Functional Ecology

Ecological variation drives morphological differentiation in a highly social vertebrate

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Author contributions

TT, FH and JGF conceived the study; JGF coordinated the project; MT and JGF planned the breeding line, which was maintained by AF and DJ; AF, DJ, and JGF planned the data collection; DJ, JMF and JGF collected data and fish samples in the field; AF prepared F1 and F2 fish for analyses; AF and TT produced the x-ray pictures; DJ and AF conducted the microsatellite analyses; AF analysed the data and was supported by DJ, JMF and DAM. AF wrote the manuscript and was supported by all authors. All authors approved the final version of the manuscript.

Data availability

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.fj6q573v8 (Freudiger et al. 2021).

Ethical note

Field data were collected within the framework of a Memorandum of Understanding between the Department of Fisheries, Ministry of Agriculture and Livestock of Zambia and the University of Bern, and followed the regulations of the 'Zambian prevention of cruelty to animals' act. The breeding line was established in accordance with the ASAB/ABS guidelines for the treatment of animals in behavioural research and teaching, and it was done under the control of the Veterinary Service of the Kanton Bern (Facility number BE 4/11).

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Abstract

1. Animals may respond to ecological heterogeneity by genetic differentiation or phenotypic plas-
   ticity. Responses of organisms to their ecology can include adaptation at various levels of organ-
   ization, including morphology, behaviour and social structure. Adaptations at one level might
   constrain or enhance adaptations on other levels, which highlights the importance of under-
   standing their interactions.

2. In highly social animals, understanding the influence of ecology on the evolution and mainte-
   nance of complex social organization poses an intriguing challenge. Predation risk and habitat
   characteristics determine social structure in the cooperatively breeding cichlid *Neolamprologus
   pulcher*. Here we examine how varying combinations of these ecological factors across eight
   distinct populations influence morphological differentiation.

3. We investigated the relationship between body shape and ecological parameters for 137 wild-
   caught individuals from eight distinct populations. Furthermore, we examined the genetic struc-
   ture and differentiation among these populations using microsatellites. Finally, to disentangle
   heritable from plastic responses we raised two successive generations from six populations in
   the laboratory under common garden conditions and screened 188 individuals for morphologi-
   cal differentiation.

4. We found that body shape of *N. pulcher* strongly correlates with the measured ecological parameters. Low predation risk, low habitat structure and small shelter size favoured shallow bodies, whereas at the opposite end of these environmental gradients deep body shapes prevail. These consistent morphological differences persisted over two laboratory-reared generations, revealing a heritable basis. In contrast to the significant effect of local ecology on morphological differentiation between populations, both geographic and genetic distance had little explanatory power, suggesting that morphological differentiation between populations is not a simple by-product of genetic isolation. Remarkably, the largest difference in body depth emerged between the two populations located closest to each other, but differing strongly in their ecological niche.

5. These results highlight that morphological variation is a key component of local adaptation in neighbouring populations of a highly social species. Such morphological differentiation has the potential to influence individual cooperative behaviour, which will eventually feedback on group structure and mediate the evolution and maintenance of complex social systems.

Abstract

1. Tiere können auf eine ökologisch heterogene Umwelt mit genetischer Differenzierung oder phänotypischer Plastizität reagieren. Diese Reaktionen können Anpassungen auf verschiedenen Organistationsebenen betreffen, einschließlich Morphologie, Verhalten und Sozialstruktur. Anpassungen auf einer Ebene können Anpassungen auf anderen Ebenen einschränken oder verstärken, was aufzeigt, wie wichtig ein Verständnis der Wechselbeziehung dieser Ebenen ist.

2. Komplexe soziale Organisation macht es speziell anspruchsvoll den Einfluss der Umwelt auf die Evolution und Aufrechterhaltung der Sozialstruktur zu verstehen. So bestimmen zum Beispiel sowohl Raubdruck und als auch spezifische Habitateigenschaften die Sozialstruktur des kooperativ brütenden Buntbarsches *Neolamprologus pulcher.* In der vorliegenden Studie untersuchen wir anhand von Tieren aus acht Populationen, wie Kombinationen unterschiedlicher ökologischer Faktoren die morphologische Differenzierung beeinflussen.

3. Zu diesem Zweck analysierten wir die Beziehung zwischen Körperform und ökologischen Parametern bei 137 im Tanganjikasee gefangenen Individuen aus acht Populationen. Außerdem untersuchten wir die genetische Struktur und Differenzierung zwischen Populationen mit Hilfe von DNS-Mikrosatelliten. Um vererbbare von plastischen Reaktionen zu unterscheiden, zogen wir zwei aufeinanderfolgende Generationen von Elterntieren aus sechs dieser Populationen im Labor unter den gleichen Bedingungen („common garden") auf und untersuchten 188 Nachkommen auf morphologische Unterschiede.

4. Die Körperform von *N. pulcher* ist stark mit den gemessenen ökologischen Parametern korreliert. Niedriger Raubdruck, geringe dreidimensionale Habitatstruktur und geringe Höhlengröße begünstigen flache Körper, während am anderen Ende dieser Umweltgradienten hohe Körperformen vorherrschen. Diese konsistenten morphologischen Unterschiede blieben über zwei im Labor aufgezogene Generationen bestehen, was eine hohe Erblichkeit aufzeigt. Im Gegensatz zum signifikanten Effekt der lokalen Umwelt auf die morphologische Differenzierung zwischen den Populationen hatten sowohl die geographische als auch die genetische Distanz nur eine geringe Erklärungskraft, was darauf hindeutet, dass die morphologische Differenzierung zwischen den Populationen kein einfacher Nebeneffekt genetischer Isolation ist. Bemerkenswert ist, dass der größte Unterschied in der Körperhöhe zwischen denjenigen zwei Populationen auftrat, die am nächsten beieinanderlagen, sich aber in ihrer ökologischen Nische stark unterschieden.

5. Im Zusammenhang mit den markanten Unterschieden in der Sozialstruktur zwischen den untersuchten Populationen weisen unsere Ergebnisse auf ein faszinierendes Wechselspiel

zwischen morphologischen Anpassungen an ökologische Bedingungen hin. Das Verständnis dieser Wechselwirkung ermöglicht uns Einblicke in die Evolution sozialer Komplexität.

Introduction

Adaptations to ecological conditions may include morphological and physiological attributes, behavioural traits, social structure or a combination thereof (Koenig, 1981; DeWitt et al., 1999; Willmer et al., 2005; Heynen et al., 2017). These adaptations may reinforce or compensate each other (DeWitt et al., 1999; Heynen et al., 2017; Marshall & Wund, 2017; Mushagalusa et al., 2019). For instance, under high predation pressure freshwater snails (*Physia gyrina*) evolved narrow shell apertures rendering them less vulnerable to shell-entry predation. Along with the resulting reduced vulnerability, individuals with narrow shell apertures were also found to be bolder and to show more antipredator behaviour (DeWitt et al., 1999). In social animals, variable ecological factors may directly affect social interactions by changing the costs and benefits of group living (Curry, 1989; Arnold & Owens, 1999; Koenig et al., 2011; Marshall et al., 2016). In addition, ecological variation may trigger adaptations in other phenotypic traits, which may in turn feed-back on social organization (Zuk et al., 1998; Cannizzo et al., 2019). For example, parasite infections lead to changes in female red junglefowl's (*Gallus gallus*) comb size, which is accompanied with a shift in hierarchy, so that infected females lose their dominance and consequently attain a lower social status (Zuk et al., 1998). Therefore, it seems appropriate to study ecological effects on different attributes of organisms to understand their mutual influence on the striking diversity of social structures.

Adaptations to the local ecology may lead to consistent phenotypic differences between populations. Two distinct processes are commonly assumed to be responsible for such divergence. First, physical or geographical restrictions to gene flow may cause genetic differences between populations through genetic drift, which may result in phenotypic differentiation as a by-product (Wright, 1943; Slatkin, 1993; Bolnick & Otto, 2013). Second, genetic differentiation may result from divergent natural selection between populations leading to phenotypic adaptations to the local ecology (Slatkin, 1993; Schluter, 2000; Nosil et al., 2005; Wang & Summers, 2010; Sexton et al., 2014). However, in fluctuating habitats the fitness effects of different phenotypes may considerably diverge within or between generations (Bradshaw, 1965; West-Eberhard, 2003; Miner et al., 2005; Ruehl & DeWitt, 2005). Fluctuating selection pressures should hence diminish genetic differentiation between populations by maintaining heritable phenotypic variation within populations (DeWitt & Scheiner, 2004; Nosil et al., 2018). The ability to plastically respond to a particular environment by expressing appropriate phenotypes (Bradshaw, 1965; McCollum & Buskirk, 1996; DeWitt & Scheiner, 2004) can happen either within the lifespan of an individual (Young et al., 2003) or across generations (DeWitt, 1998; Agrawal et al., 1999). In any case, adjustments of this sort typically involve individual behavioural responses that may strongly affect social organization (Groenewoud et al., 2016; Cantor et al., 2020).

Morphological attributes of organisms seem less plastic than, for instance, behaviour. They might hence be assumed to vary primarily due to genetic differentiation (Falconer, 1981). Still, it has been well established that phenotypic plasticity may be similarly important for local morphological adaptation (Krueger & Dodson, 1981; West-Eberhard, 1989; Rohner & Moczek, 2020). Fishes are one of the best studied groups showing remarkable differentiation in morphology as response to various ecological factors, which can be linked to heritable or plastic responses (e.g. Imre et al., 2002; Olsson & Eklöv, 2005; Eklöv & Jonsson, 2007; Ruehl et al., 2011; Riesch et al., 2018). Among the ecological factors driving morphological differentiation are predation risk and habitat complexity, which may induce changes in body shape (Taylor & McPhail, 1985, 1986; Brönmark & Miner, 1992; Ghalambor et al., 2004; Stauffer & van Snik Gray, 2004; Frommen et al., 2011). For example, under high risk, fishes often show a deep body rendering various potential benefits, including reduced handling success by gape-size limited predators (Brönmark & Miner, 1992; Nilsson et al., 1995; Nilsson & Brönmark, 2000; Weber et al., 2012), or a higher burst swimming performance and enhanced turning rate (Webb, 1982, 1984; Domenici et al., 2008; Langerhans & Reznick, 2010). Improved swimming performance is especially advantageous in highly structured environments (Webb, 1984; Webb & Weihs, 1986; Webb et al., 1996; Walker, 1997; Schrank et al., 1999; Langerhans, 2009). On the contrary, in species relying on access to shelters to hide and breed, body depth may be constrained by the size of available shelters (Gashagaza et al., 1995; Takahashi et al., 2009).

Fishes are also renowned for their impressive diversity in social organization, ranging from solitary life or assembling in loose shoals to long-lasting, complex societies (Hara & Pitcher, 1986; Godin, 1997; Taborsky, 2016). This social diversity is linked to variation in biotic (e.g. predation pressure) and abiotic factors (e.g. habitat type; for review see Taborsky & Wong, 2017). For example, fishes living in long-lasting social groups may adjust group size (Taborsky, 1985; Bergmüller et al., 2005; Zöttl et al., 2013) or group structure (Groenewoud et al., 2016; Tanaka et al., 2016) to adapt to the prevailing conditions. Such adjustments in behaviour and social organization may coincide with divergence in other traits varying with local conditions, including morphology. The covariance between behavioural and morphological attributes across different populations exposed to deviating ecological conditions is hitherto not well understood.

The cooperatively breeding cichlid *Neolamprologus pulcher* offers great opportunities to study the interplay between morphology and sociality in animals. *N. pulcher* is endemic to Lake Tanganyika where it breeds colonially in ecologically diverse benthic habitats along the sublittoral zone (Balshine et al., 2001; Heg et al., 2005; Groenewoud et al., 2016). Colonies are stable over several years (Stiver et al., 2004; Heg et al., 2005) and individual dispersal distances rarely exceed 20 meters (Stiver et al., 2004, 2007). Groups consist of a breeding pair, which is on average assisted by five to six helpers of

different age cohorts (Balshine et al., 2001; Stiver et al., 2004; Groenewoud et al., 2016; Taborsky, 2016). Within groups, helpers engage in direct brood care, territory maintenance and defence (Balshine-Earn et al., 1998; Bergmüller et al., 2005; Taborsky, 2016). Territories usually contain either clefts in rocks, burrows under stones or empty gastropod shells providing shelter and serving as breeding chambers. The territory is defended against hetero- and conspecific intruders, which is mainly done by large group members (Bruintjes & Taborsky, 2011; Jungwirth et al., 2015; Groenewoud et al., 2016). Variation in ecological settings (including predation risk, habitat complexity and substrate quality) has pronounced impact on the behaviour and group structure of *N. pulcher* in populations living in different habitats (Groenewoud et al., 2016).

Here we ask whether multidimensional differences in the ecological niches of distinct populations affect morphological attributes, which may relate to the established variation in social structure of *N. pulcher*. We used three different approaches. First, we quantified the morphological differentiation of wild-caught individuals from eight populations varying in predation risk and structural complexity. We compared morphological and ecological data to assess whether local adaptation might explain potential morphological differentiation. Second, we tested for neutral genetic differentiation (i.e. variance in 13 microsatellites distributed across the genome that are unlikely to have fitness effects) between these populations to examine whether potential morphological differentiation might be a by-product of restricted gene flow and genetic drift. If morphological differentiation arose by restrictions in gene flow, we would expect to find a correlation between morphological and neutral genetic differentiation between populations. Third, to disentangle plastic responses from heritable variation, we collected specimens from the different populations and compared wild-caught, F1 and F2 fish raised under common garden conditions in the laboratory.

Methods

Study Species

Study populations

We selected eight ecologically distinct populations of *N. pulcher* at the southern tip of Lake Tanganyika, near Mpulungu, Zambia, according to marked differences in rock cover and predator abundance (for details see 'Methods – Ecological Measures'; Table 1 and Fig. 1B). Throughout the manuscript, we refer to these populations by the numbers 1 – 8, which correspond to the scores of the ecological niche axis in an ascending manner (for details see 'Methods – Ecological Measures'; Fig. 2). The eight populations were 140 m to 21 km apart from each other (Table S1). Two of these populations are geographically clearly separated from the others, while the other six cluster in two groups of three populations each (Fig. 1A).

Samples

For genetic analysis, we collected tissue samples of 34 breeder-sized individuals in each population from September to November 2014 and 2015. Individuals were caught haphazardly using fence nets over the complete colony, and a small part (approx. 1 by 2 mm) of their dorsal or anal fin was removed and preserved in 98% ethanol. Afterwards, they were released again.

For geometric morphometric analyses, we collected 151 individuals of these eight ecologically distinct populations from September to November 2012. We haphazardly selected fish from all parts of the study populations using fence nets. We measured the standard length (SL) of each specimen and determined its sex by visual inspection of the genital papilla. Males only have one opening (urinary pore), while females have two (urinary pore and oviduct; Popma & Masser, 1999). The fish were then euthanized with an overdose of KOI MED® Sleep and preserved in buffered 4% formalin. Determining the sex was not possible for one small individual, which was therefore excluded from further analyses.

Common garden experiment

We brought wild-caught individuals from six of the eight study populations (populations 1, 3, 4, 5, 7 and 8) to the lab at the Ethologische Station Hasli (Bern, Switzerland), in December 2012. We used these fish to breed first F1 and subsequently F2 individuals, which we raised under common garden conditions (see 'Appendix' for detailed description of housing conditions). We sampled 119 F1 individuals originating from 75 families (specimens per family: range = 1-4, median = 2) and 102 F2 individuals originating from 49 families (specimens per family: range = 1-8, median = 2). We noted SL and sex of each individual and euthanized them with an overdose of KOI MED® Sleep before fixing them in buffered 4% formalin. Sexing was not possible for three F2 individuals, because they had not reached sexual maturity, and were hence excluded from further analyses.

Ecological measures

From September to November in the years 2012-2015, we measured ecological parameters of the eight populations by SCUBA diving. We focussed on predation risk, shelter size and structural complexity, as these parameters had been shown to influence the morphology of Lake Tanganyika cichlids and other fishes (e.g. Nilsson and Brönmark 2000; Domenici et al. 2008; Takahashi et al. 2009; Langerhans and Reznick 2010). The methods have been described in detail by Groenewoud et al. (2016) and Josi et al. (2018). In brief, four transects of 1 x 10 m^2 were established by laying sisal fibre ropes on the lake bottom, starting from the centre of each population. Predation risk was assessed by counting the number of predatory fishes (*Lepidiolamprologus elongatus*, *Lepidiolamprologus attenuatus* and *Lamprologus lemairii*) along the four transects. Each transect was surveyed between 6

and 10 times per population on different days, to account for variation in fish activity. The mean number of large (> 10 cm) *L. elongatus* and *L. attenuatus* per population served as a proxy for predation risk, as these were the most common species, posing a risk to *N. pulcher* of all sizes. We used sand cover as a measure of structural complexity of the habitats, with sandy stretches being flat and showing little variation in the third dimension, while rocky patches exhibit much more variation in the third dimension. The percentage of sand cover was determined in every square meter of the four transects, and we calculated the means per site. Within each colony, we randomly selected shelters inhabited by *N. pulcher* (n_{tot} = 127, range: n = 10 – 21 per population) and measured the size of the shelter entrances. Following the methods described in Tanaka et al. (2016), a transparent plastic sheet was rolled to a cylinder and inserted into the shelter entrance. There, it was slowly unrolled, until the diameter of the cylinder equalled the diameter of the shelter entrance, and measured with a ruler (Table 1). We constructed a model of the ecological niches by combining these three factors into principal components (PCs). Given the strong correlation of the three ecological parameters (see 'Results – Ecological niche axis'), we used the first PC as one-dimensional niche axis in the analysis and refer to it as 'ecological niche' in the following.

Data analyses

Morphological differentiation

We created a set of radiographs of all specimens using a Faxitron LX 60 x-ray device. We excluded specimens that had visible bone fractures leading to deformations of their body shapes (13 wild-caught, 18 F1, 15 F2; see Table 1 for final sample sizes). All observed bone fractures occurred at the skull, except for one individual that had a distorted spine. To quantify the morphology, we used a set of 19 landmarks that were placed on homologous skeletal structures across the body (Fig. S1) using the TpsDig 2.3.2 software (Rohlf, 2018).

We used the software R (R Core Team, 2018) with the packages geomorph (Adams et al., 2019) and Morpho (Schlager, 2017) to perform statistical analyses. First, we performed a generalized Procrustes analysis to correct for size, position and orientation of the specimen on the radiograph. Then, we ran a Procrustes ANOVA with random residual permutation procedure (1'000 iterations) to identify effects of population and sex on overall body shape of wild-caught fish. We used shape as response variable and population and sex as explanatory variables. Subsequently, we applied a post-hoc pairwise comparison to identify morphological disparity between populations and estimated its significance by a permutation procedure (1'000 iterations). We conducted a principal component analysis (PCA) to identify the main axes of variance in body shape. PC1 explained 25.2% of the variation and mainly accounted for shape changes at the head and caudal peduncle (Fig. S3) known as typical artefacts in fishes. Such changes are not biologically meaningful, as they arise during fixation or result

from slight posture differences and are typically captured by PC1's in intraspecific comparisons (i.e. a slight dorsolateral bending of the body; Valentin et al., 2008; Fruciano, 2016; Larochelle et al., 2016). PC1 also contained dorsoventral variation in pelvic fin insertion (Fig. S3), a signal that likely represents the current condition of the fish (e.g. stomach fullness and spawning condition). Even though PC1 also contained subtle signatures of anteroposterior elongation and shortening that might be biologically relevant, these cannot be disentangled from bending and condition artefacts. In contrast, the signal captured in PC2 (explaining 21.9% of the variance; covariation of landmarks describing variation in body depth) was considered informative with respect to the focus of this study, as it mainly captured variation in body depth and elongation (Fig. S3). A sharp drop of explained variance occurred after PC2. We therefore proceeded with PC2 as a measure for body shape variation for the following analyses while not considering PC1 to avoid bending and condition artefacts to affect the results. This corresponds to a standard practice in fish geometric morphometrics using principal component analyses (e.g. Albert et al., 2008; Hudson et al., 2013; Fruciano, 2016; Hüllen et al., 2021).

The effect of ecological niche on morphology of wild-caught fish was tested with a linear mixed-effect model (LMM) testing for a relation between body shape and ecology. We used body shape (PC2) as response variable, the ecological niche axis as explanatory variable and included population as random effect. We tested if morphological pairwise distance between populations can be explained by restricted gene flow and genetic drift, or by local adaptation (Hereford, 2009), by fitting a multiple regression on distance matrices (MRM) using the R package ecodist (Goslee & Urban, 2007). Morphological distance between populations was set as response variable, and geographic, ecological and genetic distances between populations as explanatory variables. For morphological distance, we calculated the Euclidean pairwise distance of mean body shape between populations. For geographic distance, we used the shortest physical distance through water between populations (Fig. 1A and Table S1). For ecological distance, we calculated the Euclidean pairwise distance of the ecological niche between populations (Table S1). For genetic distance we used the population pairwise F_{ST} values (Table 2B). Significance was assessed by running 1'000 permutations.

Comparison across generations

To test if body shape differentiation persists in the F1 and F2 generations, we projected the Procrustes shape data of F1 and F2 into the PCA morphospace of wild-caught fish. Subsequently, we tested if body shape correlated between populations and generations in three separate linear models (LM). For the first LM, we fitted the mean body shape score per population of F1 as response variable and the mean body shape score per population of wild-caught fish as explanatory variable. In the second LM, we fitted the mean body shape score per population of F2 as response variable and the mean

body shape score per population of wild-caught fish as explanatory variable. In the third LM, we used the mean body shape score per population of F2 as response variable and the mean body shape score per population of F1 as explanatory variable. We corrected for multiple testing by implementing the Benjamini-Hochberg method (Benjamini & Hochberg, 1995).

Genetic differentiation

The processing of genetic material followed Josi et al. (2019): We used a set of 13 microsatellite markers on polymorphic loci (UNH154, UNH106 (Lee & Kocher, 1996); NP007, NP773, ULI2 (Schliewen et al., 2001); TmoM11, TmoM13, TmoM25, TmoM27 (Zardoya et al., 1996); Pzeb4 (Van Oppen et al., 1997); UME003 (Parker & Kornfield, 1996); UNH1009 (Carleton et al., 2002); Ppun21 (Taylor et al., 2002)). Some of these sequences have been optimized for the genome of *N. pulcher* (Kotrschal et al., 2012). Using the QIAGEN® Multiplex PCR Kit, we amplified the DNA allowing co-amplification of several locus-specific, fluorescently labelled primer pairs in one single PCR reaction. We used two different primer sets containing 6 and 7 primer pairs, respectively. PCR reactions took place in a 10 µl cocktail consisting of 1 µl genomic DNA, 5 µl 2x QIAGEN Multiplex PCR Master Mix, 3 µl H_2Odd and 1 µl of 10x primer mix. Primer mix contained fluorescent-labelled forward and non-labelled reverse primer pairs with end concentrations of 0.04 – 0.06 µM each, according to the intensity of the respective amplification products. The fluorescent dyes used were 6-FAM (blue), Yakima Yellow (green), HEX (green), ATTO550 (yellow), ATTO565 (red) (Microsynth), PET (red) and VIC (green) (Thermo Fisher). We amplified the DNA in a GeneAmp® 9700 PCR System (Applied Biosystems) using the following cycling parameters: 15 min at 95°C, 35 cycles at 95°C for 30 s, 57°C for 3 min and 72°C for 60 s followed by a final elongation step of 72°C for 15 min. Fluorescent PCR fragments were visualized by capillary electrophoresis on an ABI 3100® Genetic Analyser (Applied Biosystems). GeneScan 500 LIZ (Thermo Fisher) was used as an internal size standard and the fragments were analysed using GeneMarker® Analysis software version 2.4.0 (SoftGenetics).

We used the software GenAlEx version 6.5 (Peakall & Smouse, 2006, 2012) to conduct genetic analyses. We tested each marker for Hardy-Weinberg equilibrium (HWE) within each population. After correcting for multiple testing by implementing the Bonferroni method (Bland & Altman, 1995), 11 microsatellite markers remained within HWE in each population, which we used for subsequent analyses (Table S2). First, we computed the global and pairwise fixation index (F_{ST}) by running an Analysis of Molecular Variance (AMOVA) with 1'000 permutations to assess significance. Second, we calculated six standard measures of genetic diversity per population: percentage of polymorphic loci, mean alleles per locus, effective number of alleles, observed and expected heterozygosity, and the fixation index over loci. Third, we performed Bayesian clustering assignment using STRUCTURE version 2.3.4 (Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009). We performed ten

independent runs for each K between 1 and 10, with a burn-in period of 100'000 iterations and subsequent 100'000 iterations of the Markov Chain Monte Carlo algorithm. We identified optimal cluster numbers using STRUCTURE Harvester (Earl, 2012) following the Evanno method (Evanno et al., 2005). Additionally, we tested if genetic differentiation is explained by geographic or ecological distance, by running a MRM with genetic distance between populations as response variable, and geographic and ecological distance between populations as explanatory variables. For this purpose, we used the population pairwise distances as explained above.

Results

Ecological niche axis

The three ecological parameters of interest (predation risk, shelter size and sand cover) correlated with each other across the eight populations (Spearman correlation, n = 8 populations; predation risk – shelter size: r = 0.83, p = 0.015; predation risk – sand cover: r = -0.69, p = 0.069; sand cover – shelter size: r = -0.90, p = 0.004). We therefore combined them into a single, one-dimensional ecological niche axis. This axis accounted for 83.5% of the variation in the natural habitat. Each variable loaded strongly on the PC (|loading| > 0.52), where loadings of predation risk and shelter size were positive, and sand cover was negative. The eight populations were distributed continuously across this ecological niche axis (Table 1 and Fig. 2).

Morphological differentiation

Body shape of wild-caught fish significantly differed between populations (Procrustes ANOVA, df = 7, Z = 9.91, p < 0.001; for post-hoc pairwise comparison see Table S3). Males tended to have a deeper body than females, though this effect was not statistically significant (df = 1, Z = 1.33, p = 0.095). Comparing the overall mean body shape with the means of each population showed that populations 1, 4 and 5 had shallow bodies, while populations 3 and 6 had deep bodies (Fig. S4). Populations 4, 6, 7 and 8 showed variation in head and eye size (Fig. S4). PC2 accounted for body depth and elongation (Fig. S3). Body shape of wild-caught fish was significantly correlated with the ecological niches (LMM, intercept: -0.0002, β ± SE: 0.0047 ± 0.0018, df = 1, t = 2.59, p = 0.014). Morphological distance between populations tended to show a relationship with ecological distance (MRM, intercept: 0.0095, β: 0.0022, df = 1, p = 0.09; Fig. 4A), but neither with genetic distance (β: -0.1157, df = 1, p = 0.35; Fig. 4B) nor geographic distance (β: 0.0003, df = 1, p = 0.34; Fig. 4C).

Comparison across generations

Average body shape per population of wild-caught fish correlated with that of the F1 generation (LM, intercept: -0.0016, β ± SE: 0.648 ± 0.190, df = 1, t = 3.41, p = 0.013; Fig. 3A). Body shape scores of F1 and F2 fish correlated with each other (LM, intercept: -0.0076, β ± SE: 0.823 ± 0.330, df = 1, t =

2.49, p = 0.027; Fig. 3C), whereas there was no significant correlation of body shape scores between wild-caught and F2 fish (LM, intercept: -0.0091, β ± SE: 0.462 ± 0.323, df = 1, t = 1.43, p = 0.115; Fig. 3B).

Genetic differentiation

The AMOVA revealed significant genetic differentiation among populations (F_{ST} = 0.037, p = 0.001). Population pairwise comparisons revealed that all populations were significantly differentiated from one another, except populations 1 and 7, where we found a non-significant trend (F_{ST} range: 0.004 (populations 1 and 7) to 0.071 (populations 2 and 4); Table 2B). Microsatellite variation revealed moderate levels of allelic diversity and heterozygosity, with no evidence of allele fixation (Table 2A). The STRUCTURE analysis suggested that there are five genetic clusters (see Fig. S5 for results for all Ks calculated). Populations 1, 6 and 7 showed similar allele frequencies, whereas 2, 3, 4 and 8 all had distinct allele compositions and frequencies (Fig. 1C). Genetic distance neither correlated with geographic (MRM, intercept: 0.0313, β: 0.0008, df = 1, p = 0.51; Fig. 5A) nor with ecological distance (β: -0.0009, df = 1, p = 0.76; Fig. 5B).

Discussion

Animals can adapt to their environment by changes at different levels, such as social organization, behaviour or morphology. Adaptations to environmental variation at one level potentially constrain or enhance adaptations at other levels, which may lead to a complex interplay of adaptive strategies. Our data revealed significant differentiation between populations linked to their ecological niche in a highly social cichlid. Morphological differentiation between populations was inherited by following generations, suggesting that it reflects heritable adaptation to the local ecology of these fish. We found no link between morphological and neutral genetic differentiation, indicating that morphological differences are not a simple by-product of restricted gene flow and consequential drift.

Body depth significantly increased with ecological niche differences representing enhanced predation risk, high structural complexity and large shelter size (Fig. 2). Deep bodies have been predicted to evolve under high risk and high structural complexity, and are expected to be counter-selected in habitats with limited shelter sizes (Webb, 1982, 1984; Brönmark & Miner, 1992; Nilsson et al., 1995; Ruehl & DeWitt, 2005; Domenici et al., 2008; Takahashi et al., 2009; Langerhans & Reznick, 2010; Ruehl et al., 2011; Larouche et al., 2020). The finding that the fish had the deepest bodies in habitats featuring high predation pressure suggests that this may serve as a morphological protection against predation risk, especially if shelter access is not hampered by a deep body. The access to shelters is crucial in *N. pulcher*, which defend territories containing rocky crevasses, excavated chambers underneath stones or empty gastropod shells (Heg et al., 2005; Groenewoud et al., 2016; Taborsky,

2016). Interestingly, the largest differences in body depth were found between populations 1 and 6, which are located only 140 m apart from each other but strongly differ in ecological settings. In population 1, predators are largely absent, shelters are small and large rocks are scarce. In population 6 in contrast, risk of predation is intense, the structural complexity is high and large rocky crevasses and excavated cavities are used as shelters. This shows that morphological differentiation took place on a small spatial scale in absence of neutral genetic differentiation (cf. Conover et al., 2006). Comparable results have been described for the lamprologine cichlid *Telmatochromis temporalis*, which also inhabits two ecologically distinct habitats, namely rocky-sandy bottoms and shell beds (Takahashi, 2004; Takahashi et al., 2009). In this species, divergent natural selection apparently induced the evolution of two distinct morphotypes in dependence of their ecological niches. A large morph occurs on rocky-sandy bottoms with large shelters, while a small dwarf morph inhabits the shell beds where shelter sizes are limited (Takahashi et al., 2009). In the present study, available shelter size and predation risk were positively correlated with each other, and hence we cannot disentangle their individual effects on the morphology of *N. pulcher*. To do so, it would be necessary to include populations with small shelter sizes and high predation risk in the analyses. This combination did not appear in any of our study populations, which might indicate that habitats with small shelter sizes and high predation risk may either be less suitable for this species or be scarce in general.

Morphological differentiation tended to increase with increasing ecological differentiation between populations, suggesting that natural selection might lead to body shape differences as a response to habitat variation. In contrast, neither geographic distance nor neutral genetic divergence between populations explained significant amounts of morphological differentiation, corroborating that populations appearing homogenous for neutral loci may still exhibit local adaptation (Conover et al., 2006). Morphological adaptations to local ecology can either result from phenotypic plasticity or from heritable genetic differences between populations (Bradshaw, 1965; Slatkin, 1993; McCollum & Buskirk, 1996; Schluter, 2000; DeWitt & Scheiner, 2004; Nosil et al., 2005; Wang & Summers, 2010; Sexton et al., 2014). We show that the effect of ecology on morphological differentiation in *N. pulcher* persists in two subsequent, common garden reared laboratory generations (Fig. 3). Body depth of F1 strongly correlated with body depth of wild-caught fish originating from the same site. Furthermore, body depth of the F2 generation significantly correlated with that of the F1 fish, even if the correlation between F2 and the wild-caught generation was not statistically significant (Fig. 3). This finding suggests that shape differences have a heritable component, while the exact mechanism of inheritance remains elusive. In addition to changes in the DNA sequence, morphological differences can also be inherited via epigenetic variation, which can be influenced directly by ecological interactions (Fieldes & Amyot, 1999; Anway et al., 2005; Cropley et al., 2006; Richards, 2006;

Whitelaw & Whitelaw, 2006), and hence serve as an additional, more dynamic pathway for evolutionary change (Bossdorf et al., 2008). Genome screenings for epigenetic variation are required to check whether this would explain at least part of our results (Bossdorf et al., 2008).

The microsatellite analyses revealed genetic differentiation among populations, indicating limited gene flow. Populations 1, 6 and 7 form one spatial cluster and are also genetically very similar, while all other populations are genetically differentiated (Fig. 1C). Testing for correlations between genetic, geographic and ecological pairwise distances between these populations revealed that genetic distance neither correlated with geographic, nor with ecological distance (Fig. 5). However, the Structure plot shows that populations 3 and 4 are genetically as well as geographically isolated from the others, which likely arises by limited dispersal possibilities and prevailing short distance dispersal (Stiver et al., 2004). Besides geographic distance, dispersal of bottom-dwelling cichlids is also hampered by ecological barriers, such as large muddy or sandy stretches, which do not provide any shelter from predators and are therefore risky to cross (Koblmüller et al., 2007). Such barriers may account for the genetic differentiation found between the populations 2, 5 and 8, which are genetically distinct even though they are located in close proximity to each other. Hence, it is likely the combination of short dispersal distances and unsuitable habitat between populations that leads to limited gene flow. Interestingly, populations 1 and 6 exhibit strong morphological differentiation in the absence of neutral genetic differentiation, which shows that morphological differentiation is not simply a by-product of restricted gene flow between populations.

A potential alternative explanation for morphological differentiation in the wild-caught fish might be age related morphological effects (cf. Frommen et al., 2011; Meuthen et al., 2018). We cannot exclude the possibility that the individuals sampled in different populations differed in age, even if only dominant breeder-sized fish were collected, as individuals of some populations (e.g. population 4) were much smaller than those of others (e.g. population 6; Table 1). Age in this species was shown to correlate with body size (Skubic et al., 2004). Additionally, our sampled wild-caught fish were not balanced with respect to sex, which had a weak though non-significant effect on body shape. However, we still found comparable morphological differentiation in the laboratory-reared fish that were similarly aged when sampled and balanced with respect to sex (Table 1). Hence, age and sex differences are unlikely to be responsible for the observed morphological differentiation.

In combination with previously demonstrated differentiation in social structure between the study populations (Groenewoud et al., 2016), our data suggest that this cooperatively breeding vertebrate adapted to its habitat on various levels, which may interact with each other. While morphological adaptations reflect rather slow responses that are difficult to reverse, changes in social structure and

behaviour might be more dynamic and adjustable within an individual's lifetime (Gabriel, 2006). Consequently, group structure and social behaviour might be affected both directly by local ecological conditions and indirectly by the impact of morphological adaptations. For example, in *N. pulcher*, subordinates need to show helping behaviour (i.e. territory maintenance and defence, direct brood care) to be allowed to stay in the dominant's territory (Bergmüller & Taborsky, 2005; Fischer et al., 2014; Naef & Taborsky, 2020). The amount and quality of such help might depend not only on the individuals' size (Heg & Taborsky, 2010; Bruintjes & Taborsky, 2011), but also on their morphology, as exemplified by other cooperative breeders (Young & Bennett, 2010; Simpson et al., 2011; Molet et al., 2012). Eventually, differences in morphology might lead to strict task specialisation, as is the case in many eusocial insects (Hölldobler & Wilson, 1998).

Morphological differences could eventually have an impact on group structure (Young & Bennett, 2010; Simpson et al., 2011; Molet et al., 2012). For example, among cooperatively breeding fishes, large and deep-bodied helpers might be beneficial under high risk of predation, as they are efficient in antipredator defence and less prone to predation. On the other hand, shallow-bodied individuals may be more vulnerable to predation, and their shallow bodies might be less impressive when performing visual threat displays like spread fins or raised opercula lids (Balzarini et al., 2014). This could lead to a trade-off between the number of deep-bodied helpers versus shallow-bodied helpers in relation to the availability of large shelters in the territory, eventually shifting the predator avoidance strategy from direct defence to hiding in a shelter (Heg & Taborsky, 2010), which may feedback on group structure. In line with this argument an earlier study on our eight *N. pulcher* populations showed adaptive responses in social structure to ecological differences (Groenewoud et al., 2016), with groups from high risk populations with large shelters containing more large helpers and groups from low risk populations with small shelters having less and/or smaller helpers. An intriguing task for future studies would be to elucidate how cooperatively breeding species adjust their negotiations about group memberships and help (Quiñones et al., 2016) in dependence of the local ecology and the resulting morphological differences between populations.

In summary, this study highlights that in a cooperatively breeding vertebrate morphological attributes show adaptation to their local ecological niche. This coincides with systematic variation in social structure and behaviour, which may interact among one another. To clarify the importance of morphological adaptation to local ecological conditions for the evolution of behaviour and social organization is a worthwhile challenge for future studies of highly social animals.

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Table 1: Summary of sample sizes used for morphological analyses, with additional information on the numbers of females (F) and males (M) and the

respective range of body sizes (SL), and ecological parameters measured for each study population. Given are arithmetic means ± standard deviations.

Populations are sorted by spatial distance from West to East, population numbers indicate their position on the ecological niche axis.

population	wild-caught n and SL [mm]	F1 n and SL [mm]	F2 n and SL [mm]	predation risk	shelter size [mm]	sand cover [%]	ecological niche
4	5F (26 − 30) 10M (25 − 37)	9F (50 − 73) 7M (55 − 66)	5F (28 − 42) 6M (29 − 40)	3.58 ± 1.86	17.63 ± 2.71	38.06	-0.590
3	28F (31 − 56) 25M (31 − 65)	9F (50 − 61) 8M (55 − 70)	9F (29 − 43) 9M (31 − 42)	1.31 ± 0.38	19.14 ± 2.69	36.60	-0.779
8	4F (38 − 54) 9M (34 − 56)	9F (44 − 62) 8M (53 − 70)	4F (30 − 39) 0M (NA)	5.56 ± 1.13	28.60 ± 2.29	6.17	2.254
5	5F (33 − 49) 1M (39)	9F (48 − 69) 9M (54 − 71)	5F (30 − 40) 4M (40 − 42)	0.75 ± 0.50	22.16 ± 3.20	10.83	0.444
2	8F (31 − 50) 10M (33 − 52)	NA	NA	0.17 ± 0.30	15.56 ± 1.81	44.17	-1.691
1	11F (29 − 45) 6M (29 − 45)	7F (43 − 64) 8M (54 − 78)	13F (30 − 42) 6M (29 − 41)	0.00 ± 0.00	11.84 ± 1.85	45.76	-2.212
6	0F (NA) 11M (57 − 70)	NA	NA	4.65 ± 2.30	24.03 ± 5.07	22.00	0.957
7	3F (27 − 49) 1M (37)	8F (55 − 71) 10M (53 − 80)	14F (28 − 41) 10M (32 − 43)	7.75 ± 3.21	23.21 ± 3.08	17.19	1.618

Table 2: (A) Genetic diversity observed at 11 microsatellite loci in eight populations of *N. pulcher*. Populations are sorted by spatial distance from West to East, population numbers indicate their position on the ecological niche axis. Given are proportion of polymorphic loci (P), mean number of alleles per locus (N_a), effective number of alleles (N_e), observed (H_o) and expected (H_e) heterozygosity and inbreeding coefficient (F_{IS}). (B) Pairwise population F_{ST} values resulting from the AMOVA.. F_{ST} values are shown above the diagonal, p-values were calculated using a permutation procedure and are displayed below the diagonal. Significant p-values ($p < 0.05$) are highlighted in bold and a non-significant trend ($p < 0.1$) is indicated in italics.

A

population	P	N_a	N_e	H_o	H_e	F_{IS}
4	1.00	14.91	8.41	0.781	0.779	-0.025
3	0.91	12.00	5.78	0.727	0.691	-0.066
8	0.91	10.18	5.63	0.737	0.719	-0.029
5	1.00	13.46	7.51	0.746	0.742	-0.003
2	0.91	8.64	4.60	0.668	0.675	0.006
1	1.00	14.73	8.09	0.711	0.750	0.048
6	1.00	41.55	8.57	0.770	0.762	-0.016
7	1.00	14.36	8.20	0.740	0.737	-0.011

B

	4	3	8	5	2	1	6	7
4		0.070	0.053	0.038	0.071	0.039	0.027	0.030
3	**0.001**		0.048	0.027	0.063	0.049	0.054	0.042
8	**0.001**	**0.001**		0.014	0.061	0.026	0.035	0.027
5	**0.001**	**0.001**	**0.001**		0.045	0.010	0.021	0.012
2	**0.001**	**0.001**	**0.001**	**0.001**		0.045	0.045	0.040
1	**0.001**	**0.001**	**0.001**	**0.005**	**0.001**		0.013	0.004
6	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		0.012
7	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	*0.081*	**0.001**	