

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 the *Campylomormyrus* radiation is caused by 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, that is fauna at the bottom, the water surface and the water column. A 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 indicate that different snout morphologies and the associated divergence in the EOD could translate into different prey spectra. This suggests that the diversification in EOD and/or morphology of the feeding apparatus could be under functional adaptation.

KEYWORDS

dietary metabarcoding, ecological speciation, feeding behaviour, next generation sequencing, weakly electric fish

1 | 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), a phenomenon where electric fishes produce electric discharges using a specialized electric organ composed of multiple electrically charged cells called electrocytes. The sum of action potentials propagated by the electrocytes is referred to as the externally measurable EOD. These discharges play a key role in social communication and pair formation as well as for object location and foraging. Even closely

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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 morphologies regarding their feeding apparatus, that is the snout, regarding the snout's length, thickness, and curvature (Feulner et al., 2008). Within the genus *Campylomormyrus*, there is no apparent linkage between duration of the EOD and the length of the snout, that is both long and short EODs can co-occur with snouts of different lengths (Figure 1; see also Lamanna et al., 2016), yet both traits directly relate to foraging. Therefore, the diversification in these species-specific traits makes *Campylomormyrus* a valuable 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 behaviour (Amen et al., 2020; Nagel, Kirschbaum, Engelmann, et al., 2018; Nagel, Kirschbaum, Hofmann, et al., 2018). In combination, these studies suggest an ecological speciation scenario that the *Campylomormyrus* radiation is driven by adaptation to exploit different microhabitats and/or food sources, associated with diversification of the EOD. Indeed, behavioural 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*) favoured 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, Kirschbaum, Engelmann, et al., 2018; Nagel, Kirschbaum, Hofmann, et al., 2018) 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 the diet composition of *Campylomormyrus* is so far limited to only two studies on single species: Roberts and 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, that is the Congo river, seems unfeasible and microscopic examination of gut contents may yield incomplete results, as food items may be digested to various degrees, 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 did not work for our samples (F. Lamanna & R. Tiedemann, unpublished), which is indicative of low DNA quality. This low DNA quality may be attributed to logistical constraints during field work in Congo, that is *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

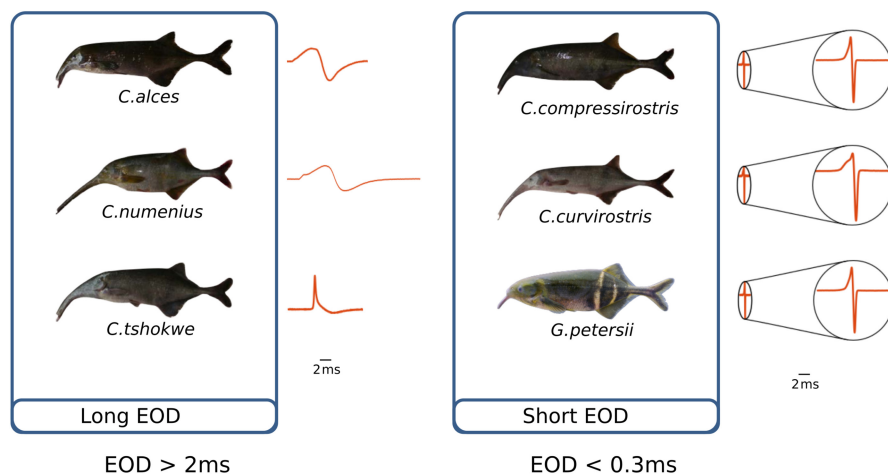


FIGURE 1 Fish species studied here and their species-specific Electric Organ Discharge (EOD), in orange; note that—for the short EODs—the length scale applies for the non-enlarged (left; oval) pattern, while the enlargement (right; circle) is presented to better visualize EOD characteristics.

enrich low-concentration and highly degraded DNA-fragments from environmental DNA (Shokralla et al., 2012) or ancient sedimentary DNA (Krueger et al., 2021). 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.

2 | MATERIALS AND METHODS

2.1 | 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*, $n=9$; *C. numenius*, $n=1$; Figure 1). 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 extended snout, but a trunk-like protrusion on the head (Figure 1). 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 analysed stomach samples were extracted from sympatric fish living in the Congo River rapids south of Brazzaville [$S4^{\circ}18.788'$ $E15^{\circ}13.790'$]. 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 Île 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 File S1). After dissection, the stomach samples were stored in Queen's tissue buffer solution (20% dimethyl sulfoxide, 0.25 M EDTA, saturated with NaCl, pH 8.0; Seutin et al., 1991) at 4°C. 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 (Section 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, that is *C. compressirostris* ($n=10$) and *C. tshokwe* ($n=9$), were considered. Regarding EOD duration, we formed two groups: Long EOD (>2 ms) and short EOD (<0.3 ms; 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.

2.2 | Lysis and DNA extraction

DNA extraction was performed in 2019, approximately 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 -20°C .

2.3 | 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 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 1 mL of a 1:20 dilution of each library with 3 μL of nuclease free water, 5 μL of 2 \times SYBRTM green qPCR master mix (ThermoFisher Scientific), 0.5 μL of qPCR primer IS7, and 0.5 μL of primer IS8 (both 10 μM ; from Gansauge & Meyer, 2013). For each library three reaction replicates were analysed. The PCR reactions were performed with 7 min at 95°C, followed by 40 cycles of 10 s at 95°C denaturation, 30 s annealing at 60°C, and 1 min 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 44.8 μ L of nuclease free water, 8 μ L of 10 \times AccuPrimeTM Pfx reaction mix (ThermoFisher Scientific), 0.8 μ L of 2.5 U AccuPrimeTM Pfx polymerase, 3.2 μ L of P5 indexing primer, 3.2 μ L of P7 indexing primer (both 10 μ M) and 20 μ L of each library. Thermocycling started with 2 min at 95°C, followed by the number of cycles calculated according to Basler et al. (2017) consisting of 15 s at 95°C denaturation, 30 s 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.

2.4 | 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 File S2. The sequence capture probes were custom-made at Arbor Biosciences (formerly MYcroarray) 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 15,284 probes, each with a length of 80 bp. 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 100 ng to 500 ng. This was equivalent to 7 μ L of the library material with a DNA concentration of 14–72 ng/ μ L. 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 2 \times 150 bp paired end of double indexed libraries (Biosciences, 2016).

2.5 | Bioinformatics, data analysis and statistics

Before further analysing 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) was used to quantify occurrence across samples. The relative read abundance (RRA) was used as a proxy for relative biomass consumed. Details on the calculations of these measures are given in the 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 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, that is 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.

3 | RESULTS

3.1 | Sequencing results

The sequencing for all samples generated 6.9Gb raw sequencing data of FASTQ formatted reads. The sequence data have been submitted to the GenBank SRA database under accession number PRJNA1041468 (Amen, 2023a). 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 filtering, ranging from 16,703 to 828,735 per individual (Mean = 314,421; SD = 222,465; see 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.

3.2 | 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 revealed 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, that is 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 (Figures S5.1 and S5.2) and tabulated in Tables S5.1 and S5.2 (File S5).

3.3 | 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 2). 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 2). 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 (species, EOD duration,

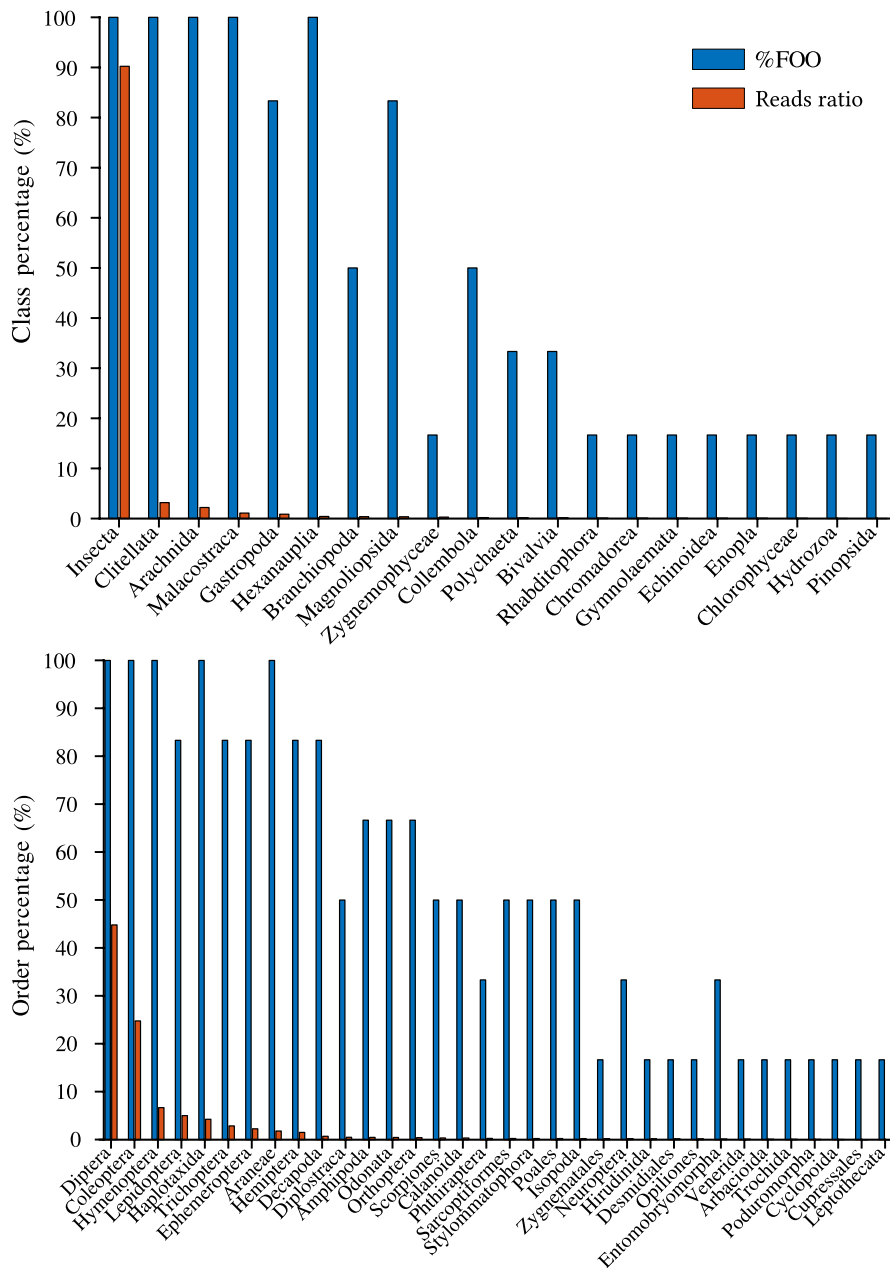


FIGURE 2 Overall diet composition among all samples (*Campylomormyrus*, $n=24$, and *Gnathonemus*, $n=3$) based on frequency of occurrence (%FOO, shown in blue bars) at class (upper panel) and order (lower panel) levels. Taxa are ordered based on the reads percentage abundance (shown in orange bars) 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.

and snout length) are depicted in [Figure 3](#). Most groups share similar food taxa. Interestingly, the most dominant prey taxon, that is insects (Diptera, Coleoptera, Hymenoptera and Lepidoptera), annelid worms (Haplotoxida), spiders (Araneae), crustaceans (Amphipoda, Diplostraca, Decapoda and Calanoida) and plants (Cupressaceae), appeared in different proportions in the diet among these groups. Most notably, with increased snout length and EOD duration, the relative proportion of Coleoptera increased and that of Diptera decreased. The RRA for individual samples are given in the [Figure S6.1](#) in [File S6](#).

The RRA data were used to assess the dietary niche width by calculating the Shannon diversity index ([Figure 4](#) and [Table S7.1](#) in the [File S7](#)). The Shannon diversity index differed considerably among the studied groups, pointing towards increased dietary niche widths in species with elongated EODs and long snouts.

3.4 | 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 1000 null models (see [Figures S8.1](#) and [S8.2](#) in [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. However, as many reads could not be assigned to family, genus or species level (see [Table S4.1](#) in [File S4](#)), we performed further statistical analyses on read assignments at the order level. Diet overlap is further confirmed by the Bray-Curtis dissimilarity index (0: similar; 1: dissimilar), as shown in [Table S7.2](#) in the [File S7](#), revealing a higher

FIGURE 3 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, $n=11$).

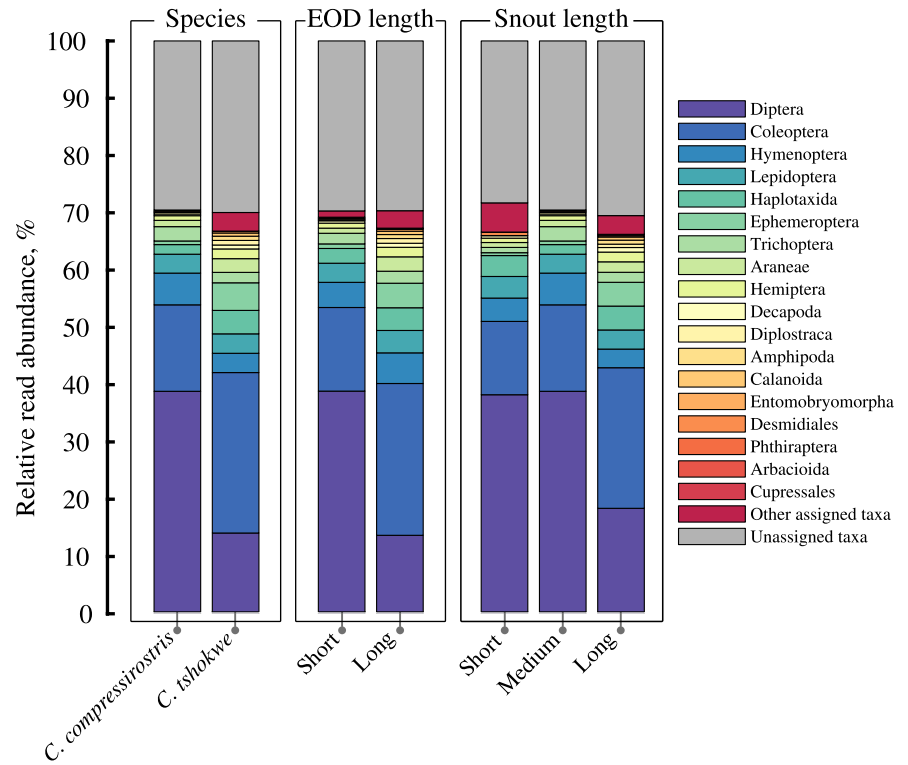
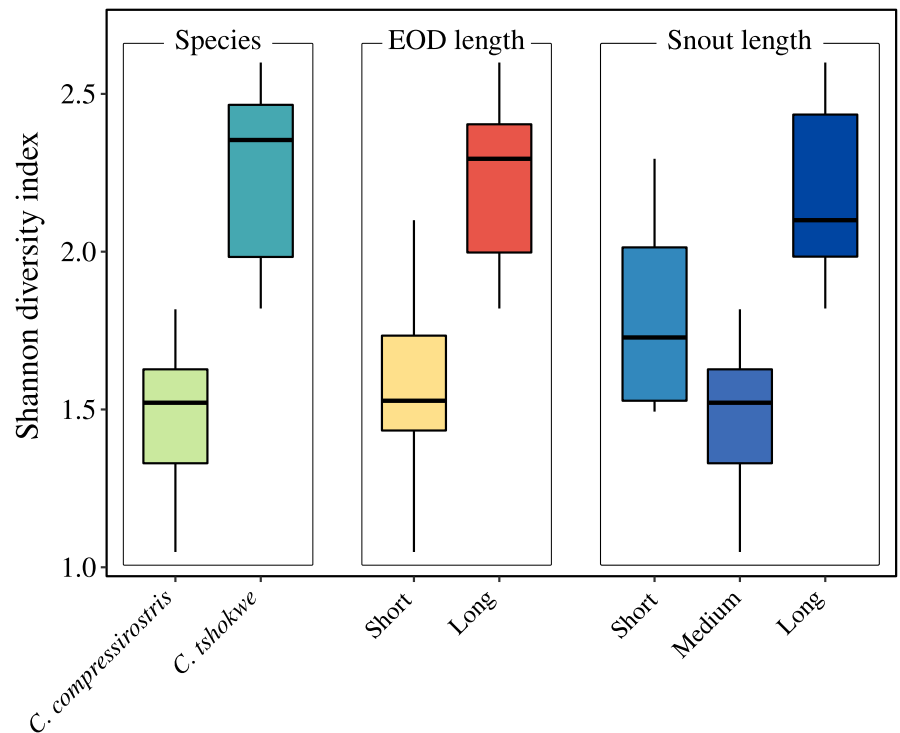


FIGURE 4 Boxplot of Shannon diversity indices of food taxa from dietary metabarcoding data sets of stomach contents of 25 samples of *Campylomormyrus* and one sample of its sister genus *Gnathomemus* based on RRA at order level for species, EOD duration and snout length groups (cf. Figure 2). Shannon diversity indices are increased in species with elongated EOD and long snouts.



overlap on the class than the order level. We further investigated whether niche overlap was significantly lower across our groups (species, EOD duration, snout length) than within them, by performing a perMANOVA on a dissimilarity matrix (based on Bray-Curtis dissimilarity indices) derived from the RRA values at order level. This analysis indicates significant dietary differences among

C. compressirostris and *C. tshokwe* ($F=4.64$, $r^2=.215$, $p \leq .001$, $df=1$), among the EOD duration groups (long vs. short EOD; $F=5.34$, $r^2=.182$, $p \leq .001$, $df=1$) and relative to snout morphology (short vs. medium vs. long; $F=2.50$, $r^2=.182$, $p \leq .01$, $df=2$). Regarding snout length, 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 ($p \leq .001$ and $p \leq .05$, respectively).

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.

Diet overlap among fish with different EOD duration [short EOD and long EOD]				
Class	Order		Family	Genus
0.99/0.92	0.74/0.63		0.61/0.46	0.55/0.36
Diet overlap among fish with different snout length [short snout (S), medium snout(M) and long snout (L)]				
Class	Order		Family	Genus
	M	L	M	L
S	0.99/0.92	0.99/0.94	S	0.94/0.89
M		0.99/0.91	M	0.84/0.67
Family	Genus		Family	Genus
	M	L	M	L
S	0.93/0.60	0.76/0.41	S	0.29/0.27
M		0.83/0.53	M	0.52/0.38

Note: The comparisons are done based on EOD duration (short EOD, $n=5$, and long EOD, $n=11$) and snout length (short snout, $n=5$, medium snout, $n=10$, and long snout, $n=11$). Bold numbers indicate statistically significant niche overlap (that is Pianke index is greater than that expected by chance based on comparison with 1000 null models, $\alpha=0.05$, and Schöner index is >0.6).

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

The patterns of dietary difference among samples are visualized in Figure 5 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.

4 | DISCUSSION

4.1 | Diet profile

The DNA metabarcoding method, specifically our hybrid capture/HTS DNA approach, 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 also 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, that is benthic invertebrates, allochthonous invertebrates, and macrophyte material. The most

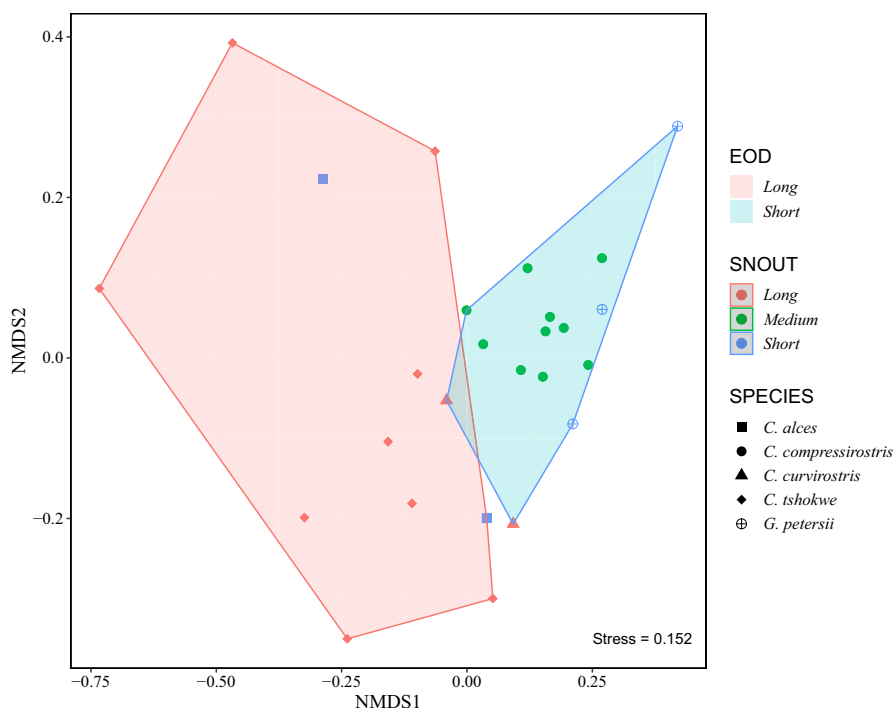


FIGURE 5 Dietary niche partitioning within and among individual samples of *Campylomormyrus* and its sister genus *Gnathonemus* by nMDS of RRA-based Bray–Curtis dissimilarity indices of samples on the order level (adonis: comparison across EOD phenotypes $F=5.34$, $R^2=.182$, $p \leq .002$; across snout phenotypes $F=2.50$, $R^2=.179$, $p \leq .002$). The symbols represent the species and the colours represent snout length. The shaded convex hulls indicate short versus 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.

dominant prey taxa found in 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 odonatan (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 and 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.

4.2 | Diet comparison among *Campylomormyrus* phenotypes

The dietary compositions among *Campylomormyrus* and *Gnathonemus* suggest that their feeding behaviour 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, *Campylomormyrus* species may exhibit high trophic flexibility and diverse feeding behaviours. However, the RRA results revealed significant differences among the dietary compositions among the species, 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 preferentially fed on other taxonomic groups (i.e. coleopterans, ephemeropterans, spiders and 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 among the *Campylomormyrus* species analysed here, species with long EOD also exhibit a longer snout (Figure 1; cf. Lamanna et al., 2016).

4.3 | Dietary analysis and the radiation scenario

Our study was motivated by potentially providing further support for the hypothesis that the 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 provides 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 exploit 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 behaviour. 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.

4.4 | Potential limitations of our dietary analysis

4.4.1 | Sample sizes and quality of DNA

Our study was limited by the availability of stomach content samples we collected from the field, that is the Congo River. Despite of a 4 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 analysed 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.

4.4.2 | 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., 2023) 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 implicitly 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.

4.4.3 | Taxonomic resolution of dietary items

The dietary differences among the species and phenotypes are more visible in the low taxonomic levels, that is at genus and species levels. However, the relatively low percentages of reads assigned to the family, genus or species level (see Section 3.4) may compromise the confidence in the inferred prey spectra at these taxonomic levels. The unassigned reads may be due to a potential bias stemming from limited availability of reference sequences from the global South, particularly from regions like the Congo River, which poses challenges in accurately identifying certain prey items. 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 attested 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.

5 | CONCLUSIONS

We analysed 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.

AUTHOR CONTRIBUTIONS

Rahma Amen participated in the design of the study, carried out the experiments, analysed the data and drafted the manuscript. Katja Havenstein participated in carrying out the experiments and revised the manuscript. Ralph Tiedemann and Frank Kirschbaum 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.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicting interests.

DATA AVAILABILITY STATEMENT

Raw sequence reads are deposited in the Genbank SRA repository (BioProject PRJNA1041468). The codes used to analyse the data

and generate the results can be found in the open repository Zenodo DATMetaB (DOI: [10.5281/zenodo.10148506](https://doi.org/10.5281/zenodo.10148506)) (Amen, 2023b).

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 from making the codes developed to analyse the data publicly available.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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