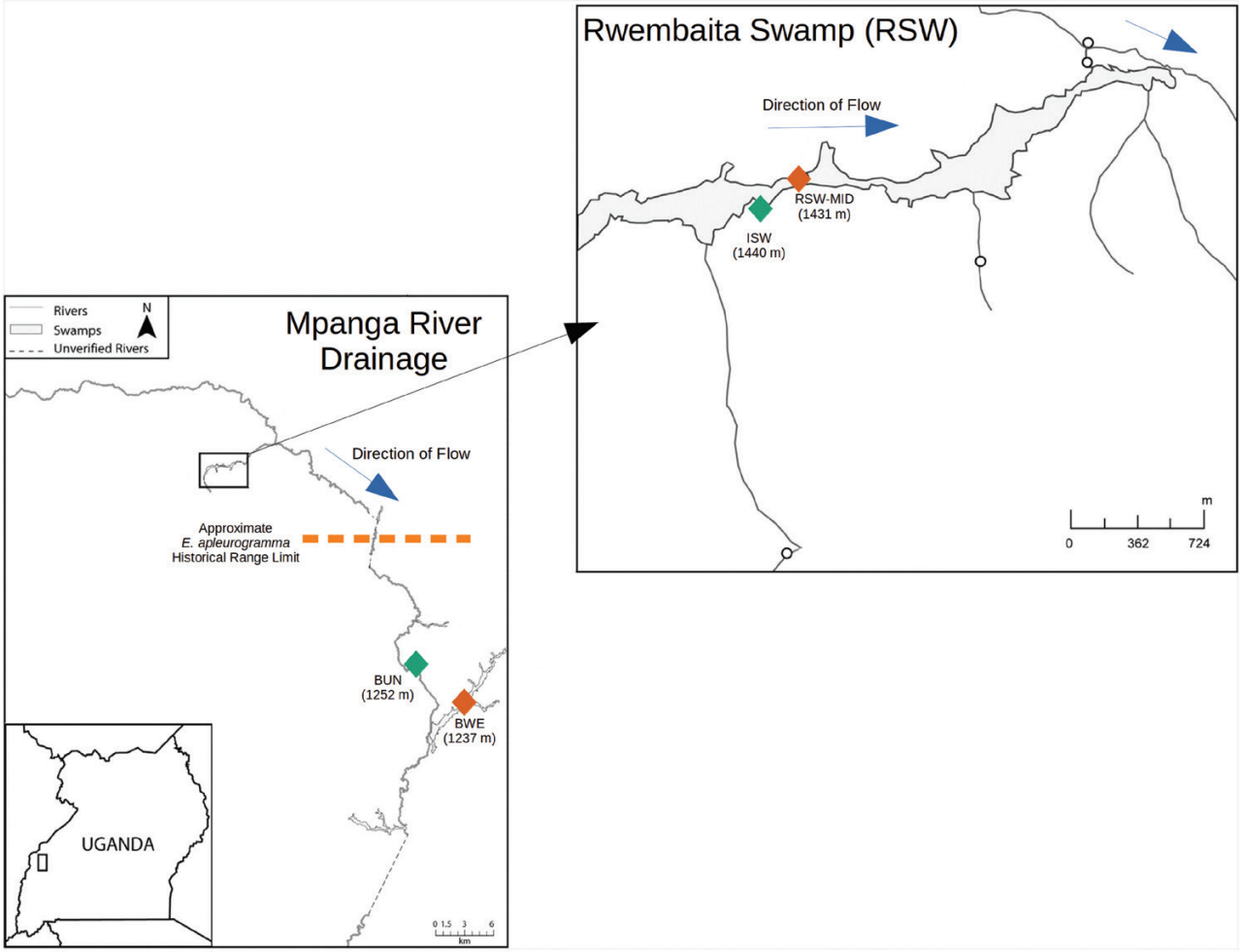

Figure 2. Map of study system. Hypoxic swamp sites are identified in orange, normoxic stream sites are identified in green. Letter codes on the map are arbitrary site names. Sites are referenced in text by their location and environmental characteristics.

local adaptation, as migration between DO regimes would otherwise homogenize adaptive genetic change.

Historically, *E. neumayeri* was the only cyprinid species present in the Rwembaita Swamp system (Chapman *et al.*, 1995). However, *E. apleurogramma* was first observed in the Rwembaita Swamp system in 2015 and has since spread throughout the entire swamp and tributary streams (L. Chapman, unpub. data). It is one of three native 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). The non-native *Poecilia reticulata* has also been introduced to the system (first observed in 2012). We should note that between 1994 and 2016, there has been an increase in water temperature in the Rwembaita Swamp of 1.41 °C, an increase that aligns well with local air temperature increases of 1.45 °C over the same period (L. Chapman, unpub. data). Unfortunately, long-term temperature data for the lower elevation sites are not available. However, the historical *E. apleurogramma* populations are almost exactly 200 m lower in elevation; at a standard atmospheric lapse rate of 6.50 °C/ km (International Civil Aviation Organization, 1993), this would predict an average difference in temperature between the sites of approximately 1.3 °C, although forest cover differences inside and outside the park are likely to also affect temperature, accentuating this difference. Thus, climate warming has likely facilitated or driven range shifts, though there may be other contributing factors. Since fish are often at the upper limit of their thermal niche (McDonnell & Chapman, 2015), even this small change in water temperature could pose a significant challenge for them.

Collection techniques

All collections and post-collection procedures were carried out in accordance with our approved animal care protocol (McGill University animal care protocol # MCGL-5029) and relevant legislation in both Canada and Uganda.

Fish were collected using barrel minnow traps with a mesh size of 6.35 mm square and throat openings of 25.4 mm (Gees Feets model G-40). Traps were baited with bread (home-made Ugandan style chapati). Traps were placed by hand in established monitoring sites with expert judgment used to maximize catch of the target species. Trapping was continued until a sample size of 40 individuals for each site and species combination was obtained, or no more fish could be caught at that site.

Gill analysis (Q1 and Q2)

For *E. apleurogramma*, the left side of each fish was photographed using a dissecting microscope with an attached camera (Lumenera Scientific Infinity). Gills from the same side of the fish were dissected out of the branchial basket and the gill arches (hemibranches) separated. Each side of the four arches was photographed using the same camera and microscope as above. The photos were examined using ImageJ v.1.50i to quantify various metrics measured to the nearest 0.001 mm or 0.01 mm^2. From pre-dissection photos, opercular area and subopercular area were measured on the left side as single value for each fish. From post-dissection photos, gill hemibranch area, total filament length and total filament number were obtained as two values for each of the four gill arches for each fish (one from each of the two hemibranchs on an arch) before being summed. Total filament length was not measured by each individual filament; rather every fifth filament was measured, and the average between measured filaments used for the four in between. Data for *E. neumayeri* were derived from fish collected in 2003 (collection protocol described in Langerhans *et al.*, 2007); gill metrics were measured following the identical protocol used here for *E. apleurogramma*. See Fig. 3 in Langerhans *et al.* (2007) for an illustration of the gill metrics.

All statistical transformations and analyses were done using R v.4.0.5 (R Core Team, 2018). All gill metrics were common logarithm (i.e. log 10) transformed and standardized to the mean body mass of the samples in the analysis according to the formula below (Reist, 1986; see Hendry & Taylor, 2004) where β is the slope of the decimal logarithm transformed trait value by decimal logarithm transformed mass, incorporating population (and thus DO environment) origin as a non-interacting co-variate, i.e. the β used for standardization is a common within-group slope, generated from a linear model with varying intercepts by population.

$$Traitvalue_{adj} = Traitvalue_{raw} \times \left(\frac{mass_{mean}}{mass_{individual}}\right)^{\beta}$$

The analysis was first run using only *E. apleurogramma* samples for gill filament length, hemibranch area, filament number and opercular area. The analysis was then run again for both *E. apleurogramma* and *E. neumayeri* data, but only for gill filament length. Since the samples are standardized to a mean mass of all samples in a given analysis, the same samples may have slightly different values in the two analyses.

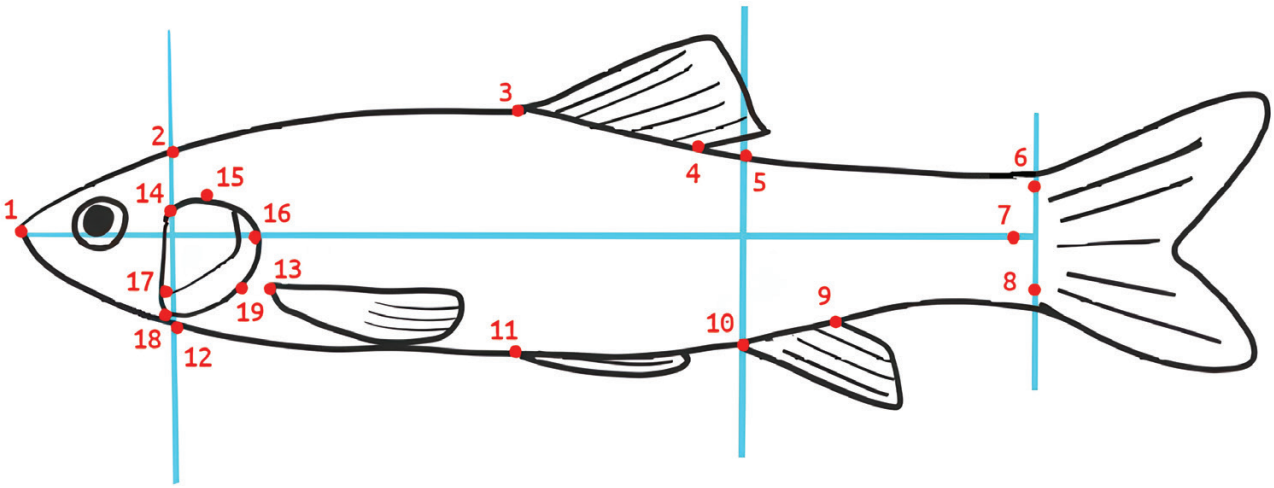

Figure 3. Landmarks for geometric morphometrics of *E. neumayeri* and *E. apleurogramma*. In addition to the general body shape landmarks (points 1–13), the operculum is characterized in detail (points 14–19). Landmarks 2 and 5 are sliding landmarks; they are generated by first establishing a straight line along the length of the fish (in blue), and then setting two more lines orthogonal to that line which intersect point 14 (for point 2) and point 10 (for point 5).

Mean group values were calculated using the package emmeans v.1.8.4-1 (Lenth, 2022), and Tukey-adjusted *P*-values were calculated using the package multcomp v.1.4-20 (Hothorn *et al.*, 2008). For further detail, R code is presented in D4.

Mass adjusted total gill filament lengths were compared using a Pearson correlation coefficient to opercular area values to see how well the two metrics measured the same trait (i.e. gill size). This was done using the cor.test() function in base R (R Core Team, 2018).

Geometric morphometrics (Q3 and Q4)

All fish were euthanized with clove oil before being photographed with their left side visible on a 17 mm square grid with a ruler visible for additional reference. Attempts were made to fully splay the fins prior to photography. All photography was done using a Nikon Coolpix S9100 from a distance of 90 mm to the background in natural light. Examples can be seen in Figure 1. Landmarks were digitized using tpsDig2 v.2.31 following the guide given in Figure 3 and saved as individual .tps files before being later combined into a single file for import into R.

All statistical analyses were done using R v.4.0.5 (R Core Team, 2018). Geometric morphometric analyses were done using the packages geomorph v.4.0.0 (Adams *et al.*, 2021), shapes v.1.2.6 (Dryden, 2021) and Morpho v.2.8 (Schlager, 2017). All visualization was done using ggplot2 v. 3.3.1 (Wickham, 2016) or base R (R Core Team, 2018).

Detailed R code is available as D3 via Zenodo, but in short, the methodology of Zelditch *et al.* (2012) was followed. First, landmark files were imported into R and then a Procrustes superimposition was performed where all samples are aligned and standardized to a common size, retaining shape information as well as providing a separate metric of size (centroid size). We used the resulting shape data in a Principal Component Analysis (PCA) [using the gm.prcomp function from the package geomorph v.4.0.0 (Adams *et al.*, 2021)], which isolates independent (i.e. orthogonal) axes of variation through the data (which has dimensions equal to the number of landmarks multiplied by the number of original spatial dimensions, i.e. 38 in this case: 19 × 2). The shape data post-PCA can also be used in a Linear Discriminant Analysis (LDA) [using the lda() function from the package MASS v.7.3-58 (Venables & Ripley, 2002)]. An LDA uses a training dataset and an applied dataset, where the data in the training set is used to establish a linear function to discriminate between two (or more) a priori groups (in this case, hypoxic and normoxic populations). The formula is then applied to a new dataset, and one or more LD scores is generated for each sample in the dataset.

The above procedure was performed on two combinations of the data: the *E. apleurogramma* data alone from both its expanding and historical ranges; as well as the *E. apleurogramma* data from its expanding range combined with the *E. neumayeri* data from its historical range. The dataset without *E. neumayeri* was used to analyse trends in *E. apleurogramma* body and operculum shape across the four sites (and thus the range expansion) as a bare PCA. It was also used in an LDA using the historical *E. apleurogramma* populations as a training dataset and the expanding populations as an applied dataset. The combination of both species was used to perform an LDA with the historical *E. neumayeri* populations as the training dataset and the expanding *E. apleurogramma* populations as an applied dataset.

RESULTS

Gill analysis (Q1 and Q2)

Mean total filament length and mean total hemibranch area in *E. apleurogramma* showed similar patterns (Fig. 4). In historical populations the fish from hypoxic swamps were characterized by a greater total gill filament length and larger gill hemibranch areas than fish from normoxic tributary streams ($P < 0.05$, exact values in Supporting Information, Table S1). The fish from the range-expanding populations were intermediate to the two historical populations, but divergent from each other in the same direction as the historical populations (i.e. larger gills in hypoxic populations). In contrast, total filament number showed no clear pattern; the historical populations were not different from each other ($P > 0.05$, exact values in Supporting Information Table S1), and the range-expanding populations were indistinguishable from the normoxic historical populations ($P > 0.05$, exact values in Supporting Information, Table S1). However, the historical hypoxic population was somewhat greater in total filament number than the range-expanding populations ($P < 0.05$, exact values in Supporting Information, Table S1). Opercular area showed a weaker pattern: the historical populations were well diverged ($P < 0.0001$), with hypoxic populations having larger opercula, but the range-expanding populations were indistinguishable from the historical hypoxic population and each other ($P > 0.05$, exact values in Supporting Information, Table S1). Opercular area and total gill filament length were positively correlated (Pearson's r = 0.6863, n = 36, $P < 0.0001$).

When both species were analysed together for total gill filament length (Fig. 5), the pattern in total gill filament length was broadly recapitulated. However, the difference between the hypoxic and normoxic

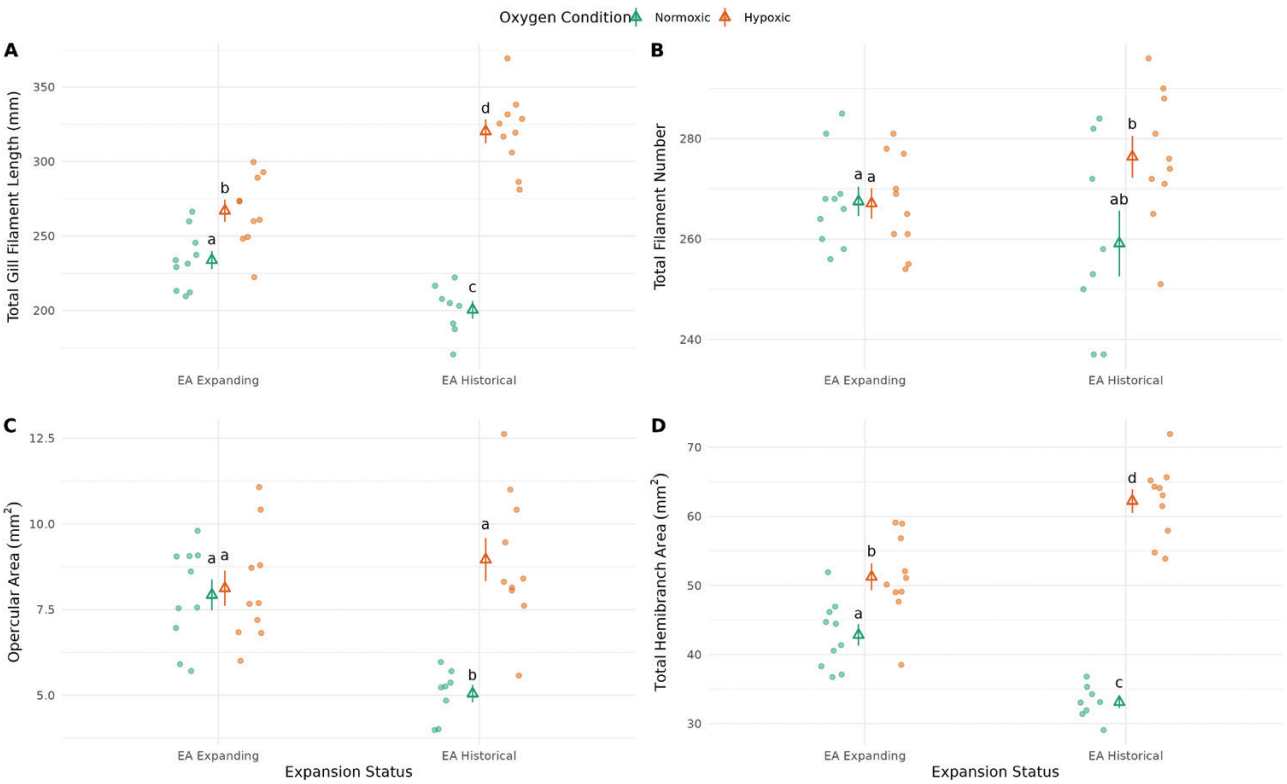

Figure 4. Detailed gill metrics of *E. apleurogramma* with normoxic and hypoxic populations from expanding and historical ranges. Panels show (A) total gill filament length (mm) per gill arch, (B) gill filament number per gill arch, (C) opercular area (mm^2), and (D) gill hemibranch area (mm^2) per gill arch. All values are standardized by mass of fish as per Reist (1986) and Hendry & Taylor (2004). Triangular points with bars are means ± SE. Within a panel, means sharing a common letter are not significantly different at $P < 0.05$. Precise P-values are presented in Supporting Information, Table S1.

populations of range-expanding *E. apleurogramma* became statistically non-significant ($P = 0.0655$) under the new standardization. Total gill filament length did not differ between the hypoxic *E. neumayeri* population and the historical hypoxic *E. apleurogramma* population ($P = 0.7668$). However, total gill filament length was greater in *E. neumayeri* from the normoxic site than *E. apleurogramma* from the normoxic site in its historical range ($P = 0.0124$). The range-expanding normoxic *E. apleurogramma* were intermediate to these two groups, with no differences from either ($P > 0.05$, exact values in Supporting Information, Table S2).

Geometric morphometrics

Principal Component 1 (PC1) (32.4% of total variation) of the analysis of *E. apleurogramma* data showed a trend mostly comprised of changes in opercular area (Fig. 6, inset). Fish from hypoxic populations had larger opercula than their paired normoxic populations (i.e. within expanding or historical ranges), but both of the historical range populations actually had even larger opercula (based on PC1 scores) than either of the range-expanding populations (Fig. 6).

The LDA trained on the historical *E. neumayeri* populations (i.e. a sympatric but interspecific comparison, Fig. 7B) showed similar results to the LDA trained on historical *E. apleurogramma* populations (i.e. a parapatric but intraspecific comparison, Fig. 7A). In the case of the *E. neumayeri* trained LDA, the population mean for the normoxic range expanding *E. apleurogramma* was the same as the mean for the normoxic training population, with the hypoxic *E. apleurogramma* population somewhat intermediate to the normoxic and hypoxic *E. neumayeri* populations but more similar to the normoxic population. In short, the normoxic populations lined up, and the range-expanding hypoxic population was 'on the way' to the hypoxic training population. However, when trained on historical *E. apleurogramma* populations, this trend was flipped in the range-expanding *E. apleurogramma* populations. In this case, the hypoxic range-expanding population had the same mean as

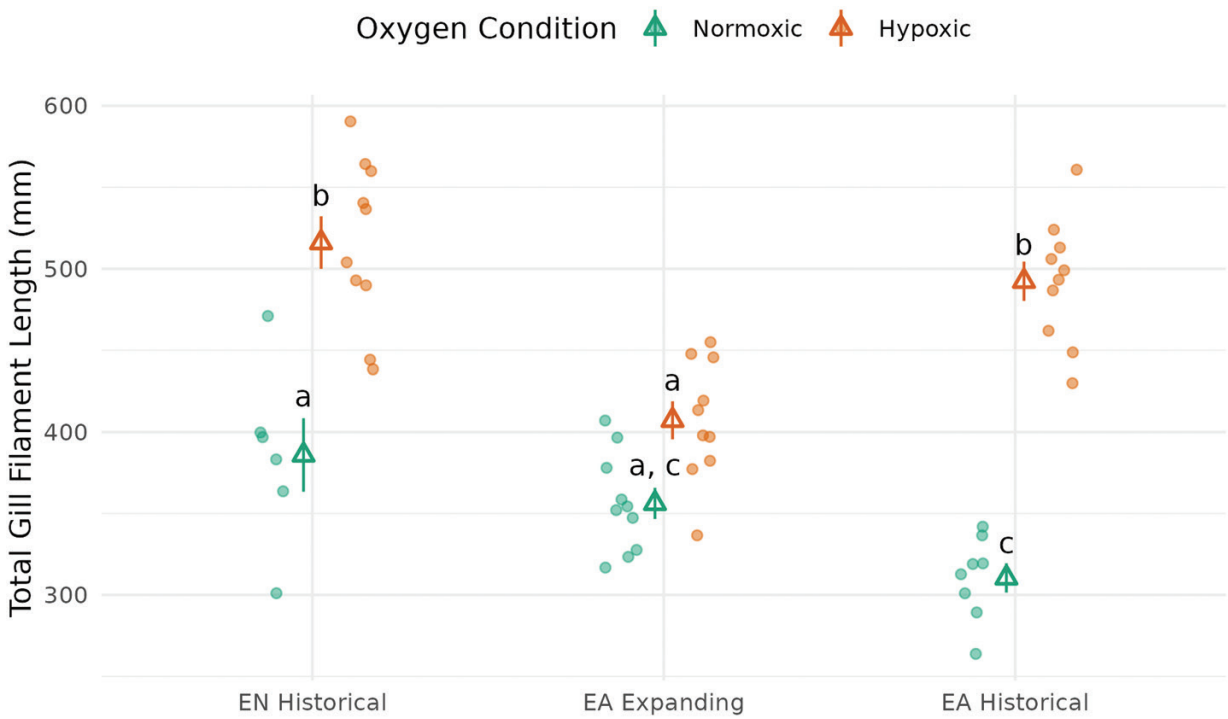

Figure 5. Comparison of total gill filament length (mm) per gill arch in *E. neumayeri* and *E. apleurogramma* from normoxic and hypoxic populations from historical and range expanding (*E. apleurogramma* only) populations. All values are standardized by mass of fish as per Reist (1986) and Hendry & Taylor (2004). Triangular points with bars are means ± SE. Within a panel, means sharing a common letter are not significantly different at $P < 0.05$. Precise *P*-values are presented in Supporting Information, Table S2.

the normoxic historical population, with the normoxic range-expanding population intermediate, but more similar to the normoxic historical population than the hypoxic one. In short, the range-expanding hypoxic and normoxic populations 'flipped position' when compared to the previous analysis.

DISCUSSION

In summary, our analysis confirmed four points. First (Q1), performance associated traits of gills were divergent across environmental DO gradients in the directions predicted from previous work (Chapman & Liem, 1995, 1999; Langerhans *et al.*, 2007) and functional considerations; *E. apleurogramma* from historical populations were well divergent, with larger gills in the hypoxic population (Fig. 4). Second (Q2), more detailed gill traits were correlated with a grosser morphological trait, namely opercular area. Third (Q3), opercular area as measured by geometric morphometrics was strongly divergent in historical *E. apleurogramma* populations in the direction expected (larger opercula in hypoxic fish), but the historical populations had even larger opercula (Fig. 6). Fourth (Q4), using an interspecific sympatric training dataset for an LDA produced results close to that produced using an intraspecific parapatric training dataset (Fig. 7).

GILL DIVERGENCE (Q1 AND Q2)

Larger gill size in hypoxic populations relative to normoxic populations mirrored previous work both in *E. neumayeri* (Chapman & Liem, 1995, 1999; Schaack *et al.*, 2003; Langerhans *et al.*, 2007) as well as in diverse other taxa (Saroglia *et al.*, 2002; Crispo & Chapman, 2010) where gill filament length was well correlated with DO gradients, but weaker or no association was found for filament number. Although the actual surface area of the gill requires integrating measures of filament length and number with lamellar area; total gill filament length has been found to correlate with gill surface area within and between species (Hughes, 1966; Chapman & Hulen, 2001; Chapman *et al.*, 2008; Fernandes, 2007). What is novel about our new analysis is the inclusion of range-expanding populations presumably not fully adapted to their new environmental conditions. The traits of these new populations were intermediate to historical

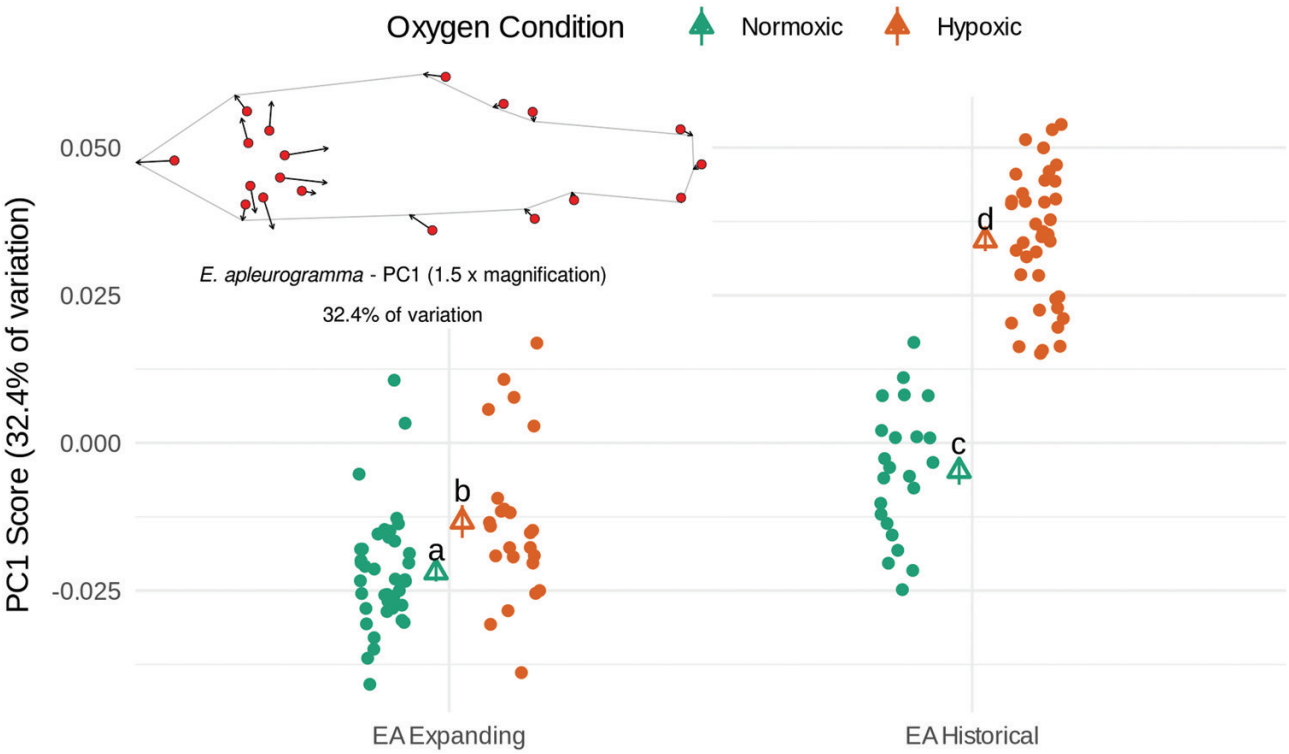

Figure 6. Principal Component 1 of a geometric morphometric analysis of *E. apleurogramma* data, with normoxic and hypoxic populations from expanding and historical ranges. Inset is a visualization of PC1; this PC mainly corresponds to opercular area. Minimum PC score corresponds to red circles, and maximum PC score to tip of arrow. Triangles and bars are mean values +/- standard error. All pairwise differences are significant (EA Expanding Normoxic vs. Hypoxic: $P = 0.0102$, all others: $P < 0.0001$).

parapatric populations of both *E. apleurogramma* as well as sympatric populations of *E. neumayeri*, but with divergence in the direction expected. Our comparison of gill size between *E. apleurogramma* and *E. neumayeri* may reflect both site and time effects—gill data for *E. neumayeri* were collected from the same sites as data for *E. apleurogramma*, but much earlier, in 2003. However, comparisons of gill size of *E. neumayeri* between low- and high-DO sites measured at various time points (Chapman & Liem, 1995 1999; Langerhans *et al.*, 2007) have revealed similar patterns of divergence between the Rwembaita Swamp and inflowing well-oxygenated streams. Based on the geography of the system (Fig. 2), it is presumed that the source population of *E. apleurogramma* for the range expansion is from a stream, and thus largely normoxic adapted. The intermediate nature of the range-expanding populations is consistent with the idea that generalist fish will be more successful in a warming world and associated range expansions (Kingsbury *et al.*, 2020; Stefani *et al.*, 2020), especially as the wavefront populations (Colautti *et al.*, 2017).

Opercular area showed a very similar trend as gill size and was well correlated with more directly performance related traits such as gill filament length. Direct measurement of opercular area or measurement of a geometric morphometrics principal component largely comprised of opercular markers gave broadly the same result: a clear differentiation between hypoxic and normoxic populations in an intuitively sensible direction (larger opercula in hypoxic fish due to larger underlying gills). However, in recently range-expanding (and thus re-adapting) populations, the direct measurements of opercular area described the range-expanding fish as intermediate to the well-established populations, regardless of oxygen condition. This validates the much more rapid (and potentially non-lethal) method of geometric morphometrics as a larger scale supplement or even substitute for the direct measurements of gill traits.

Body shape (Q3 and Q4)

PC1 of the geometric morphometric analysis (utilizing only *E. apleurogramma* populations) shows the range-expanding fish as 'even more normoxic' than well-established normoxic populations. However, PCAs are non-constrained analyses, and DO is not the only thing

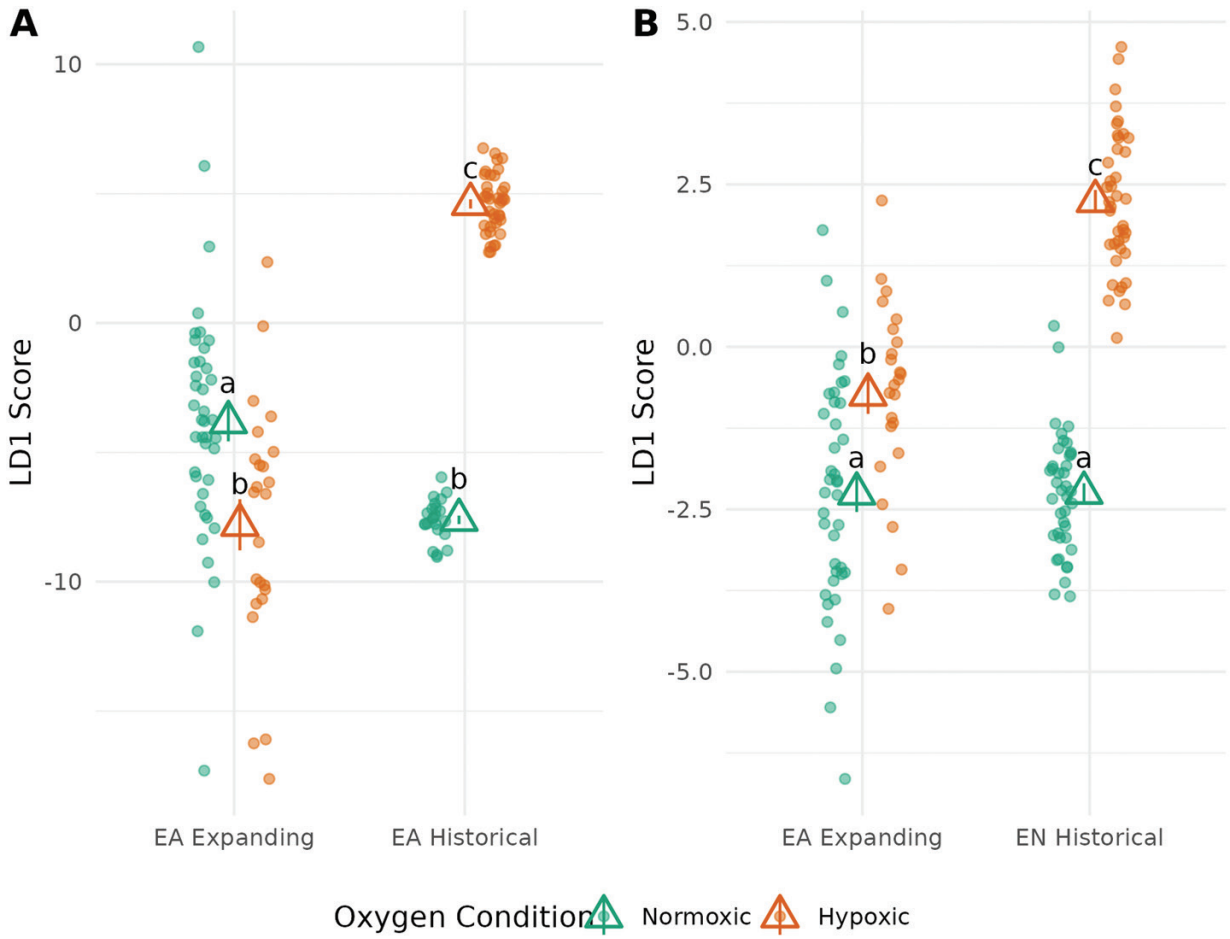

Figure 7. LDA classification of recently range-expanding *E. apleurogramma* between normoxic (stream) and hypoxic (swamp) populations, trained on data from long-adapted historical populations of (A) conspecifics from an analogous gradient from the historical range, or (B) contemporaneous congener heterospecifics (*E. neumayeri*) from the same gradient. Triangular points are means ± SE. Within a panel, means not sharing a letter in common are significantly different at $P < 0.001$.

that differs between swamp and stream sites; other environmental factors such as flow vary as well, which is known to have effects on body shape (Langerhans *et al.*, 2007).

The more focused and pertinent analysis of the geometric morphometric data is to use an LDA, with the well-established populations serving as 'reference points' and training data for the LDA. No matter if we used parapatric conspecifics or sympatric heterospecifics as the training data, the broad pattern was the same: the range-expanding fish were largely comparable to the long-established normoxic populations, but with a large variation in trait values within populations. Interestingly, training on the sympatric heterospecifics produced more intuitively expected results, with the range-expanding normoxic population the same as the well-established normoxic population, and the range-expanding hypoxic population 'on the way' to the reference point of the well-established hypoxic population. In contrast, an LDA trained on the parapatric conspecifics described the hypoxic range-expanding population as similar to the well-established normoxic one, with the range-expanding normoxic population somewhat intermediate (although still closer to the normoxic reference).

Phases of adaptation

Divergence between historical and expanding populations was generally in the direction predicted based on environmental DO; however, this system is not without its issues in analysing the effects of hypoxia specifically. The geography of the system (see Fig. 2, map) means range expanding fish will have had to move through rounds of normoxic and

hypoxic environments in order to reach sites classified as normoxic (i.e. appreciably flowing streams). Since this study considered the forefront of the wave, it is possible, even probable, that our study has captured the initial colonization by generalist phenotypes. Anthropogenic disturbance is known to result in the homogenization of fish community assemblages (Scott & Helfman, 2001), including the specific phenomenon of range expansion by generalist fish native to the same watershed, but not normally present in the upper reaches of the system. Literature posits that these shifts will result in fundamentally and permanently changed aquatic community structures, with environmental temperature and DO often being primary determinants of these shifts (Ficke *et al.*, 2007). Generalist species and phenotypes often have an advantage here, as they can better cope with these rapidly changing environments; a 2017 meta-analysis found that habitat breadth was one of the most important traits in explaining range shifts under climate change (MacLean & Beissinger, 2017). Other studies have found that generalist freshwater fish are most likely to shift their ranges under climate warming (Stefani *et al.*, 2020). Thus, our results of the range-expanding populations showing much more variable trait values than in the well-established populations is not unreasonable.

As *E. apleurogramma* move into the new habitat, we follow general expectations (Price *et al.*, 2003; Gilchrist *et al.*, 2004; Crispo, 2007; Lande, 2009; Mazzarella *et al.*, 2015; Oke *et al.*, 2016) and assume there will be three phases of adaptation to divergent DO habitats within the Rwembaita Swamp system: (1) plasticity, (2) rapid selection from standing genetic variation if present, and (3) a longer period of slow evolution working from very low-frequency variants, new mutants, introduced alleles, linkage breaking or compensation for initial trade-offs. Understanding these phases requires a brief diversion to consider generation times.

Although the specific lifespan of these species is unknown, nor their time to maturity, a single specimen of *E. neumayeri* collected during this study in 2017 showed elastomeric tags from previous work by C. Baltazar and L. Chapman in 2012, meaning they can potentially live up to 5 years. Breeding in *E. neumayeri* is synchronized with wet seasons (Chapman & Frank, 2000), and thus a 6 month generation time is supposed, with a similar 6 month time to maturity (Kambikambi *et al.*, 2021). Since the first range-expanding fish was observed in the Rwembaita Swamp in 2015, and the fish in this study were sampled in 2017 and 2018, it is likely that the population still has at least some of the original colonizers, with their original source population derived trait values. The presence of original colonizers is likely at least a partial explanation for the large variety in morphology seen in the LDA analysis, where even though the means were divergent, individual samples showed a huge range of values, including some consistent with the 'wrong' reference population. It is likely that resampling of the system (something which was prevented by the COVID-19 pandemic) at a later date would start to show less of this spread, as plasticity and rapid selection from standing variation begin to assert themselves and eliminate this spread of phenotypes.

Given the timeline, it is almost certain that the traits observed in this study fall into the first two phases described above. Regarding the first phase, some of the response to environmental change will be due to the phenotypic plasticity. Organisms may respond to novel environments partially through mechanisms such as the so-called 'Baldwin effect' (Crispo, 2007); here the initial response is due to phenotypic plasticity, and over time this phenotypic change is 'taken over' by genetic change, allowing the trait to become inherited, through a mechanism termed genetic accommodation. As an example, changes in body size in *Drosophila* can occur rapidly in new thermal environments, and is known to be based on both plastic and standing genetic variation (Gilchrist *et al.*, 2001, 2004). This plasticity will often result in populations in similar environments being more similar than their underlying genetic architecture would generally indicate, although plasticity can also actually result in less similarity (Oke *et al.*, 2016). Other research has found that, perhaps counterintuitively, adaptive plasticity actually constrains evolution, since such plastic change can obviate selection and thus adaptation, whereas non-adaptive plasticity potentiates it (Ghalambor *et al.*, 2015).

The plastic nature of fish gills in a number of species is well established, such as African cichlids (Rutjes, 2006; Chapman *et al.*, 2008; Crispo, 2010), the cyprinid *Notropis anogenus* (Potts *et al.*, 2021), as well as the sympatric comparison in this study to *E. apleurogramma*, *E. neumayeri* (Chapman, unpublished data). Thus, it is likely that plasticity has played a role in divergence of gill traits in the range expanding fish. However, our results do not contradict the hypotheses of rapid adaption: Stuart *et al.* (2014) described rapid adaptation after 20 generations. Leaver & Reimchen (2012) described a system where eight generations in fish resulted in differentiation one-third the magnitude of differentiation between long-established populations. In our study, we might only have six generations (assuming a 6-month generation time over 3 years). Furthermore, overlapping generations can buffer genetic change, even against cyclical or extended selection (Zhdanova & Frisman, 2021). In short, some of the fish we caught could still be the original adults

to enter the swamp, meaning no phenotypic change due to developmental plasticity or selection due to environment could have occurred. This buffering effect is not necessarily maladaptive, as it can contribute to retention of genetic diversity even in the face of bottleneck events (Lippé *et al.*, 2006). The fact that some traits such as gill filament length and hemibranch area have shown statistically significant divergence even under such limited timescales argues in favour of rapid adaptation and/or high plasticity in these traits, but our study cannot separate these two processes. What is clear in our study is that plasticity alone cannot provide complete differentiation. If it could, then the traits in even the range-expanding populations should already be much more, or even 'fully', divergent. Since they are not, it is clear that further divergence must be the province of phases (2) and (3) described above. Further resampling of the system would not just reduce the influence of the original colonizers, but also capture these phases.

Finally, our data argues in favour of a high degree of inter- and intraspecific parallelism across DO gradients. Whether using parapatric conspecifics or sympatric heterospecifics as a 'reference point', the broad pattern remained very similar. This is perhaps not unexpected, given the strong evolutionary pressure DO can put on fishes (Chapman, 2015), but still provides an important example for the literature as to the strength of such a pattern, even over short time frames.

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AUTHOR CONTRIBUTIONS

All authors conceived the ideas and broad experimental design. D.A.G.A.H. and L.J.C. conducted the fieldwork and collected the data. D.A.G.A.H. analysed the data. D.A.G.A.H. wrote and edited the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

CONFLICT OF INTEREST

The authors declare that no conflicts of interest exist.

DATA AVAILABILITY

The data and code underlying this article are available in the Dryad Digital Repository and Zenodo, respectively, at https://doi.org/10.5061/dryad.02v6wwq7c and https://doi.org/10.5281/zenodo.7786517 (Hunt *et al*. 2023). Datasets and code are referenced in the manuscript as follows:

D1. tps format data file for geometric morphometric analyses.

D2. csv format—data file for detailed gill analysis.

D3. R format—R script for geometric morphometric analyses.

D4 . R format—R script for detailed gill analysis.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article on the publisher's website.

Table S1. Pairwise *P*-values for Figure 4. Tukey-adjusted *P*-values were calculated using the package multcomp (Hothorn *et al.*, 2008), detailed code is in D4.
Table S2. Pairwise *P*-values for Figure 5. Total gill filament length (mm) per gill arch in both *E. apleurogramma* and *E. neumayeri*. Tukey-adjusted *P*-values were calculated using the package multcomp (Hothorn *et al.*, 2008), detailed code is in D4.
Table S3. Biometric values for fish utilized for geometric morphometric analysis in this study.
Table S4. Biometric values for fish utilized for gill analysis in this study.