Ecological character displacement among *Nothobranchius* annual killifishes in Tanzania

David. O. Alila[1,2,3]   (David.Alila@eawag.ch)

Hanna ten Brink [2,4]   (hannatenbrink@gmail.com)

Marcel Haesler[1,2]   (marcel.haesler@unibe.ch)

Ole Seehausen[1,2]   (ole.seehausen@eawag.ch)

[1] Department of Fish Ecology and Evolution, Centre of Ecology, Evolution, and
    Biogeochemistry, EAWAG Swiss Federal Institute of Aquatic Science and Technology,
    Seestrasse 79, CH-6047 Kastanienbaum, Switzerland.

[2] Division of Aquatic Ecology and Evolution, Institute of Ecology & Evolution, University of
    Bern, 3012 Bern, Switzerland.

[3] Department of Biological Sciences, Mkwawa University College of Education, University of   Dar
es salaam, P.O Box 2513 Iringa, Tanzania.

[4] Royal Netherlands Institute for Sea Research (NIOZ), 1790 AB Den Burg, Texel, Netherlands.

Corresponding author:

David. O. Alila

David.Alila@eawag.ch

Department of Fish Ecology and Evolution,

EAWAG Swiss Federal Institute of Aquatic Science and Technology,

Seestrasse 79, CH-6047 Kastanienbaum,

Switzerland.

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Abstract:

Divergent ecological character displacement (ECD) is the competition-driven divergence in resource use-related phenotypic traits between coexisting species. It is considered one of the primary drivers of ecological diversification and adaptive radiation. We analyzed phenotypic and ecological variation in two African annual killifish species of the genus *Nothobranchius*; *N. eggersi* and *N. melanospilus* in sympatry and *N. melanospilus* in allopatry. Our aim was to test if allopatric and sympatric populations of *N. melanospilus* differ morphologically from each other and from *N. eggersi*, and examine if these differences are consistent with the predictions of ECD. We find that sympatric *N. melanospilus* differ from allopatric *N. melanospilus* and differ from *N. eggersi* more strongly than the latter. Our data satisfy four criteria for demonstrating ECD: Differences in phenotypes between allopatric and sympatric *N. melanospilus* are greater than expected by chance; the divergence pattern between allopatric and sympatric *N. melanospilus* results from an evolutionary shift rather than from ecological sorting; morphological differences observed reflect differences in resource use and, lastly, sites of allopatry and sympatry do not differ in food resource availability or other ecological conditions. Our results suggest that competition is the main driver of the observed divergence between two *N. melanospilus* populations.

Introduction

Knowledge of the ecological forces causing population divergence and species diversification in resource use are central topics in evolutionary ecology and key to understanding speciation, adaptive radiation, and community assembly (Gray et al., 2005; Komine et al., 2019; Pfennig & Pfennig, 2010). Competition between species for resources is one such force with potentially large impacts on ecological diversification, community assembly, and community diversity. The "competitive exclusion principle" (CEP) states that two species with identical niches that compete for a single resource cannot coexist in the same space indefinitely (Gause, 1934; Hardin, 1960). However, one possible evolutionary outcome of resource competition is divergent ecological character displacement (ECD): heritable divergence in resource-related phenotypic traits between coexisting species due to frequency-dependent outcomes of competition for a shared resource (Brown & Wilson, 1956; Dayan & Simberloff, 2005; Grant, 1972; Schluter & McPhail, 1992). This mechanism leads to exaggerated trait differences among species living in sympatry compared to where either species is found alone (i.e. in allopatry). This divergence in traits will reduce competition for shared resources and introduce negative frequency dependence into the trait-dependent interaction dynamic, potentially stabilizing coexistence. Schluter & McPhail (1992) predicted that this process should lead to well-separated trophic niches of species when in sympatry.

Despite the importance of ecological character displacement, there are only a few convincing examples of ECD. Schluter & McPhail (1992) outlined six criteria that must be tested and met in order to qualify a case as likely ECD (Schluter, 2000; Schluter & McPhail, 1992). The few examples that meet all, or most, of these criteria include Darwin's finches (Brown & Wilson, 1956; Grant & Grant, 2006), sticklebacks in lakes in coastal British Columbia (Gray & Robinson, 2002; Schluter &

McPhail, 1992), American spadefoot toads (Pfennig & Pfennig, 2010), and *Anolis* lizards (Stuart & Losos, 2013).

Here, we assess whether competition between annual killifish species in the genus *Nothobranchius*, subgenus *Adinia*, may have led to ecological character displacement between sympatric species sharing small ephemeral habitats in Eastern Africa. *Nothobranchius* is a genus of small, annual freshwater fish in the family Nothobranchiidae, and part of the order Cyprinodontiformes (Blažek et al., 2013; Reichard et al., 2022; Wildekamp, 2004). Their distribution and occurrence are limited to the sub-tropical and tropical parts of central, Eastern, and South-eastern Africa that are subject to pronounced seasonality in rainfall. All *Nothobranchius* habitats receive seasonal rainfall (Blažek et al., 2013; De Wet van der Merwe et al., 2020; M. Reichard et al., 2022), but they vary greatly in terms of size, patterns of inundation, and connectivity (Bartáková et al., 2020; Nagy & Watters, 2021).

The coastal region of Tanzania harbors more than 20 endemic species of *Nothobranchius* and has the highest incidence of sympatry observed anywhere in the distribution range of the genus (Reichard et al., 2022; Terzibasi et al., 2009; Watters et al., 2019). This raises the question how so many closely related species can coexist in the region. Interestingly, there is also a lot more ecomorphological variation among species in this region than anywhere else in the wide distribution of the genus. This includes a wide range of body sizes and multiple feeding specialists, such as surface and open water feeding fish, piscivores, as well as the widespread benthic generalists (Nagy & Watters, 2021). Given the extensive sympatry of closely related species, combined with larger than elsewhere ecomorphological variation, we hypothesize a role for ecological character displacement (ECD) among sympatric species. *Nothobranchius* are confined to ephemeral water

bodies where few if any other fish compete with them or prey on them. The resulting patchy distributions that all *Nothobranchius* species have, across numerous small waterbodies with different local species richness and community compositions, makes this system especially suitable to explore the role of ECD among populations of closely related species in facilitating ecomorphological diversification.

The main objective of our study was to examine if allopatric and sympatric populations of *N. melanospilus* displayed morphological differences and then test if these differences were consistent with the predictions of the ECD hypothesis. We focused on the most widespread and most common species in the coastal lowlands, *Nothobranchius* (*Adinia*) *melanospilus* and its interaction with the next most widespread and common species in the region in the same subgenus, *N. (A.) eggersi*. Morphologically, *N. melanospilus* is overall larger (known max length: 60 mm SL male) than *N. eggersi* (known max length: 39 mm SL male) (Watters, 2009; Wildekamp, 2004).

Nothobranchius melanospilus has a wider and more continuous geographical distribution range than *N. eggersi,* the range of which is geographically more narrowly confined and the distribution more patchy within the range (Bartáková et al., 2020; Nagy, 2010; Nagy & Watters, 2021).

In the spring of 2019, we undertook a field study to determine resource use, prey availability, and morphological traits of *N. melanospilus* and *N. eggersi* living in either allopatry or sympatry. Of the six ECD criteria summarized in Schluter & McPhail (1992) our data allowed us to test four (numbers refer to the numbering in Schluter & McPhail 1992): (1) The observed pattern of exaggerated phenotypic difference in sympatry is not due to chance; (3) The divergence pattern between sympatric species results from an evolutionary shift (in situ displacement in trait values) rather than ecological sorting; (4) Observed morphological differences must reflect differences in resource use;

(5) Sites of allopatry and sympatry should not differ significantly in available food resources or other ecological conditions which may affect the phenotype.

Materials and Methods

Study area and species

Between 19–23rd June 2019, we sampled 44 ponds in the Tanzanian lowlands and found 13 ponds with *N. melanospilus* only, 11 ponds having both species but no other species, 20 ponds in which both coexisted with one or more additional *Nothobranchius* species and not a single pond in which *N. eggersi* occurred alone. For this study, we focus on 5 ponds with *N. melanospilus* alone and 5 ponds with both *N. melanospilus* and *N. eggersi* present, but no other killifish species. Due to constrains on time and resources, we could not analyse all the 24 ponds with *N. melanospilus* and/or *N. eggersi* and therefore focused on 10 of them. We chose these 10 ponds based on their proximity from each other, so as to minimize variation in abiotic factors that could otherwise confound our results.

Sample collection

Samples of the two *Nothobranchius* species were collected from temporary pools in the Ruvu River basin (5°S-7°S and 36°E-39°E) in Eastern Tanzania (Figure 1). The two species are listed as species of Least Concern by the IUCN and are endemic to Tanzania (Watters et al., 2020). To avoid the influence of seasonal variation, all fish and invertebrate samples were collected within a period of five consecutive days (19th-23rd, June 2019). All ten ephemeral ponds that we chose have relatively similar sizes (66-120 m^2). Population samples of *N. melanospilus* and *N. eggersi* were sampled using a seine net (depth 0.7 m, length 2.7 m, and its stretched mesh-size 4 mm) and a triangular dip-net

(450 mm × 450 mm and its mesh size 5 mm). The mesh-size was sufficiently fine to retain the smallest adult female *Nothobranchius.* (Frost et al., 1971; Watters, 2009). All collected fishes were identified to species level in the field. In total, we collected 384 adult individual fishes as sample specimens from the ten sites, five ponds with *N. melanospilus* only (allopatric *melanospilus* from now onwards) and five with the two species in sympatry (sympatric *melanospilus* and sympatric *eggersi* from now onwards). These individuals were euthanized in clove oil and then fixed in 70% ethanol (Gray & Robinson, 2002). Sample specimens were shipped to the Institute of Ecology and Evolution; at the University of Bern. Field sampling, export, and import procedures followed the regulations of both the Tanzania and Swiss governments. Research permits, export, and import licenses were issued by the responsible authorities (research permit: DAARS/R/1/37; export license SL195/2019; Import license: 2407/19).

In the laboratory, fish were weighed by total mass to the nearest 0.01 g using an electronic balance. Standard length (SL) was measured with a digital caliper (to the nearest 0.01 mm) (Laufer et al., 2009; Önsoy et al., 2010). For stomach content analysis, we took a random sample of 10 individuals per species (5 of each sex), resulting in 10 (allopatric sites) or 20 (sympatric sites) individuals per sampling site, and a total of 150 individuals. The digestive tracts were taken out from the fishes. Prey items from the stomach were identified to order or, wherever possible, to family level. A small number of prey items that were highly digested were excluded from the analysis due to identification difficulties. References for zooplankton and other aquatic invertebrates were consulted for identification (Marrero & Lopez-Rojas, 1995; Motta & Uieda, 2004; Ruppert & Barnes, 1994).

Three morphological traits considered to be relevant for foraging efficiency on different prey types (Bouton et al., 1997; Gray & Robinson, 2002; Olivier et al., 2019) were measured on the left side of each individual fish: in addition to standard length (SL) we measured lower jaw length (JL) and eye diameter (ED) using a digital caliper (to the nearest 0.01 mm) (Costa, 2018, 2019; Schluter & McPhail, 1992).

A sample of aquatic invertebrates was collected at each sampling site at the time of fish sampling. We used a rectangular hand-net (20 cm × 15 cm, 500 μm mesh-size) for sampling both pelagic and benthic invertebrates (Meintjes, 1996; Žák et al., 2019). A total of 12 hauls (6 benthic and 6 pelagic) were completed at each site. The entire sampled contents were fixed in 70% ethanol. In the laboratory, all sampled invertebrates were carefully picked up from the organic debris, classified into their respective taxonomic groups, and counted (Meintjes, 1996). During the invertebrate identification process, observers were unaware of the sympatric/allopatric status of the ponds. Based on the total number of invertebrate individuals in each sample taken at a site, the proportional contribution of each taxonomic group to the invertebrate sample, and by inference, the assemblage was calculated for each site (Ruppert & Barnes, 1994).

Hypothesis testing

We assessed the evidence for ecological character displacement in our study system by testing four of the six criteria for ECD suggested by Arthur (1982) and synthesized by Schluter and McPhail (1992). In this study, we were not able to test if the phenotypic differences between the allopatric and sympatric populations have a genetic basis (criterion 2). Neither could we provide independent evidence for resource competition among individuals with similar phenotypes with our field data (criterion 6). These two criteria will need to be addressed experimentally in the future. Below, we describe how we tested the other four criteria for ECD. In the following, we summarize the

predictions that each criterion makes and the tests we used to examine support for each. We prepared all graphs and statistical tests with R version 4.2.2 (R Core, 2020).

Criterion 1: The observed pattern of exaggerated character differences in sympatry is not due to chance.

We visualized phenotypic character variation within and differences between the three different types of fish populations (*N. melanospilus* in allopatry, *N. melanospilus* in sympatry, and *N. eggersi)* using split histograms. We used non-parametric Kruskal-Wallis tests to assess if the trait values of SL, JL, and ED differ between the three population groups. Post-hoc comparisons were performed using the Dunn's test to determine which population groups differed significantly. We predicted that trait values for SL, JL, and ED of *N. melanospilus* populations in sympatry would be significantly greater than those of allopatric populations. This expectation is based on the premise that an increase in these trait differences would enhance the contrast with the putative competitor, *N. eggersi*.

To test the null hypothesis that the trait overlap (in standard length) between *N. eggersi* and sympatric *N. melanospilus* is the same as the trait overlap between *N. eggersi* and allopatric *N. melanospilus*, we used a randomization test. Firstly, we calculated the difference between the mean values of the SL of *N. eggersi* and sympatric *N. melanospilus* for each of the five sympatric population sites. Next, we obtained a hypothetical difference in mean SL between *N. eggersi* and allopatric *N. melanospilus* by randomly sampling 30 individuals from all allopatric *N. melanospilus*, and calculating the mean SL of this sample. We then calculated the difference between the mean SL from each *N. eggersi* site and the random allopatric *N. melanospilus* sample, resulting in five values. We repeated this process 10,000 times to generate a null distribution of hypothetical mean SL differences. Finally, we compared the observed mean SL difference between *N. eggersi* and

sympatric *N. melanospilus* with the null distribution of mean SL differences between *N. eggersi* and allopatric *N. melanospilus* to determine the probability of observing a similar or larger trait difference in the null distribution. If the result was less than 5%, we rejected the null hypothesis that the trait overlap between *N. eggersi* and sympatric *N. melanospilus* is the same as the trait overlap between *N. eggersi* and allopatric *N. melanospilus*.

To explore morphological differences other than size, we performed linear regression using log-transformed jaw length (JL) or eye diameter (ED) with log-transformed standard length (SL), including the interaction between SL and fish group. Pairwise comparisons were then made on the slopes of the JL-SL and ED-SL relationships to assess variation in allometric scaling across the three groups. In addition, the estimated means of these traits were compared between groups at mean standard length

Criterion 3: The differences between allopatric and sympatric N. melanospilus are the outcome of an evolutionary shift (in situ displacement of mean trait values).

Ecological sorting through biased colonization or extinction of populations predicts that the trait means observed in sympatry are contained within the range of trait means that can also be observed in allopatry. An evolutionary shift, on the other hand, predicts that trait means observed in sympatry lie outside the range of means observed in allopatry (Grant, 1972; Gray & Robinson, 2002; Losos, 2000; Pfennig & Pfennig, 2010; Schluter & McPhail, 1992). This is because enhanced differences between sympatric species should not be the outcome simply of the inability of similar populations to coexist. We used frequency distribution plots to visualize trends of the trait mean values in allopatry and sympatry.

Criterion 4: Morphological differences reflect differences in resource use.

Based on this criterion we predicted that, relative to the sympatric *N. eggersi*, sympatric *N. melanospilus* would have greater differences in their diet compared to allopatric *N. melanospilus*. Because *N. eggersi* is the smaller species, we can also predict the direction of the shift: sympatric *N. melanospilus* should feed on larger dietary items than allopatric populations do and the differences with *N. eggersi* should be larger still.

The stomach contents of a subsample of 150 fishes were taxonomically identified to the level of the order. Prey organisms were assigned to one of three major functional prey categories: Pelagic Soft-Bodied Crustaceans, SBC (Copepods and Cladocerans); Epiphytic and benthic Hard-Bodied Crustaceans, HBC (Ostracods and Conchostraca); and insect larvae, INL (Coleoptera, Odonata, Ephemeroptera, Hymenoptera, Hemiptera, Culicidae, and Chironomidae,) (Ruppert & Barnes, 1994). The total number of prey items in each category was counted. We then visually compared the diet between three different types of fish populations; *N. melanospilus* in allopatry, *N. melanospilus* in sympatry, and *N. eggersi* using box and whisker plots. We used PCA to visualize dietary niche separation among the three killifish types. The PCA was constructed using the absolute values of individual prey types found in the stomach of each fish. Furthermore, we used a non-parametric Kruskal-Wallis test followed by Dunn's post hoc test to assess the statistical significance of dietary variation among the three types of killifish. To account for the potential influence of size differences on total prey intake, we used proportions rather than absolute values in our statistical analysis.

Criterion 5: Sites of allopatry and sympatry do not differ significantly in food resource availability nor other abiotic ecological conditions affecting the phenotype.

To test the assumption that allopatric and sympatric sites do not differ in the dietary resources available to killifish, we quantified and compared the invertebrate samples obtained by hand netting in the benthic and open water zones. We used non-parametric Mann-Whitney U test to test the null hypothesis of no differences in the three functional invertebrate categories between allopatric and sympatric ponds. Furthermore, we also used non-parametric Mann-Whitney U test to assess the variation in the ecosystem size variables (pond size and depth) and physicochemical variables (pH, conductivity, and temperature). All samples were collected within a short period of time (5 days) to ensure that any temporal effects of sampling day were minimized.

Results

Criterion 1 – Chance

Chance is unlikely to be the main cause of the observed pattern of exaggerated trait differences in sympatry. Standard length, uncorrected jaw length, and uncorrected eye size were all significantly larger in sympatric *N. melanospilus* compared to allopatric *N. melanospilus* and *N. eggersi* (Kruskal-Wallis test (SL: H(2) = 220.4, p < 0.001; JL: H(2) = 163.6, p < 0.001; ED: H(2) = 131.6, p < 0.001), (Figure 2 & 3; Supplementary Figure 1). Dunn's post hoc test was then used to determine which of these groups differed significantly from each other. For all three traits, the post hoc test showed that sympatric *N. melanospilus* had significantly larger values compared to both *N. eggersi* and allopatric *N. melanospilus* (SL: p < 0.001; JL: p < 0.001; ED: p < 0.001, see Supplementary Table 1). Furthermore, randomization test showed that the mean body size (SL) overlap between *N. eggersi* and sympatric *N. melanospilus* is significantly smaller (p < 0.001) than that between *N. eggersi* and allopatric *N. melanospilus* (see for an example figure S1).

To understand the interplay among jaw length (JL), eye diameter (ED), and body size (SL) across our three killifish types, we performed size corrections for JL and ED. The pairwise comparisons between the slopes of regression lines for JL against body size (SL) did not reveal any significant differences between the three killifish types, including allopatric *N. melanospilus* vs sympatric *N. melanospilus* (p = 0.43), allopatric *N. melanospilus* vs sympatric *N. eggersi* (p = 0.63), and sympatric *N. melanospilus* vs *N. eggersi* (p = 0.87). Additionally, the regression of ED against SL did not consistently show any differences between the slopes of regression lines for allopatric *N. melanospilus* vs sympatric *N. melanospilus* (p = 0.4), allopatric *N. melanospilus* vs *N. eggersi* (p = 0.27), and sympatric *N. melanospilus* vs *N. eggersi* (p = 0.96) (Supplementary Table 3: Supplementary Figure 2 & 3).

With the aim of comprehensively examining the variation in each morphological trait (SL, JL, and ED) among the three killifish types, we conducted an analysis of differences in estimated marginal means (EMM). The pairwise comparisons of the estimated marginal means of JL at the mean SL value for each of the three types of populations showed that the JL of allopatric *N. melanospilus* was significantly different from that of sympatric *N. melanospilus* (p=0.001), with allopatric *N. melanospilus* having a higher estimated marginal mean (Supplementary Table 3). There was marginally significant difference between the JL of *N. eggersi* and allopatric *N. melanospilus* (p=0.07) and no significant difference between the JL of *N. eggersi* and sympatric *N. melanospilus* (p=0.64).

The pairwise comparisons of estimated marginal means showed that the eye diameter (ED) of allopatric *N. melanospilus* was significantly different from that of sympatric *N. melanospilus* (p=0.04), with allopatric *N. melanospilus* having a higher estimated marginal mean at the mean SL. There was no significant difference between the eye diameter of *N. eggersi* and either allopatric *N. melanospilus* (p=0.92) or sympatric *N. melanospilus* (p=0.21) at the mean SL.

Criterion 3 – Evolutionary shift

The frequency distribution plots show clear trends in trait values between sympatric *N. eggersi*, allopatric *N. melanospilus,* and sympatric *N. melanospilus* (Figure 3). The result reveals that in all three traits *N. eggersi* are smaller followed by allopatric *N. melanospilus* and sympatric *N. melanospilus.* Only *N. melanospilus* from one sympatric site are exceptionally small, but even here *N. melanospilus* are significantly larger than the co-occurring population of *N. eggersi*.

Standard length and jaw length observed in populations of *N. melanospilus* that are sympatric with *N. eggersi* are not only shifted to significantly larger means, but they exceed the range observed in all allopatric populations combined by a large margin. While the largest fish in sympatric populations measured over 67 mm SL, and the fish with the longest jaws measured 5.7 mm JL, none of the fish in allopatric populations exceeded 55 mm SL and 4.6 mm JL; (Figure 2 & 3). This implies that ecological sorting of trait variation that exists in allopatry and hence likely predates the establishment of sympatry is unlikely to explain the exaggerated divergence in trait mean values between *N. melanospilus* and *N. eggersi* in sympatry.

Criterion 4 – Resource use

Stomach content analysis showed that both species of *Nothobranchius* had diets based on aquatic crustaceans (Copepoda, Cladocera, Conchostraca, and Ostracoda) and insect larvae (INL) (Coleoptera, Odonata, Ephemeroptera, Hymenoptera, Hemiptera, Culicidae, and Chironomidae) (Figure 3). We found significant dietary niche separation between allopatric *N. melanospilus,* sympatric *N. melanospilus* and *N. eggersi* (Figure 4&5).

The Kruskal-Wallis test results revealed the statistical significance of differences among the three types of populations for all the three diet types: SBC, HBC, and INL, (SBC: (H2) = 67.96, p < 0.001; HBC: (H2) = 104.66, p < 0.001; INL: (H2) = 88.06, p < 0.001). The results of the post hoc Dunn's test showed significant differences between each all three pairwise contrasts of population types for all three diet types (p < 0.001 for all 9 comparisons).

The diet of sympatric *N. melanospilus* was found to have shifted towards a higher proportion of hard-bodied crustaceans (HBC) (mean values for SBC=6, HBC=12.3, INL=10) compared to *N. eggersi* and allopatric *N. melanospilus* (Figure 4&5).

The major dietary component of the sympatric competitor *N. eggersi* consisted of soft-bodied crustaceans (mean values for SBC=14.4, HBC=2.54, INL=1.76). In contrast, allopatric *N. melanospilus* in all five single-species ponds were found to be generalists, feeding on both HBC and SBC in relatively equal proportions (mean values for SBC=10.4, HBC=8.24, INL=6.84). *N. melanospilus* from sympatric populations consumed larger and harder-bodied prey compared to both *N. eggersi* and *N. melanospilus* from allopatric populations. Therefore, the observed morphological differences between allopatric and sympatric populations reflect clear differences in resource use.

Criterion 5– Ecological conditions

The Mann-Whitney U test results indicate that there were no significant differences in the densities of any of the three invertebrate groups (SBC, HBC, and INL) between the allopatric and sympatric sites (SBC: W = 3.5, p = 0.07; HBC: W = 13.5, p = 0.92; INL: W = 7.5, p = 0.34, see Supplementary Table 2).

All five single-species ponds and all five species pair ponds are located in the same river basin and have similar microclimatic conditions. Mann-Whitney U test results reveal no significant differences between single-species and species pair ponds in terms of ecosystem size variables (pond size: $W = 15$, $p = 0.69$, and depth: $W = 11.5$, $p = 0.88$), nor in other physicochemical variables (pH: $W = 12$, $p = 0.94$, conductivity: $W = 15.5$, $p = 0.59$, and temperature: $W = 9.5$, $p = 0.6$, see Supplementary Table 4). Therefore, environmental factors other than the presence or absence of a competitor seem unlikely to account for the observed differences between allopatric and sympatric *N. melanospilus*.

Discussion

Ecological character displacement (ECD) has been fully demonstrated in only a few examples. Most studies of ECD succeeded to demonstrate only some of the six ECD criteria (Arthur, 1982; Grant, 1972; Schluter & McPhail, 1992), with the first (chance ruled out) and fourth criteria (shift in trait matches the shift in ecology) having been demonstrated more often than the others (Stuart & Losos, 2013). The results of our study satisfy four out of the six widely accepted criteria for ECD while the remaining two require additional investigation with common garden and quantitative genetics experiments. The support that we showed here for four of the criteria suggests a considerable possibility of the occurrence of ECD between closely related annual killifish species.

In support of the first ECD criterion, stating that an observed divergence pattern does not arise by chance, we show that all three morphological traits, (Standard Length, Jaw Length, and Eye Diameter) were significant different among sympatric *N. melanospilus*, allopatric *N. melanospilus*, and *N. eggersi*. Subsequently, Dunn's post hoc analysis consistently revealed that sympatric *N. melanospilus* exhibited significantly larger values in all three traits when compared to both *N.*

eggersi and allopatric *N. melanospilus*. This makes chance a very unlikely explanation for the shifts in body size between *N. melanospilus* that do co-occur with *N. eggersi* and those that do not.

The results for size correction of JL and ED suggest that body size (SL), rather than jaw length (JL) or eye diameter (ED), may have been the trait that experienced ecological character displacement (ECD), and it is the main trait distinguishing sympatric and allopatric *N. melanospilus*.

Nothobranchius grow extremely fast (Dorn et al., 2014; Polačik & Reichard, 2010), and hence size differences between populations could be due to different hatching or sampling dates. However, hatching is determined by the onset of the seasonal rainfall. All of our ponds are in close proximity to one another and experience synchronous start of the rainy season. Furthermore, it is expected that fish in sympatric and allopatric ponds hatch at the same time (Blažek et al., 2013). We avoided confounding time effects by samplings populations in random order within a timespan of five days. The observed trend of larger body size (SL) in sympatric *N. melanospilus*, in contrast to allopatric *N. melanospilus*, is consistently replicated across all five sampled ponds. Our results could also be caused due to phenotypic plasticity. Therefore, a common garden study is needed to further elucidate the underlying mechanisms and provide a more comprehensive understanding of the observed patterns.

In support of the third criterion of ECD, the divergence pattern between sympatric species likely results from an evolutionary shift (in situ displacement in trait values) rather than ecological sorting. We find no overlap in the ranges of population mean standard length between allopatric and sympatric sites, i.e. all five sympatric populations have mean standard lengths larger than the mean of the largest allopatric population. This implies that the pattern of exaggerated size differences

between the species in sympatry cannot result from sorting of *N. melanospilus* populations by body size.

In support of the fourth criterion of ECD, observed morphological differences must reflect differences in resource use, stomach content analyses showed dietary niche separation between the three groups. Freshwater crustaceans form a large percentage of *Nothobranchius* diet, followed by insect larvae. In terms of volume, insect larvae are in general much larger than crustaceans. Our data show that in allopatry, *N. melanospilus* feeds to roughly equal parts on both pelagic soft-bodied crustaceans and benthic hard-bodied crustaceans, while in sympatry with *N. eggersi, N. melanospilus* shifts its feeding niche to mainly Ostracods and Conchostraca (epiphytic and benthic hard bodied crustaceans) and insect larvae. On the other hand, *N. eggersi* in sympatry mainly feeds on soft-bodied pelagic crustaceans (Copepods and Cladocerans). This is consistent with dietary character displacement permitting these phenotypically divergent species to coexist.

Importantly, we find that the differences in morphology between the populations in our *Nothobranchius* spp. are closely linked to resource use. The hard-bodied epiphytic and benthic prey were mainly consumed by the large-bodied population group (*N. melanospilus* in sympatry with *N. eggersi*). The larger *N. melanospilus* exploit a larger proportion of hard-bodied epiphytic and benthic Crustaceans (Ostracods and Conchostraca) and only a small proportion of pelagic and soft-bodied crustaceans (Copepods and Cladocerans) while the small *N. eggersi* predominantly feeds on soft-bodied crustaceans.

The *N. melanospilus* in populations living in sympatry with *N. eggersi* are bigger and thus have longer lower jaws. Even though, after size correction, their jaws are not longer than those of fish in

allopatric populations, larger body and jaw size are likely responsible for an increased ability to consume and crush large hard-bodied prey. Thus, specialization on larger prey items may be enabled mainly by an increase in body size. Stomach content analysis of three other sympatric species of *Nothobranchius* in southern Africa has previously revealed that smaller species (*N. rachovii* and *N. furzeri*) feed on all types of crustaceans whereas the larger *N. orthonotus* is specialized in insect larvae and tadpoles (Blažek et al., 2013; Polačik & Reichard, 2010). This observation is also supported by other studies that have demonstrated a general correlation between fish biting force and jaw length (Gray et al., 2005; Van Wassenbergh et al., 2007).

Finally, in support of ECD criterion 5, we demonstrate that sites of allopatry and sympatry do not differ significantly in available food resources or other ecological conditions which may otherwise affect killifish phenotypes. Different prey taxa sampled (crustaceans and insect larvae) were found in similar densities in all ponds. Because all sites are located in the same river basin with similar microclimatic conditions and same seasonality and they were all sampled within a short period of time (5 days) the similarity in abiotic facts is not surprising. This criterion is an important consideration when addressing the ECD concept (Gray & Robinson, 2002; Schluter & McPhail, 1992; Stuart & Losos, 2013).

Our results show that the key factor differentiating the allopatric and sympatric ponds is the respective absence and presence of *N. eggersi*. Hence, our findings suggest that competition for food resources with *N. eggersi* may be responsible for the pattern suggestive of ecological character displacement within *N. melanospilus*. As one important ecological process, competition can provide key explanations for the evolutionary origins of morphological diversity, through mediating dynamics

within populations, evolutionary change, and change of community structure (De Lisle et al., 2018; Gray et al., 2005; Pfennig & Pfennig, 2010; Reifová et al., 2011).

Similarly, in another classical ECD study, Grant & Grant (2006) identified a compelling case of food niche separation. In sympatry, *Geospiza fortis* exhibited a specialization in consuming smaller seeds, which was attributed to competition with *G. magnirostris*. This competition led to a reduction in beak size and shape within the population of the medium ground finch, *G. fortis*, on Daphne Major island. Furthermore, the salamander species *Plethodon cinereus* and *Plethodon hoffmani* showed divergent snout-vent lengths when living in sympatry, while they were similar in size when living allopatry (Losos, 2000). We have identified a significant separation in the food niche between sympatric and allopatric *N. melanospilus* populations. This pattern of niche differentiation along the feeding axis is a recurring observation in studies investigating ecological character displacement (ECD).

In another ECD study by Gray & Robinson (2002) and Gray et al. (2005), a consistent pattern of replicated morphological character shift in sympatry was observed. This study focused on brook sticklebacks (*Culaea inconstans*) in sympatry with ninespine sticklebacks (*Pungitius pungitius*) across a series of lakes in Canada, revealing strong evidence for ECD (4 out of 6 criteria met). The findings of this study indicated that brook sticklebacks in sympatry had undergone significant morphological changes compared to their allopatric counterparts. Specifically, the sympatric brook sticklebacks exhibited a more benthic body form characterized by a larger head, subterminal mouth, and deeper body. Additionally, they possessed fewer and shorter gill rakers in comparison to allopatric brook stickleback populations. Several other studies on ecological character displacement show food niche separation when two species compete for limited food resources (Anderson & Weir, 2021; Dufour et

al., 2017; Dunham et al., 1979; Grant & Grant, 2018; Gray et al., 2005; Komine et al., 2019; Losos, 2000; Pfennig & Murphy, 2003; Reifová et al., 2011; Rice & Pfennig, 2010; Schluter & McPhail, 1992).

A total of 144 ECD case studies reported in Stuart & Losos (2013) satisfy at least one of the six ECD criteria summarized in Schluter & McPhail (1992). Among these, only a few studies were considered strong candidates for ECD (satisfying more than three criteria). These include; Darwin's finches which fulfilled all six criteria, American spadefoot toads meeting five out of six criteria, Anole lizards, satisfying five out of six criteria and three-spined sticklebacks which met four out of the six criteria. (Dayan & Simberloff, 2005; Decru et al., 2017; Grant & Grant, 2006; Gray et al., 2005; Martin & Pfennig, 2011; Schluter & McPhail, 1992; Stuart & Losos, 2013; Wilson & Brown, 1956). We suggest that our study qualifies for adding African annual killifish to this short list of strong candidates for ecological character displacement, with 4 of 6 criteria fulfilled.

We can not yet investigate the remaining two of the six criteria for ECD (Schluter & McPhail, 1992). First, testing the evidence of interspecific competition between our two *Nothobranchius* species requires experimental manipulations in the field or in laboratory enclosures (Gray et al., 2005; Pfennig & Murphy, 2003; Pfennig & Pfennig, 2010; Schluter, 2000). We have recently conducted a large outdoor enclosure experiment that we will use to address this question in the near future. Moreover, to satisfy the final ECD criterion, a heritable basis for morphological differences between the allopatric and sympatric populations, growth rate differences between the lab bred *N. melanospilus* from allopatric and sympatric populations should be evaluated in a common garden environment without a competitor (Gray et al., 2005; Grudemo & Johannesson, 1999; Losos, 2000). This aspect can be addressed in future studies. Testing to satisfy all six ECD criteria in a single study remains a challenging task, as it demands the integration of fieldwork, large mesocosm experiments,

and a variety of methods across the fields of ecology, evolutionary biology, and genetics. (Losos, 2000; Schluter, 2000; Stuart et al., 2017).

An alternative explanation for the observed differences in the body size and diet of *N. melanospilus* in the presence and absence of *N. eggersi,* might be due to differences in the strength of competition experienced during ontogenetic development. Many species, including fish, use similar resources when small but diverge in niche when larger (Nunn et al., 2012; Werner & Gilliam, 1984). Since *Nothobranchius* spp hatch around the same time (Blažek et al., 2013), it is likely that sympatric species compete strongly for similar (small) food items early in life. This may result in high mortality rates (Terzibasi et al., 2009) and subsequently little recruitment to larger size classes.

To test this alternative explanation, one approach would be to design an experiment that manipulates the strength of both intra- and interspecific competition, e.g., by varying the density of the two species. By measuring survival and growth rates, as well as the diets of individuals, it might be possible to determine the direct effect of competition on development and diet of *N. melanospilus.*

In our study, we investigated the ecological divergence between populations of *N. melanospilus* with and without sympatric *N. eggersi*, because we did not find ponds with only *N. eggersi*. The co-occurrence of *N. eggersi* with *N. melanospilus*, rather than *N eggersi* occurring alone, could be attributed to a complex interplay of ecological and evolutionary factors. One plausible explanation is the existence of a mutualistic or facilitative relationship between the two species, where *N. melanospilus* creates conditions favorable for *N. eggersi*, contributing to their coexistence (Krebs, 2014). Habitat preferences might also influence this dynamic, with *N. eggersi* showing preferences

either for a subset of the conditions preferred by *N. melanospilus* or preferences aligned with the conditions created by *N. melanospilus*. Considering that *N. melanospilus* is more common compared to *N. eggersi*, it is plausible that *N. eggersi* occasionally exists in allopatry but that we did not find such ponds. Finally, historical factors shaped by shared evolutionary processes or historical events may also contribute to the observed patterns of co-occurrence between these species (Krebs, 2014; Molles, 2010; Reichard et al., 2022).

Conclusion

Morphological divergence and the shift in resource use in sympatric *N. melanospilus* may be explained by ecological character displacement between *N. melanospilus* and its congeneric species, *N. eggersi* when they occur in the same pond. In our study, we focused on the ecological divergence between populations of *N. melanospilus* with and without sympatric *N. eggersi* since we did not find ponds with *N. eggersi* alone. Our results show a new strong case for ecological character displacement. Future studies should also examine if *N. eggersi* can be found in ponds without any other *Nothobranchius* species and if morphological divergence and resource use shifts between allopatric and sympatric populations occur in *N. eggersi* too.

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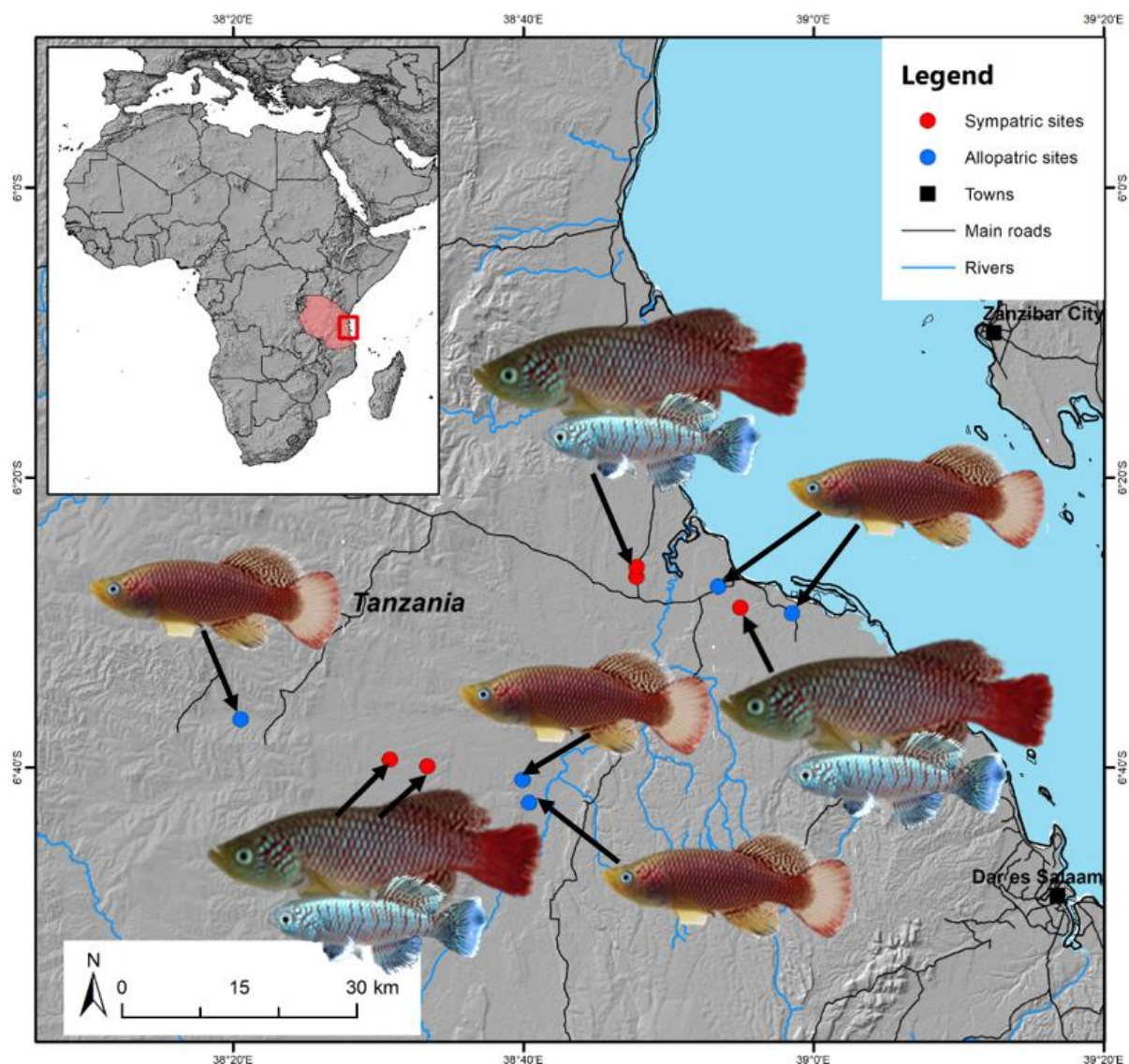
Figure 1. Map of the sampling area (Ruvu River Basin), located in Eastern Tanzania. Blue dots indicate allopatric sampling sites of *N. melanospilus*. Red dots represent sampling sites of sympatric *N. melanospilus* and *N. eggersi.*
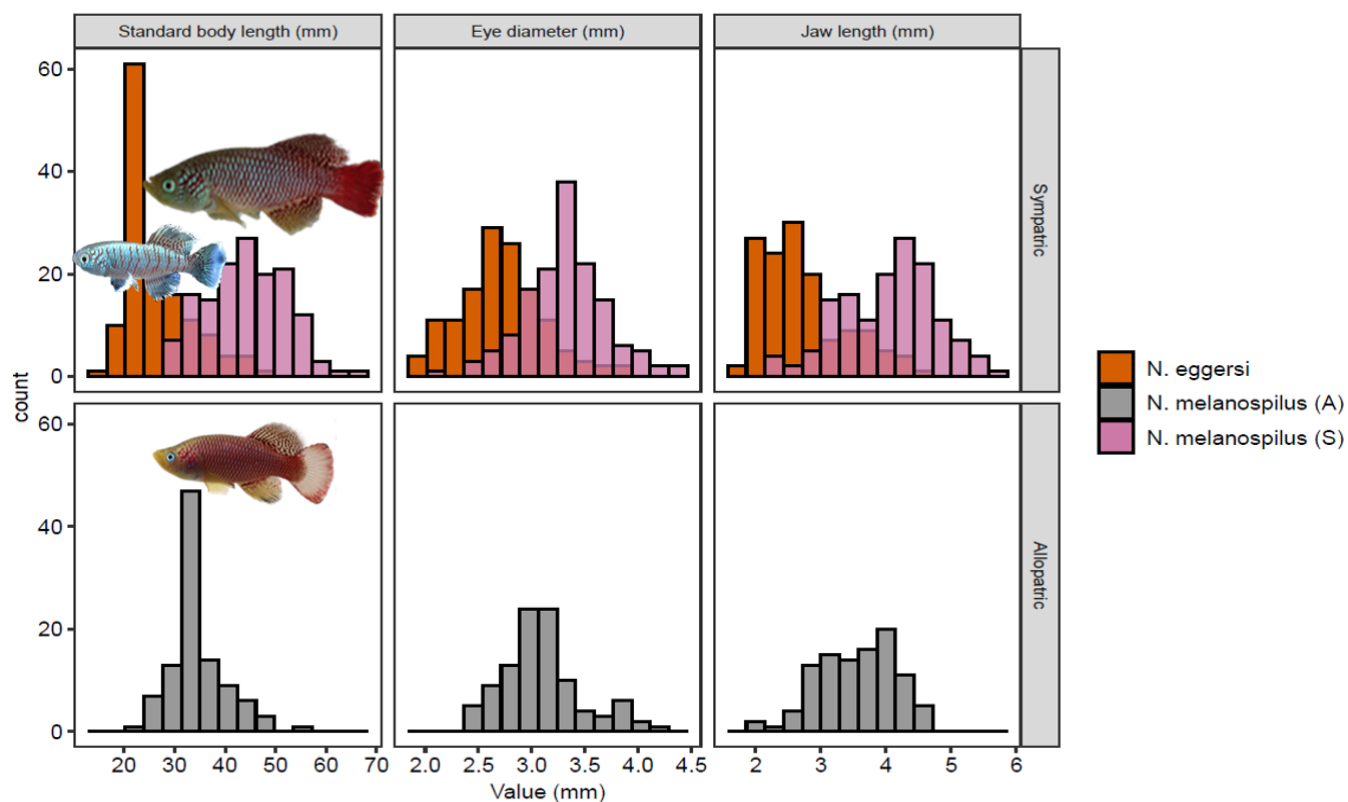
Figure 2. Frequency distributions illustrating the difference in standard length (SL) (left panels), eye diameter (ED) (middle panels), and jaw length (JL) (right panels) between sympatric (upper panel) and allopatric sites (lower panel).
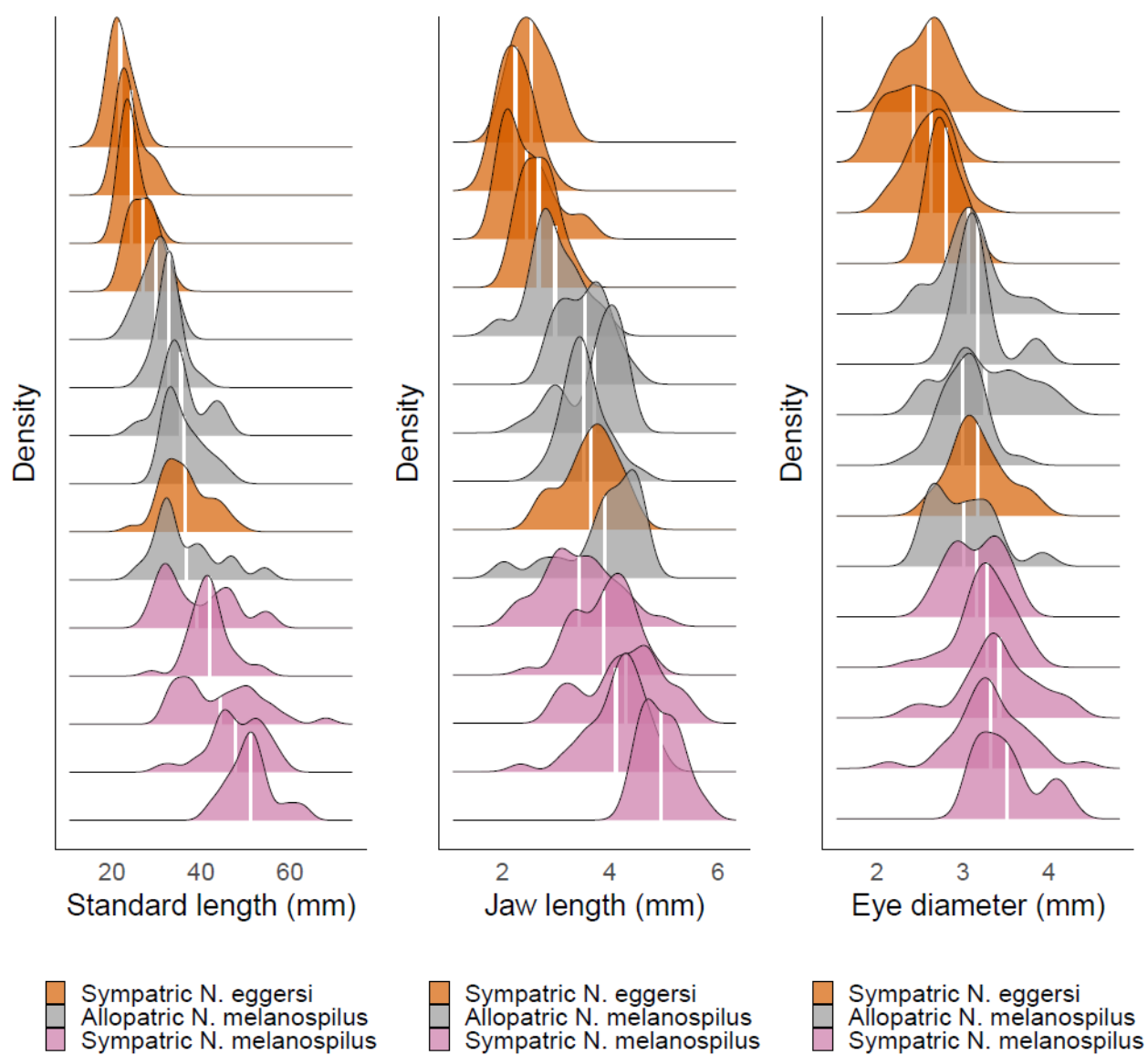
Figure 3. Frequency distribution of trait values in standard length (SL) (left panels), jaw length (JL) (middle panels), and eye diameter (ED) (right panels) for sympatric *N. eggersi*, allopatric *N. melanospilus* and sympatric *N. melanospilus* sampled in 10 sites. The white bars in each distribution indicate the mean value of the population. The sites are ordered based on ascending mean SL values.
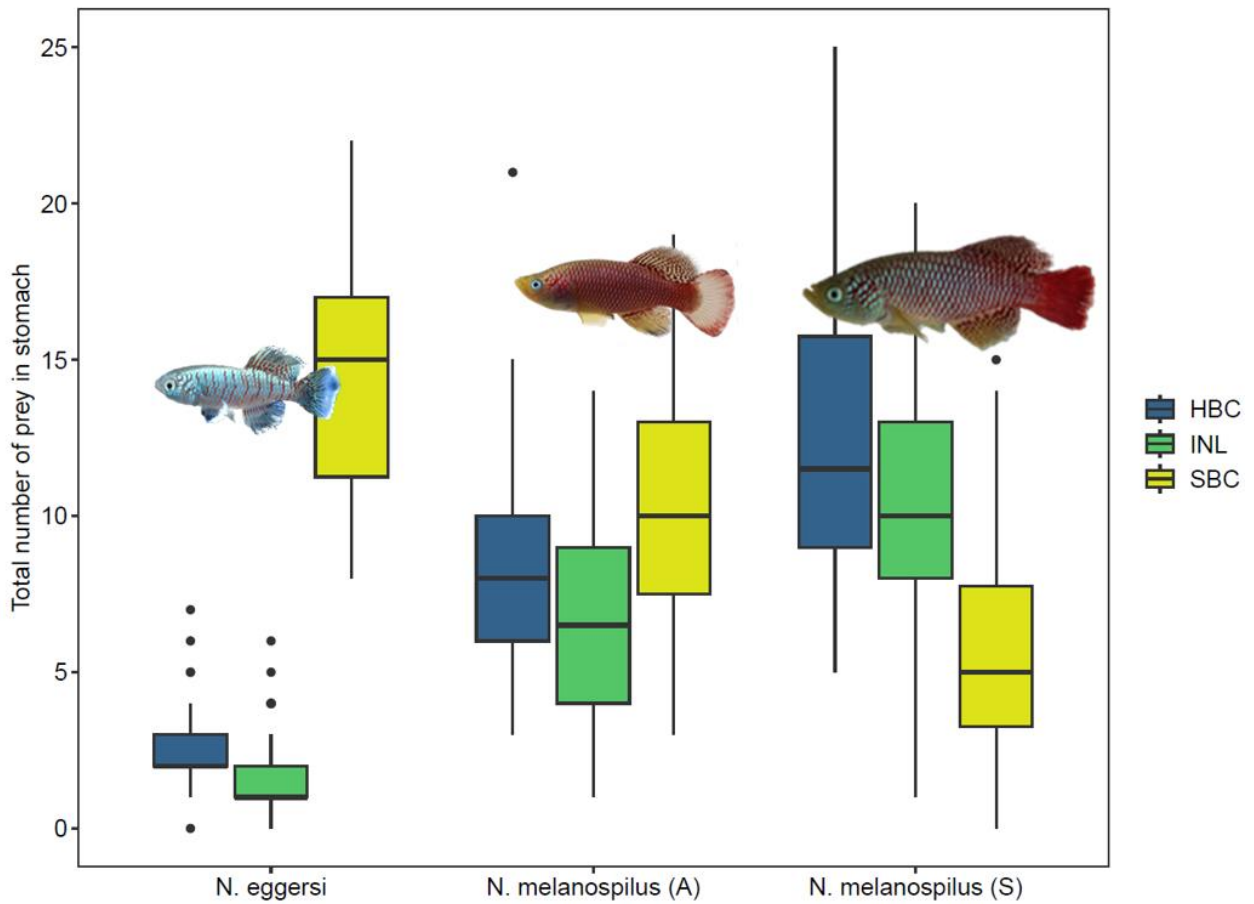
Figure 4. Stomach contents of *N. eggersi* in sympatry, *N. melanospilus* in allopatry, and *N. melanospilus* in sympatry. Box plots displays the median (horizontal line), interquartile range (box), and whiskers (1.5 times the interquartile range) to show the range of variation. Outliers are plotted individually as black points. The prey items are grouped into three major functionally different categories: hard bodied crustaceans (HBC), insect larvae (INL), and soft bodied crustaceans (SBC).
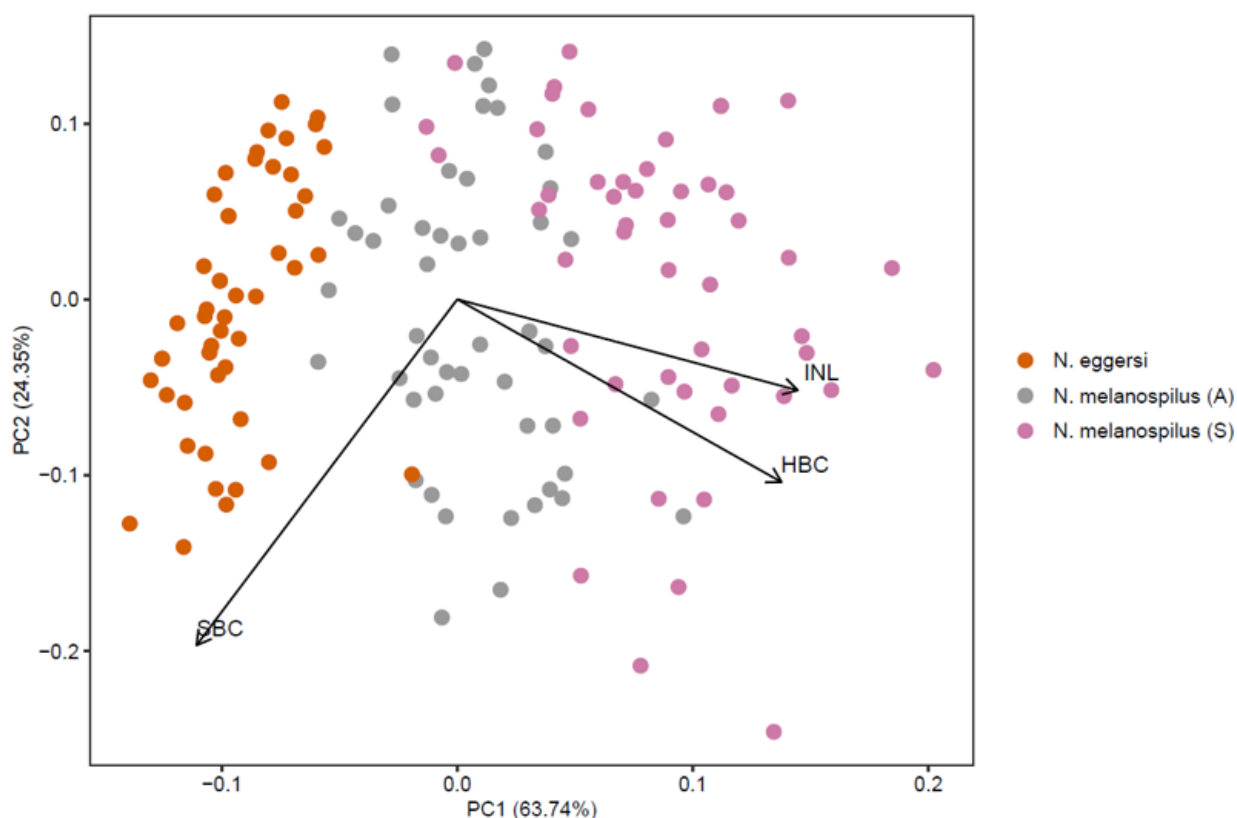
Figure 5. Principal component analysis of killifish prey (5 allopatric and 5 sympatric sites pooled by species), consistent with ecological character displacement in sympatric *N. melanospilus*: *N. melanospilus* in allopatry has a diet intermediate between that of *N. melanospilus* in sympatry and *N. eggersi* in sympatry: soft bodied crustaceans (SBC), hard bodied crustaceans (HBC), and insect larvae (INL).