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Diet in phenotypically divergent sympatric species of African weakly electric fish (genus: Campylomormyrus) – a hybrid capture/HTS metabarcoding approach

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Abstract

Ecological speciation within the mormyrid genus Campylomormyrus resulted in sympatric species exhibiting divergence in their feeding apparatus and electric organ discharge (EOD). This study documents the overall diet of the genus Campylomormyrus and examines the hypothesis that Campylomormyrus radiation is caused by an adaptation to different food sources. We performed diet assessment of five sympatric Campylomormyrus species (C. alces, C. compressirostris, C. curvirostris, C. tshokwe, C. numenius) and their sister taxon Gnathonemus petersii with markedly different snout morphologies and EODs using hybrid capture/HTS DNA metabarcoding of their stomach contents. Our approach allowed for high taxonomic resolution of prey items, including benthic invertebrates, allochthonous invertebrates, and vegetation. Comparisons of the diet compositions using quantitative measures and diet overlap indices revealed that all species are able to exploit multiple food niches in their habitats, i.e., fauna at the bottom, the water surface, and the water column. Major part of the diet is larvae of aquatic insects, such as dipterans, coleopterans, and trichopterans, known to occur in holes and interstitial spaces of the substrate. The results showed that different snout morphologies and the associated divergence in the EOD translate into different prey spectra. This suggests that the diversification in EOD and the morphology of the feeding apparatus is under functional adaptation.

Introduction

African weakly electric fish (Mormyridae) comprise a species rich group of freshwater fish endemic to Africa with more than 200 described species in 20 genera (Lavoué et al. 2003). Within the genus *Campylomormyrus* , 15 species are described native to the Congo River and its tributaries (Feulner et al. 2007). Each species exhibits a species-specific electric organ discharge (EOD). They use the EOD in social communication and pair formation as well as for object location and foraging. Even closely related species markedly differ in their pulse duration (up to 100-fold difference) and/or waveform shape (Tiedemann et al. 2010). Further, they exhibit species-specific morphological traits in their feeding apparatus, i.e. the snout, regarding the snout's length, thickness, and curvature (Feulner et al. 2008). The diversifications in these species-specific traits make *Campylomormyrus* a prime model system to study the role of ecology in driving an adaptive radiation.

The adaptive radiation within the genus *Campylomormyrus* has been studied with regard to molecular genetics (Canitz et al. 2020; Lamanna et al. 2016), electrophysiology (Feulner et al. 2006), morphometry (Feulner et al. 2007; Lamanna et al. 2016), and behavior (Amen et al. 2020; Nagel et al. 2018a,b). In combination,

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these studies suggest an ecological speciation scenario that *Campylomormyrus* radiation is caused by an adaptation to exploit different microhabitats and/or food sources, associated with diversification of the EOD. Indeed, behavioral experiments using sympatric *Campylomormyrus* species revealed an association between differing snout morphologies and preferences for certain types of substrate structure (Amen et al. 2020). Specifically, in a choice experiment, the short snouted species (*C. tamandua*) favored a sandy substrate, while the long snouted species (*C. rhynchophorus*) preferred a stone substrate for feeding.

While these trait-specific substrate preferences appear plausible from a mechanical point of view (i.e., longer snouts allow for probing further into interstitial between stones, Amen et al. 2020), there is currently no information available as to whether different trunk shapes are associated with different diets. Furthermore, it is still not known whether the diversification of EOD serves as a prezygotic isolation factor only (Nagel et al. 2018 a, b) or also is related to foraging specialization (Feulner et al. 2009). Currently, no data on substrate-specific benthofauna are available for the Congo River. However, it has been argued that the duration of the EOD plays a crucial role during food detection by determining the prey items that can be detected best (Harlan Meyer 1982).

Information on diet composition of *Campylomormyrus* is so far limited to only two studies on single species: Roberts & Stewart (1976) reported briefly, based on field observations, some food items found in the stomach of a single specimen assigned to *C. rhynchophorus* . At that time, phylogeny and species delimitation of *Campylomormyrus*was not well established. Nwani et al. (2008) reported the diet composition of *C. tamandua* based on morphological determination. Hence, so far, to the best of our knowledge, there have been no controlled studies which compare the diet composition among*Campylomormyrus* species under natural conditions. Our study aims at contributing to fill that knowledge gap by performing a dietary study for five species of *Campylomormyrus* with markedly different EODs and snout morphologies. Our purpose is not only to document the dietary ranges and components for these species, but also to infer whether species with specific traits (EOD and snout morphology) prefer specific food items.

Direct observation of feeding in the natural habitat, i.e., the Congo river, seems unfeasible and microscopic examination of gut contents may yield incomplete results, as food items may be digested to various degree, compromising their microscopic identification (Pompanon et al. 2012). High Throughput Sequencing (HTS)-based DNA metabarcoding has been successfully used to investigate the DNA extracted from highly degraded diet samples (Deagle et al. 2006; Jarman et al. 2004), however, an initial PCR amplification necessitates DNA fragments of a certain length to allow both for primer annealing and a large-enough species-specific target sequence. A pilot study revealed that this approach is not working for our samples (Lamanna & Tiedemann, unpublished), indicative of low DNA quality. This lowered DNA quality may be attributed to logistical constraints during field work in Congo, i.e.,*Campylomormyrus* are known to be nocturnal feeders, while fish catching activities were only possible at day-time, leaving time for progressed digestion which may have prevented effective direct PCR amplification with standard protocols. As an alternative, DNA hybridization capture (target enrichment) has been successfully applied to enrich low-concentration and highly degraded DNA-fragments from environmental DNA (Shokralla et al. 2012) or ancient sedimentary DNA (Krueger et al. 2022). Here, we apply a combined hybrid capture/HTS shotgun approach to quantify diet composition in gut contents taken from wild-caught African electric fish. We specifically test the hypothesis that the diversification in EOD characteristics and snout shape translates into differences in the diet compositions of*Campylomormyrus.* Thereby, we aim at contributing to our understanding of the adaptive radiation in this genus.

Materials and Methods

Stomach samples collection

Stomach content samples ($n = 24$) were collected from five*Campylomormyrus* species with markedly different EOD and snout morphologies (*C. alces* ,$n = 2$; *C.compressirostris* ,$n = 10$; *C.curvirostris* , $n = 2$;*C. tshokwe*

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, $n = 9$; *C. numenius* $n = 1$; see Lamanna et al. 2016 for phylogenetic relationships, EODs, and snout characteristics). Additionally, stomach content samples of*Gnathonemus petersii*(*G. petersii,* $n = 3$), the sister genus of *Campylomormyrus* , were used for comparison. Unlike *Campylomormyrus* , *G. petersii* has no snout, but a trunk-like protrusion on the head. The fish specimens from which these stomach content samples are extracted were collected during an expedition to the Republic of the Congo in fall 2012. All the analyzed stomach samples were extracted from sympatric fish living in the Congo River rapids south of Brazzaville [S4°18.788' E15deg13.790']. Fish were purchased from local anglers along the northern shore line of the River Congo at Rapides de Kintambo downstream of the Malebo Pool (Runge 2007) north of the island Ile de Singes, where the river bed consists of rocks up to several meters in diameter and the river is very turbid. Before stomach extracting, all fish individuals were weighed and measured for total and standard length and were assigned unique and museum identifiers (see Table S1.1 in Supplementary File S1). After dissection, the stomach samples were stored in Queen's tissue buffer solution (20% dimethyl sulfoxyde, 0.25 M EDTA, saturated with NaCl, pH 8.0; Seutin et al.1991) at 4degC. Before DNA extraction, the stomach contents were visually checked and found to be heavily digested, with very few visible tissue parts remaining.

We assembled 26 stomach contents samples, excluding the *C. numenius* sample due to its low read numbers (Sec. 3.1), to three categories, based on species, EOD duration, and snout length. For species-specific analysis, only the two species with larger sample size, i.e., *C. compressirostris* ($n = 10$) and *C. tshokwe*($n = 9$), were considered. Regarding EOD duration, we formed two groups: Long EOD (>2ms) and short EOD (<0.3ms; note the almost 10fold difference in EOD length among the two groups). The first group (long EOD; $n = 11$) included the samples of*C. alces* and *C. tshokwe* . The second group (short EOD; $n = 15$) included the samples of *C. compressirostris* , *C. curvirostris* , and *G. petersii* . EOD categorization was based on Lamanna et al. (2016). Regarding snout length, we formed three groups: Long snout (*C. curvirostris* and*C. tshokwe* , $n = 11$), medium snout (*C. compressirostris* , $n = 10$), and short snout (*C. alces* and*G. petersii* , $n = 5$). For this classification, we took advantage of a previous geometric morphometrics analysis, which stratified the respective species according to snout length along principal component 1 (Figure 3 in Lamanna et al. 2016).

Handling of animals was conducted at the University of Brazzaville in accordance with the relevant national guidelines and regulations guidelines for the care and use of animals for scientific purposes.

Lysis and DNA extraction

DNA extraction was performed in 2019, appr. 7 years after sampling. Each stomach content sample was homogenized using a Tissue Tip Homogenizer before incubating with lysis reagents. The Qiagen DNeasy Mericon Food Kit for extraction of high-quality DNA from digested foods was used to carry out the DNA extraction from the homogenized samples. Since the stomach content samples were almost digested, the DNA was suspected to be highly fragmented. Therefore, the small fragment protocol of this kit (200 mg) was used according to the manufacturer's protocol. DNA was eluted in the kit's EB buffer (10 mM Tris-Cl, pH 8.5) and stored at -20degC.

Libraries preparation, indexing and amplification

After DNA extraction, all samples underwent single-stranded DNA library preparation using T4 DNA ligase following (Gansauge et al. 2017). In this method, biotinylated adapter molecules are attached to the ends of the DNA fragments via hybridization to a stretch of six random nucleotides belonging to a splinter oligonucleotide complementary to the adapter and nick closure with T4 DNA ligase. The procedure is optimized to highly degraded DNA fragments, as expected in digested food from the stomach. An additional step of deoxyuracils removal using Uracil DNA glycolase was included. Deamination of cytosin to uracil is a typical damage pattern in degraded DNA and could cause erroneous mispairing to adenin, resulting in genotyping errors.

After library preparation, the optimal number of Polymerase Chain Reaction (PCR) cycles required for

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library indexing and amplification was estimated by Quantitative Polymerase Chain Reaction (qPCR). The rationale is to adjust the number of PCR cycles during the library amplification to different DNA concentrations (Basler et al. 2017). The reaction was performed by mixing 1ml of a 1:20 dilution of each library with 3µl of nuclease free water, 5µl of 2 x SYBRTM green qPCR master mix (ThermoFisher Scientic), 0.5µl of qPCR primer IS7, and 0.5µl of primer IS8 (both 10µM; from Gansauge & Meyer 2013). For each library three reaction replicates were analyzed. The PCR reactions were performed with 7min at 95°C, followed by 40 cycles of 10s at 95°C denaturation, 30s annealing at 60°C, and 1min extension at 72°C. Considering the differences in DNA concentration between the qPCR and the indexing PCR, the optimal number of cycles for the indexing PCR was calculated according to Basler et al. (2017).

The indexing primers P5 and P7 were chosen according to the library protocol of Gansauge et al. (2017). The reaction mix for each sample contained 44.8µl of nuclease free water, 8µl of 10 x AccuPrimeTM Pfx reaction mix (ThermoFisher Scientic), 0.8µl of 2.5U AccuPrimeTM Pfx polymerase, 3.2µl of P5 indexing primer, 3.2µl of P7 indexing primer (both 10 µM) and 20µl of each library. Thermocycling started with 2min at 95°C, followed by the number of cycles calculated according to Basler et al. (2017) consisting of 15s at 95°C denaturation, 30s annealing at 60°C and 1 min extension at 68°C. After indexing and amplification, all libraries were purified using the MinElute PCR Purification Kit (QIAGEN) following the manufacturer's instructions. The libraries were quantified with a Qubit 3.0 fluorometer after purification and the distribution of the DNA fragments lengths was measured using an Agilent 2100 bioanalyzer system.

Probe design and DNA hybridization capture

For probe design, we selected target species based on literature research on the potential taxa eaten by *Campylomormyrus* and the expected diversity of prey items in the Congo River region. We targeted 504 species across 6 animal taxa: Arthropoda, Annelida, Mollusca, Nemertea, Nematoda, and Rotatoria. Additionally, we targeted 29 species from 3 plant taxa: Streptophyta, Rhodophyta, and Chlorophyta. For these species, mitochondrial COI gene (animals) and chloroplast rbcL gene (plants) sequences were retrieved from the GenBank sequence database of the National Center for Biotechnology Information (NCBI). When a species of interest did not have sequence information available, sequence information from another species of the same genus was included. If this was not available, the taxon was excluded. The average sequence length of all collected sequences was 700 bp. A complete list of taxonomic names and NCBI accession numbers can be found in the Supplementary File S2. The sequence capture probes were custom-made at Arbor Biosciences (formerly MY croarray) and supplied in form of a MYBaits InSolution Custom Target Capture Kit (designs with 1-20k probes). The probes were designed to achieve a high overall probe count and an increased coverage in high GCcontent regions. The final probe set contained 15284 probes, each with a length of 80bp. The synthesized probes comprise short overlapping fragments representing the whole probe template sequences of COI and rbcL.

The hybridization capture enrichment reactions were performed according to the manufacturer's instructions (Biosciences 2016). Using this method, single-stranded DNA (ssDNA) fragments sharing sequence similarity with predesigned ssDNA- or RNA-baits can be enriched. Following these instructions, the recommended input of the library DNA was 100ng to 500ng. This was equivalent to 7µl of the library material with a DNA concentration of 14 to 72ng/µl. All materials (i.e., pooled across replicates of the same specimen) from the library were used for two rounds of hybridization capture reactions, thereby increasing specificity (Krueger et al. 2021). The captured and amplified libraries of the first round of capture reaction were used as input libraries for the second round. For both rounds of capture, the libraries were not pooled since pooling might produce low quality results (Biosciences 2016). The second captured and amplified libraries were pooled and underwent sequencing on an Illumina MiSeq instrument using a protocol for 2x150bp paired end of double indexed libraries (Biosciences 2016).

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Bioinformatics, data analysis, and statistics

Before further analyzing the high throughput sequencing reads, which were generated in FASTQ format, we performed a quality processing to remove the adapter sequences and the low-quality reads, using Cutadapt version 2.10 (Martin 2011). Quality cutoff, minimal sequence length, and minimal overlap between sequence and adapter were set to 20, 30, and 3, respectively. Then, we performed quality control checks to the filtered sequences using FastQC (Andrews 2010) to ensure their suitability for further analysis.

Taxonomic classification for the filtered sequences was done using Kraken version 2.0.9, a k -mer-based approach which provides a fast taxonomic classification from the sequence data (Kraken2; Wood et al. 2019). Kraken compares the reads of the metagenome to short sequences of length k, the so-called k-mers, from a database that is associated with the sequence information underlying the tree of life phylogeny. The algorithm then places the read on the tree of life, as well as the taxon's ancestors, based on its similarities to these$k$-mers, in accordance with its annotation at the lowest taxonomic level. During the taxonomic classification process using Kraken2, specific weights are allocated to each node based on the k-mer paths. This weight assignment improves the sensitivity of taxon classification, ensuring more accurate and precise results.Before applying Kraken2, a custom marker database of available eukaryotic COI and RBCl sequences from NCBI was built to map the reads. The results of Kraken2 were visualized and inspected using the online tool Pavian metagenomics data explorer (Breitwieser & Salzberg, 2019).

The read counts were used as a semi-quantitative estimate of the relative abundance of food taxa in the diet of each sample. For any sample, these count data were used to record both the occurrence (presence/absence) of a taxon and the percentage of reads assigned to that taxon. The frequency of occurrence (%FOO) is used to quantify occurrence across samples. The relative read abundance (RRA) is used as a proxy for relative biomass consumed. Details on the calculations of these measures are given in the Supplementary File S3.

The dietary niche width was assessed by calculating the Shannon diversity index (alpha diversity) for each sample using the *spaa*R package (Zhang 2016). Further, to describe the diet overlap among our categories (species, EOD duration, snout length), we used two complementary metrics: Schöner index (Schoener 1970) and Pianka index (Pianka 1974). Pairwise calculations of these indices were performed using the *spaa* R package (Zhang 2016). The equations of the indices are given in the Supplementary File S3. We used the*EcoSimR* R package (Gotelli et al. 2015) to compare the results with reference to 1000 permutations of a null model that holds the same dietary niche width, while randomizing the values for the diet items. We further calculated Bray–Curtis dissimilarity indices (beta diversity) between each pair of samples using the *vegdist* -function in the*vegan* R package (Oksanen et al. 2019).

Additionally, we performed nonmetric multidimensional scaling (NMDS) using the function *metaMDS* in the *vegan* R package to visualize the patterns of dietary dissimilarity among samples. Discriminant Analysis of Principal Components (DAPC), using the *dapc* function in the*adegenet* R package (Jombart, 2008), was performed on the three categories, i.e. species, EOD duration, and snout length, to identify dietary items that are significant contributors to dietary differences among species/phenotypic groups. We tested for differences in diet composition among the groups by performing a permutational multivariate analysis of variance (perMANOVA) with 999 permutations, using the*adonis* -function in the *vegan* R package.

Results

Sequencing results

The sequencing for all samples generated 6.9Gb raw sequencing data of FASTQ formatted reads. The raw data have been deposited to the open repository Zenodo (Amen et al. 2022). The total number of raw sequences from the 27 stomach samples is 19,531,074, ranging from 19,226 to 867,093 per individual sample ($Mean = 335,020; SD = 234,220$). Out of them, a total of 18,357,160 sequences were obtained after quality

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filtering, ranging from 16,703 to 828,735 per individual ($Mean = 314,421$; $SD = 222,465$; see Supplementary File S4 for details).

Additionally, a series of negative (library and DNA extraction) and positive controls, which were treated identically to other samples from initial processing through library preparation, was included to further filter the data and validate the obtained taxa. The positive control sample was chosen as a stomach content sample of a fish (*C. compressirostris*) raised and fed on a known food in the laboratory. Of the total sequence pool generated, we detected 0.013 % from library controls and 0.019 % from DNA extraction controls.

Taxonomic classification

The taxonomic classification of the filtered sequences against the custom marker database using Kraken2 classified all the reads into seven phylogenetic levels (domain, kingdom, phylum, class, order, family, genus, and species) or unclassified reads. The percentage of the unclassified reads reached 33.22 % of the pooled sequences of all samples. On the individual level, the percentage of unclassified reads ranged from 8.38 % to 71.67 %. The high percentage of unclassified reads is attributed to sequences outside the range of our custom marker database (eukaryotic COI and rbcL only). When the entire nucleotide database (www.ncbi.nlm.nih.gov/nucleotide/) is used instead of our custom database, the percentage of unclassified reads dropped to 10.11 % of the pooled sequences of all samples. We limited the analysis to the taxonomic classifications using the eukaryote COI/rbcL custom database, however, to keep the focus on the diet analysis (instead of prokaryotes in the gut microbiomes).

Comparing the distributions of the reads for the food taxa classified at each phylogenetic level reveals that most of the reads are assigned to few food taxa, while many of the classified taxa are represented by only a few reads. To determine which food taxa should be included in subsequent diet analyses, we subtracted the number of sequences of each taxa present in the negative controls from the sequence abundance of that taxa in the samples, following Nguyen et al. (2015). Further, we excluded records from primates and birds, which were presumably contamination. Taxa represented by less than 0.01 % of reads were further excluded, as they may constitute contamination/background noise (Alberdi et al. 2018). This approach substantially decreased the sequence yield of the *C. numenius*sample, so this sample was eliminated from the subsequent analyses.

The remaining identified prey items in each sample were assigned to different taxonomic levels, i.e., to 20 classes, 34 orders, 90 families, 115 genera, and 127 species. For the species with larger sample sizes (*C. compressirostris* and *C. tshokwe*), the classified reads are visualized using the metagenomics data explorer Pavian and tabulated in Tables S5.1 and S5.2 (Supplementary File S5).

Relative abundance

Campylomormyrus and*G. petersii* had a broad spectrum of prey items. On the class level, Insecta dominated by far. They were found in all samples, representing more than 90% of the total reads (Figure 1). Other classes were also found in all samples but with less percentage of the total reads, such as Clitellata, Arachnida, Malacostraca, and Hexanauplia. At the order level, the most abundant prey items were Diptera, Coleoptera, and Hymenoptera (all belong to the class Insecta), all of which were in all samples (Figure 1). Also, the Haplotaxida (Clitellata) and Araneae (Arachnida) were found in all samples. Further insect orders such as Lepidoptera, Trichoptera, Ephemeroptera, and Hemiptera, as well as Decapoda (Crustacea) were found in more than 83 % of the samples.

The Relative Read Abundances (RRA) of the primary food taxa (excluding taxa with RRA $<$ 0.01%potentially stemming from secondary predation,) for the three grouping categories are depicted in Figure 2. Most groups share similar food taxa, albeit in different proportions. The most dominant prey taxon are insects (Diptera, Coleoptera, Hymenoptera, and Lepidoptera), annelid worms (Haplotaxida), spiders (Araneae), crustaceans (Amphipoda, Diplostraca, and Decapoda), Copepoda (Calanoida), and plants

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(Cupressaceae). The RRA for individual samples are given in the supplement (Supplementary File S6).

The RRA data were used to assess the dietary niche width by calculating the Shannon diversity index (Figure 3 and Table S7.1 in the Supplementary File S7). The Shannon diversity index differed considerably among the studied groups, pointing towards different dietary niche widths across the two species *C. compressirostris*and *C. tshokwe* species and relative to EOD duration and snout length.

Diet overlap

The diets among groups of the three categories (species, EOD duration, and snout length) significantly overlap at class and order phylogenetic levels (Table 1). At these levels, Pianka index values showed statistically significant niche overlap based on comparison with 1,000 null models (see the Supplementary File S8 for more details) and Schöner index scored more than 0.6 for all groups. At lower taxonomic level (family, genus, species), there was much less overlap in the diet among species. The degree of diet overlap is further confirmed by the Bray–Curtis dissimilarity index (0: similar; 1: dissimilar), as shown in Table S7.2 in the Supplementary File S7.

As many reads could not be assigned to family, genus, or species level (see Table S4.1 in Supplementary File S4), we performed further statistical analyses on read assignments at the order level. A perMANOVA on the Bray–Curtis dissimilarity index data derived from the RRA values at order level indicates significant dietary differences among *C. compressirostris* and *C. tshokwe* (F = 4.64,r^2 =0.215, p [?]0.001, df =1).

A perMANOVA on the EOD duration groups (long *vs.* short EOD) indicated significant dietary differences between the two groups (F =5.34, r^2 =0.182, p [?]0.001,df =1, Bray–Curtis dissimilarity index data derived from the RRA values at order level). Similarly, for snout morphology (short*vs.* medium *vs.* long), a perMANOVA on the Bray–Curtis dissimilarity index data (derived from the RRA values at order level) showed significant dietary differences among the three groups (F =2.50, r^2 =0.182, p [?]0.01,df =2). The post hoc pairwise perMANOVA using a Bonferroni correction of the p -values indicated that only the long snout versus the medium snout and the long snout versus the short snout differences are statistically significant (p [?]0.001 and$p$ [?]0.05, respectively).

DAPC on group-specific diet discriminated across species (only *C. compressirostris* and *C. tshokwe*), EOD duration, and snout length (see Supplementary File S9). It inferred also the significant contributors to dietary differences (1) among *C. compressirostris*and *C. tshokwe* (Calanoida, Ephemeroptera, and Diptera orders), (2) among fish with long and short EOD duration (Calanoida, Diplostraca, Diptera, and Ephemeroptera), and (3) among long and short snout (Ephemeroptera, Calanoida, and Hymenoptera; Supplementary File S9).

The patterns of dietary difference among samples are visualized in Figure 4 by ordinating the Bray–Curtis dissimilarity index values in two dimensions using NMDS. The stress level for the NMDS was 0.152, which indicates a good representation (Clarke 1993). The NMDS plot shows the segregation of samples based on EOD and the degree of diet overlap/dissimilarity based on species and snout length.

Discussion

Diet profile

The DNA metabarcoding method provided a clear taxonomic resolution of the partially digested stomach content and potentially detected highly diverse food taxa. This high taxonomic resolution is in line with those of previous dietary studies based on DNA-based approaches (e.g., Harms-Tuohy et al. 2016; Pan et al. 2021; Rees et al. 2020; Sakaguchi et al. 2017). Our results indicate that the diet of *Campylomormyrus*and *Gnathonemus* species is composed mainly of three types of prey items, i.e., benthic invertebrates, allochthonous invertebrates, and macrophyte material. The most dominant prey taxa found in

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the gut contents of these species belong to benthic invertebrates, especially aquatic insects. In particular, dipterans (Chironomidae, Simuliidae, Drosophilidae, and Tephritidae), coleopterans (Zopheridae, Carabidae, Histeridae, and Scarabaeidae), trichopterans (Hydropsychidae, Lycaenidae, and Elateridae), ephemeropterans (Leptophlebiidae, Baetidae, and Ephemerellidae), and odonatans (Coenagrionidae, Chlorocyphidae, and Chlorogomphidae) are important diet constituents of all the species. The larvae of these insects live usually in holes and interstitial spaces of the riverbed. Although the DNA approach used here cannot tell the stage of the aquatic insects found in the diet, it is reasonable to assume that the aquatic larvae, rather than the terrestrial imagines, were targeted by *Campylomormyrus* and *Gnathonemus* . Indeed, larvae of aquatic insects were reported as food items found in the stomach of mormyrid fish (Blake, 1977; Hyslop, 1986).

Beside aquatic insects, annelid worms (such as Glossoscolecidae, Naididae, and Megascolecidae) were also found in the diet of all *Campylomormyrus* and *Gnathonemus* species. Similar to the insects' larvae, the annelid worms hide in mud and among aquatic vegetation in the substrate of the riverbed. Other benthic invertebrates found were freshwater snails (Gastropoda, orders Pachychilidae and Stylommatophora), and crustaceans (Malacostraca, orders Decapoda, Copepoda, Cladocera, and Amphipoda). Previous studies on the diet of mormyrids using morphological observations did not report annelid worms. The absence of this food taxon may be due to the progressed digestion before the morphological examination.

The second group of food items found in the diet of *Campylomormyrus* and *Gnathonemus* species is allochthonous invertebrates. The most abundant prey taxa from this group are Hymenoptera (including Formicidae, Mymaridae, and Braconidae) and Lepidoptera (including Nymphalidae, Lycaenidae, and Hepialidae). Additionally, Araneae (Arachnida) were frequently found in the diet. These food items are usually located in the habitat at the surface of the water.

The third group of food items is plants, including grasses, such as Poaceae of the Poales order, and flowering plants, such as Fabaceae and Asterales.

It must be noted that we cannot exclude some of these taxa having derived from the diet of the primary prey (secondary predation; Sheppard et al. 2005) or comprise small organisms and plant debris unintentionally ingested during grasp suction. However, the stomach contents of *Campylomormyrus* and *Gnathonemus* species found in our study using a DNA metabarcoding approach are compatible with *Campylomormyrus* mainly (about 90 %) feeding on aquatic insects (Nwani et al., 2008; Roberts & Stewart, 1976). A previous study, based on morphological observation (Roberts & Stewart, 1976), reported that stomach contents of some *Campylomormyrus* species contain larvae of chironomids, *Povilla* , trichopteran, ephemeropterans and odonates, dead plant debris, and decomposing animal debris. Roberts & Stewart (1976) reported also that the stomach content of a specimen of *C. rhynchophorus* had Chironomidae, Simuliidae, and trichopterans, and a few small ephemeropterans. Another study on the stomach contents of *C. tamandua* using morphological observation reported similar food taxa (Nwani et al., 2008). The few available dietary studies on other fish species inhabiting the Congo River such as *Schilbe intermedius* (Dirat et al. 2019) and *Distichodus antonii* ,*D. affinis* and *D. lusosso* (Zebe et al. 2010) showed similar prey spectra.

Diet comparison among *Campylomormyrus* phenotypes

The dietary compositions among *Campylomormyrus* and *Gnathonemus* suggest that their feeding behavior is related to phenotypical traits (EOD duration and snout morphology). Based on our results, , different prey spectra were found in the stomach of the different groups of *Campylomormyrus* species, which may relate to species exploiting diverse food niches in their habitats.

For instance, the prevalence of benthic invertebrates, such as larvae of dipterans and coleopterans and annelid worms, in the diet of all species suggests that they exploit the bottom of the riverbed, while the occurrence of allochthonous invertebrates, such as Formicidae, Nymphalidae and Arachnida spiders, may indicate a certain degree of surface feeding. Further, the diets also include food items from the water column, such as copepods. Accordingly, these species may exhibit high trophic flexibility and diverse feeding behaviors. However, the RRA results revealed significant differences among the dietary compositions among the species,

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potentially associated with EOD and snout length. For example, the diet of *C. compressirostris* contains more dipterans, while the diet of *C. tshokwe* contains more coleopterans.

Species with long EOD had preferentially fed on other taxonomic groups (i.e., coleopterans, ephemeropterans, spiders, annelids), compared to the species with short EOD, where dipterans dominated in the diet. Similar differences were found according to snout length. Note, however, that species with long EOD often also exhibit a long snout (Fig. 4; cf. Lamanna et al. 2016).

Dietary analysis and the radiation scenario

Our study was motivated by potentially providing further support for the hypothesis that radiation of *Campylomormyrus* is caused by an adaptation to different food sources, associated with diversification of the EOD. The results proved that all the species tested in the current study are able to exploit diverse niches, especially the bottom fauna, regardless of their snout shape and EOD.

The current study still provides some evidence that different snout morphologies and the associated divergence in the EOD translate into different prey spectra. As a different morphology of the feeding apparatus among the species may constitute a functional adaptation to exploiting different substrates (Amen et al. 2020), the different prey spectra could also reflect differential availability of different prey in the respective microhabitats. Our results cannot establish a causal link between EOD characteristics and prey spectra, yet they are compatible with the hypothesis that the divergence in EOD could also be of adaptive value during feeding, beside its proven function as a prezygotic isolation mechanism. In this case, EOD would be a 'magic trait' triggering both adaptation and reproductive isolation (Feulner et al. 2009). One approach to further investigate a potential dual function of the EOD divergence (feeding specialization and reproductive isolation) would be to expand on the choice experiments performed by Amen et al. (2020) by presenting a variety of food items. This will though still be confounded by the association of long EOD with long snout. Ultimately, one would like to know prey-specific detection probabilities, relative to the physical properties of the different discharges. To achieve such knowledge remains a challenge.

The presence of distinct prey spectra among *Campylomormyrus*species with different snout morphologies implies a potential association between snout morphology and feeding behavior. Specifically, the species-specific snout morphology in *Campylomormyrus* may be related to variations in their grasp suction mode of feeding, as previously described by Marrero and Winemiller (1993). Enhanced grasp suction enables them to obtain the aquatic insects that burrow into the different substrate structures. This functional foraging specialization allows for an efficient exploitation of the rich bottom fauna of benthic invertebrates, especially aquatic insect larvae, which other fish may not reach. Such trophic specializations may have triggered the observed radiation of *Campylomormyrus* in the Congo River.

Potential limitations of our dietary analysis

Sample sizes and quality of DNA. Our study was limited by the availability of stomach content samples we collected from the field, i.e., the Congo River. Despite of a four weeks effort during our 2012 expedition, we did not yield equally high sample sizes for all target species, such that the small number of individuals per species did not allow for diet comparisons among the individual *Campylomormyrus*species, except for *C. compressirostris* and *C. tshokwe* . Moreover, all species examined here are nocturnal, so the stomach contents are expected to be highly digested, due to the time between the last meal and the collection during day time. Our samples had been stored for relatively long time (7 years) prior to DNA extraction. We followed established protocols to store the samples (Seutin et al. 1991) which had been successfully applied for degraded samples (Epp et al. 2010; Stoof-Leichsenring et al. 2012). Note also that *Campylomormyrus* tissue samples taken and stored under the same conditions (i.e., during Congo expeditions in 2004, 2006, and 2012) yielded amplifiable DNA (entire cytochrome b gene, >1000bp) when analyzed up to 10 years later (Lamanna et al. 2016). While we cannot exclude that storage may have contributed to DNA degradation, our positive control (a fresh sample taken from a *C. compressirostris* raised in our lab and fed with defined

food) yielded a similar read number (419,872) as the stomach samples taken in Congo and stored for several years prior to analysis ($Mean = 335,020; SD = 234,220$). We hence conclude that progressed digestion may be the major cause of the DNA degradation encountered in our study.

Relative Read Abundance (RRA) and biomass correlation. The number of reads belonging to a particular food taxon is utilized as a proxy for the biomass of this taxon to obtain semi-quantitative measures on the relative amounts of different food taxa consumed by *Campylomormyrus* and *Gnathonemus* species. This approach is used in most of the dietary studies, and it implicitly assumes a correlation between the number of sequences and the biomass. The logic behind this assumption is that a large biomass of a particular food item in the diet would translate into a large amount of DNA in the dietary sample and, consequently, more recovered DNA sequences (reads). However, potential biases may arise from technical (such as PCR efficiency, primer choice, DNA extraction, etc.) and biological issues (such as among-prey species variation in tissue cell density, survival of DNA during digestion, and the state of digestion), obscuring the quantitative signal. Despite of these caveats, RRA has been considered valid as a proxy for abundance in eDNA (e.g., Kiemel et al. 2022) and sedaDNA (e.g., Krueger et al. 2021) studies. We hence argue that utilizing RRA as a semi-quantitative measure of prey biomass – with all caution – provides relevant additional information relative to merely reporting the presence of prey taxa, which implicitely overstates the importance of rare food items. Moreover, technical biases may affect all samples in a similar manner and hence not compromise our comparison of relative diet composition among different EOD/snout phenotypes.

Taxonomic resolution of dietary items. The dietary differences among the species and phenotypes are more visible in the low taxonomic levels, i.e., at genus and species levels. However, the relatively low percentages of reads assigned to the family, genus, or species level (see Sec. 3.4) may compromise the confidence in the inferred prey spectra at these taxonomic levels. Therefore, we limited the statistical analysis of diet overlap and assessment to the order level (see Sec. 3.4) which we argue could be more reliably inferred (as atested by a lower percentage of unassigned reads). Notwithstanding this limitation, the study confirmed significant differences in the diet among EOD and snout phenotypes. It is plausible to expect that these differences may be even more pronounced at the species/genus levels.

Conclusions

We analyzed stomach contents of wild-caught *Campylomormyrus* fish using hybrid capture and subsequent HTS DNA metabarcoding. Despite of the highly degraded nature of the dietary material, this approach provided a picture of the overall diet of this genus and revealed dietary diversity, relative to divergent phenotypical traits (EOD and snout). Although standard metabarcoding is technically less demanding and may be preferred for analysis of stomach samples if DNA quality is sufficient (Deagle et al. 2006; Jarman et al. 2004), hybrid capture provides an alternative for samples of progressed digestion and associated high DNA fragmentation. Generally, hybrid capture and subsequent HTS has been found superior to direct PCR amplification, when small fragment sizes are encountered, as in aDNA (e.g., Paijmans et al. 2016) and sedaDNA (e.g., Krueger et al. 2021). Our results confirmed that *Campylomormyrus* species can exploit multiple food niches in their habitats (fauna at the bottom, at the water surface, and in the water column) to feed on benthic invertebrates, allochthonous invertebrates, and vegetation. alpha and beta dietary diversity were significantly associated with differences in EOD duration and snout length. Divergence in these traits may constitute functional adaptations to exploiting different food sources and substrates and may underly the radiation and sympatric coexistence of the numerous species of this genus.

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Data Accessibility and Benefit-Sharing Section

Data Accessibility Statement

Raw sequence reads are deposited in the open repository Zenodo (Amen et al., 2022). The codes used to analyze the data and generate the results can be found on GitHub repository DATMetaB:

https://github.com/RahmaAmen/DATMetaB

Benefit-Sharing Statement

Benefits from this research arise from the sharing of our data and results on public databases as described above as well as the codes developed to analyze the data.

Author Contributions

RA participated in the design of the study, carried out the experiments, analyzed the data and drafted the manuscript; KH participated in carrying out the experiments and revised the manuscript; RT and FK conceived the study, organized and participated in sample collection, participated in the design of the study, revised the manuscript, and coordinated the study. All authors have read and approved the manuscript for publication.

Tables and Figures

Tables

Table 1: Pairwise diet overlap indices (Pianke index, O/ Schöner index, α) of three groupings of Campylomormyrus and one species of its sister genus Gnathonemus based on RRA data considering food taxa at different phylogenetic levels (Class, Order, Family, Genus) based on pooled samples. The comparisons are done based on EOD duration (short EOD, $n = 15$, and long EOD,$n = 11$) and snout length (short snout, $n = 5$, medium snout,$n = 10$, and long snout, $n = 11$).

Diet overlap among fish with different EOD duration [short EOD and long EOD]

Class	Class
0.99/0.92	**0.99/0.92**
Diet overlap among fish with different snout length [short snout (S), medium snout(M), and long snout (L)]	Diet overlap

Class		Class	
		M	
S		**0.99/0.92**	
M			
Family		Family	
		M	
S		**0.93**/0.60	
M			

*Bold numbers indicate statistically significant niche overlap (i.e., Pianke index is greater than that expected by chance based on comparison with 1,000 null models, $\alpha = 0.05$, and Schöner index is greater than 0.6).

Figures

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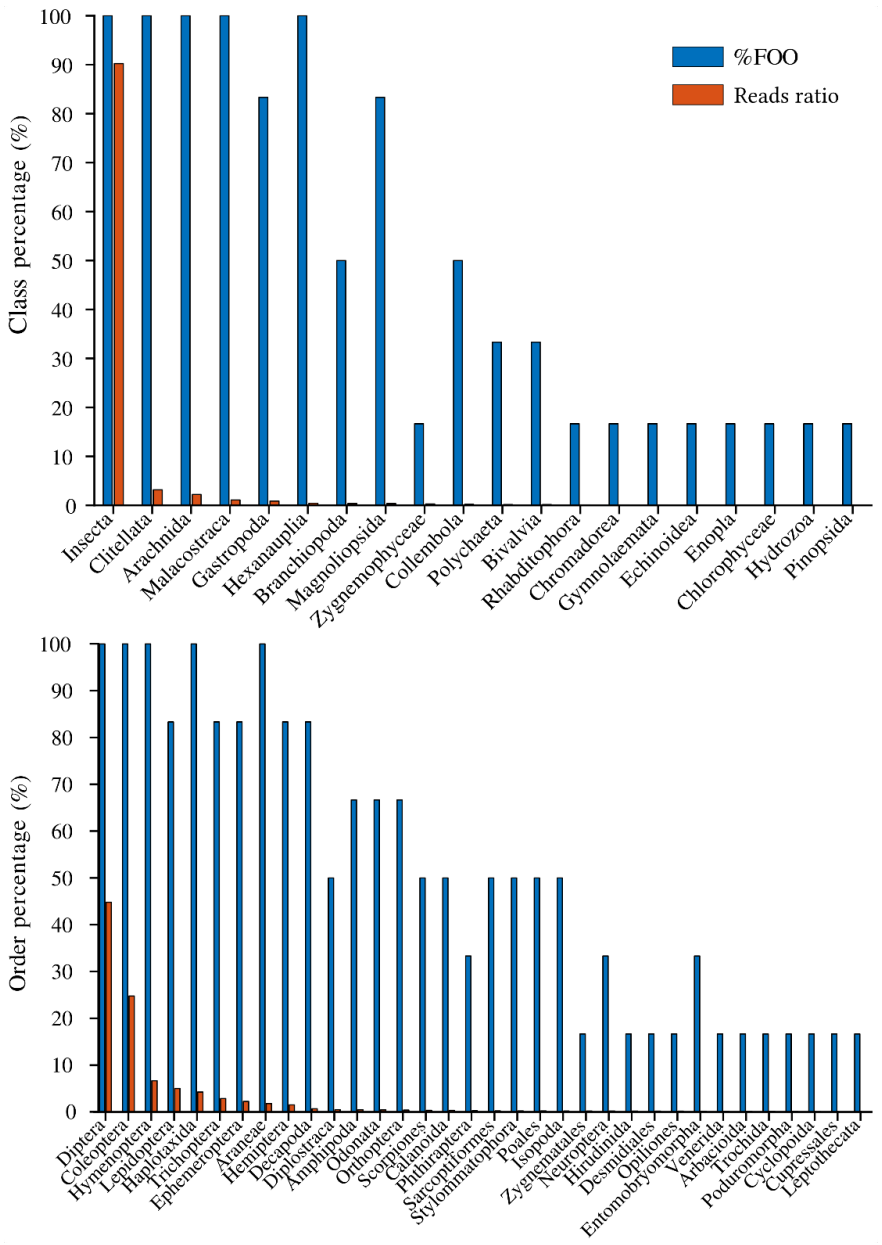

Figure 1: Overall diet composition among all samples (Campylomormyrus,n = 24, and Gnathonemus, n = 3) based on frequency of occurrence (%FOO, shown in blue bars) at class (upper panel) and order (lower panel) levels. Taxa are ordered based on the reads percentage abundance (shown in orange bars) of the total reads (4,361,220 and 18,920,020 reads for class and order levels, respectively). Only taxa which scored more than 0.01 % of the total reads after filtering are considered.

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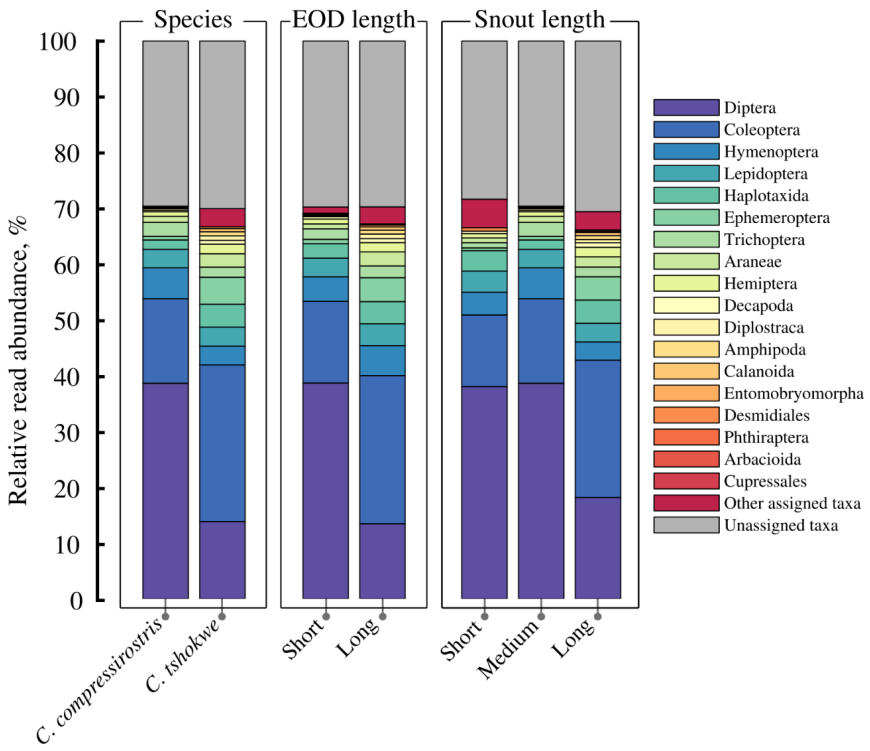

Figure 2: Comparison of relative read abundance (RRA) of food taxa from dietary metabarcoding data sets of stomach contents of Campylomormyrus and its sister genus Gnathomemus at order level. Samples are grouped based on species (only for C. compressirostris, $n = 10$, and C. tshokwe, $n = 9$), EOD duration (short duration, $n = 11$, and long duration, $n = 15$), and snout length (short snout, $n = 5$, medium snout, $n = 10$, and long snout, $n = 11$).

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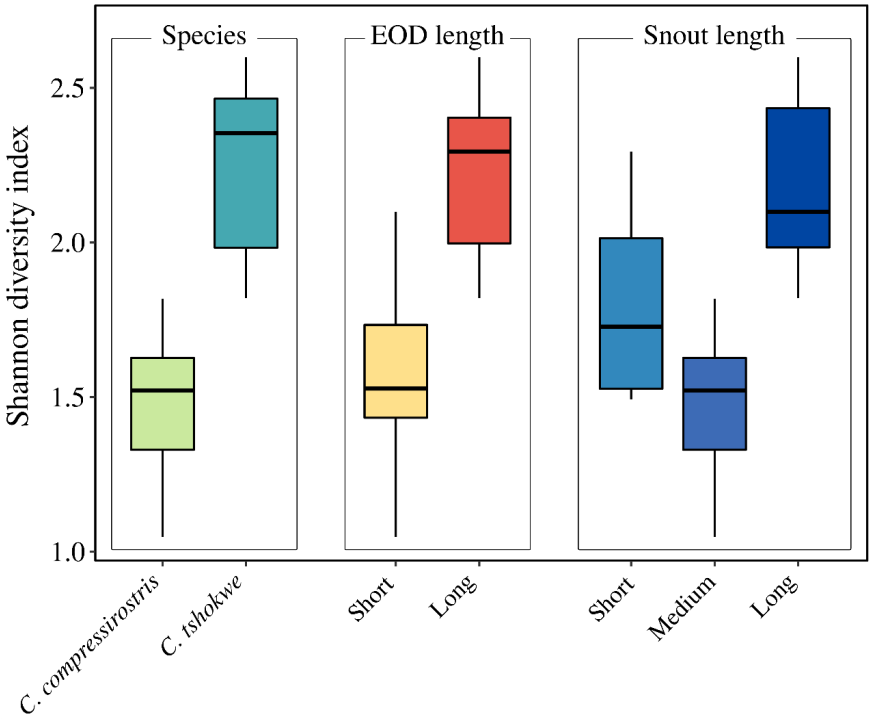

Figure 3: Boxplot of Shannon diversity indices of food taxa from dietary metabarcoding data sets of stomach contents of 25 samples of Campylomormyrus and one sample of its sister genus Gnathomemus based on RRA at order level for species, EOD duration, and snout length groups (cf. Fig. 2).

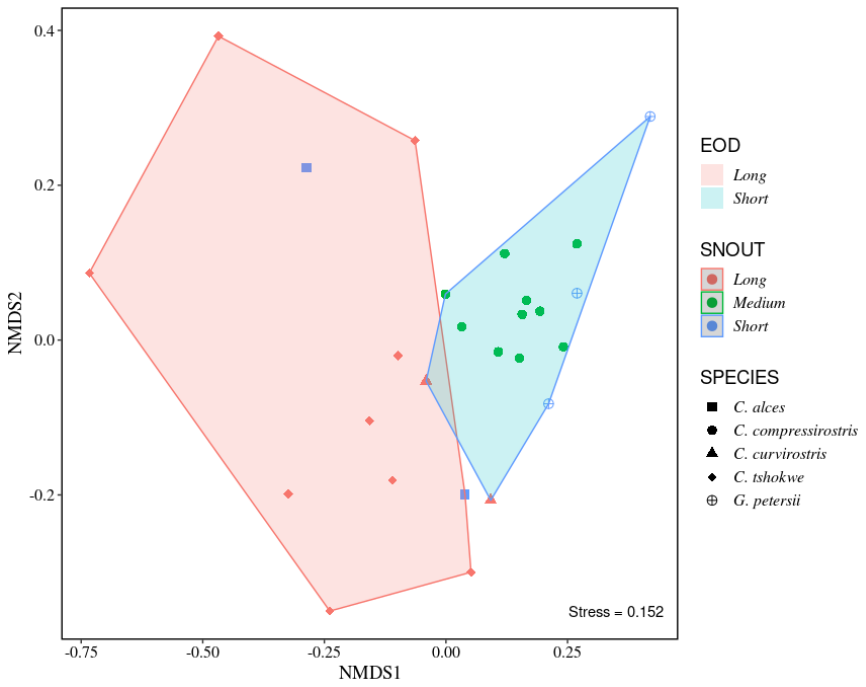

Figure 4: Dietary niche partitioning within and among individual samples of *Campylomormyrus* and its sister genus *Gnathomemus* by nMDS of RRA-based Bray–Curtis dissimilarity indices of samples on the order level (adonis: comparison across EOD phenotypes F = 5.34, R^2 = 0.182, p [?] .002; across snout phenotypes F = 2.50, R^2 = 0.179, p [?] .002). The symbols represent the species and the colors represent snout length. The shaded convex hulls indicate short vs. long EOD. The stress level of 0.152 is under the cut-off value of 0.2 as recommended by Clarke (1993) to indicate an interpretable ordination.

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