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Dietary Differentiation Between Sympatric Ecotypes of *Astatotilapia calliptera* From Lake Masoko (Kisiba), Tanzania Revealed by Metabarcoding

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ABSTRACT

Sympatric speciation is defined as the formation of new species in the absence of geographic barriers, but the genomic and life history strategy mechanisms underpinning sympatric speciation are still far from clear. It has recently been discovered that the cichlid fish *Astatotilapia calliptera* from crater Lake Masoko in Tanzania have diverged sympatrically into littoral (shallow water) and benthic (deep water) ecotypes, which differ in head and pharyngeal jaw morphology. Carbon stable isotope analysis has also broadly indicated trophic differentiation between ecotypes. Here, we explore trophic niche divergence on a finer scale, using metabarcoding of stomach contents. A combination of the mitochondrial COI region and 18S V4 region from the eukaryotic nuclear small subunit ribosomal DNA was used to target macroinvertebrate and broader eukaryotic taxonomic diversity, respectively, revealing dietary divergence between the ecotypes. Large proportions of Arthropoda (dipterans and copepod) were found in both ecotypes, indicating some food sources common to both microhabitats. However, gut contents of benthic *A. calliptera* individuals were characterized by an abundance of annelids and diatoms, while Lepidoptera, mayflies, fungi, freshwater mussels, and bivalves were common in littoral ecotypes. The variation observed in the dietary contents of the ecotypes indicates the presence of resource partitioning, facilitating adaptation to unique feeding strategies.

1 | Introduction

Cichlid fish (ca. 3000–4000 species: Turner et al. 2001; Turner 2007; Svardal et al. 2021) are one of the most iconic model systems for speciation research. In particular, the highly diverse species assemblages endemic to African lakes provide excellent natural laboratories for studying evolutionary processes

(Kocher 2004). The rapid diversification rate observed amongst cichlids has been attributed to exploitation of novel habitats (Hulsey et al. 2006), hybridisation (Seehausen 2004; Genner and Turner 2012), multiple colonization events (Loh et al. 2013; Tyers and Turner 2013), and sympatric speciation driven by natural and sexual selection (Turner et al. 2001; Kocher 2004). The pharyngeal jaw apparatus of cichlid fish is a key innovation that

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promotes diversification. Cichlid pharyngeal jaws are known to be phenotypically plastic facilitating adaptation to specific feeding ecologies (Barluenga et al. 2006; Muschick et al. 2012; Theis et al. 2014; Ronco et al. 2021). Liem (1973) hypothesized that the decoupling of pharyngeal jaw from the oral jaw promotes ecomorphological differences in cichlids leading to increased utilization of novel prey and niche partitioning, enabling a wider array of species to co-exist. Such pharyngeal jaw modifications have been observed in two *Astatotilapia calliptera* ecotypes, which are the focus of the present study.

Astatotilapia calliptera is a maternal mouth-brooding cichlid fish that occupies littoral regions of Lake Malawi and is also found more widely in the region inhabiting rivers, swamps, ponds, and shallow lakes (Parsons et al. 2017; Malinsky et al. 2018). For this reason, it is often thought of as representative of the ancestor of the Lake Malawi haplochromine radiation, which is believed to have originated via three lake specialist species diverging from a persistent *Astatotilapia* population (Malinsky et al. 2018). Populations of *A. calliptera* exhibit variation in male color and morphological traits associated with assortative mating (Tyers and Turner 2013; Nichols et al. 2014; Malinsky et al. 2015).

Astatotilapia calliptera has colonized at least six small crater lakes to the north of Lake Malawi (Malinsky et al. 2015; Turner et al. 2019), including Lake Masoko (also known as Lake Kisiba, Figure 1A,B), which formed approximately 50,000 years ago (Thevenon et al. 2003). Lake Masoko is a steep-sided freshwater lake, 700 m in diameter and reaches up to a maximum of 39 m in depth (Gibert et al. 2002). Currently, it is a closed lake and isolated from external water bodies (Turner et al. 2019).

Besides *A. calliptera*, the distantly related cichlid species *Coptodon rendalli* (redbreast tilapia) and *Oreochromis squamipinnis* along with *Clarias gariepinus* (African sharp-tooth catfish) are present in the lake. Due to the lack of closely related haplochromine species, Lake Masoko provides a simple system in which drivers of ecological speciation can be studied (Munby et al. 2021).

Malinsky et al. (2015) have shown that approximately 10,000 years ago, *A. calliptera* individuals colonized shallow littoral habitats from nearby riverine systems and extended their range into deeper benthic waters about 1000 years ago. Deepwater males appear to be uniformly blue, while shallow-water males are generally yellow, although a few blue males have been seen (Turner et al. 2019). Females from both populations, like other haplochromine cichlids, are dull sandy colored, with paler flanks and bellies. Landmark based geometric morphometrics indicate significantly distinct body shapes and lower pharyngeal jaw morphology between the ecotypes (Malinsky et al. 2015). In addition to benthic and littoral ecotypes, a few intermediate individuals, likely hybrids, are also found (Malinsky et al. 2015).

Stable isotope analysis of carbon and nitrogen shows dietary differentiation between *A. calliptera* ecotypes: benthic individuals have a planktivorous diet, while littoral individuals have a broader diet including hard-shelled invertebrates (Malinsky et al. 2015; Carruthers et al. 2022). Stable isotope analysis can classify diets into broad functional groups but does not have the

ability to elucidate prey composition to lower taxonomic levels (Maloy et al. 2013). This study aimed to investigate dietary partitioning between the ecotypes using dietary metabarcoding.

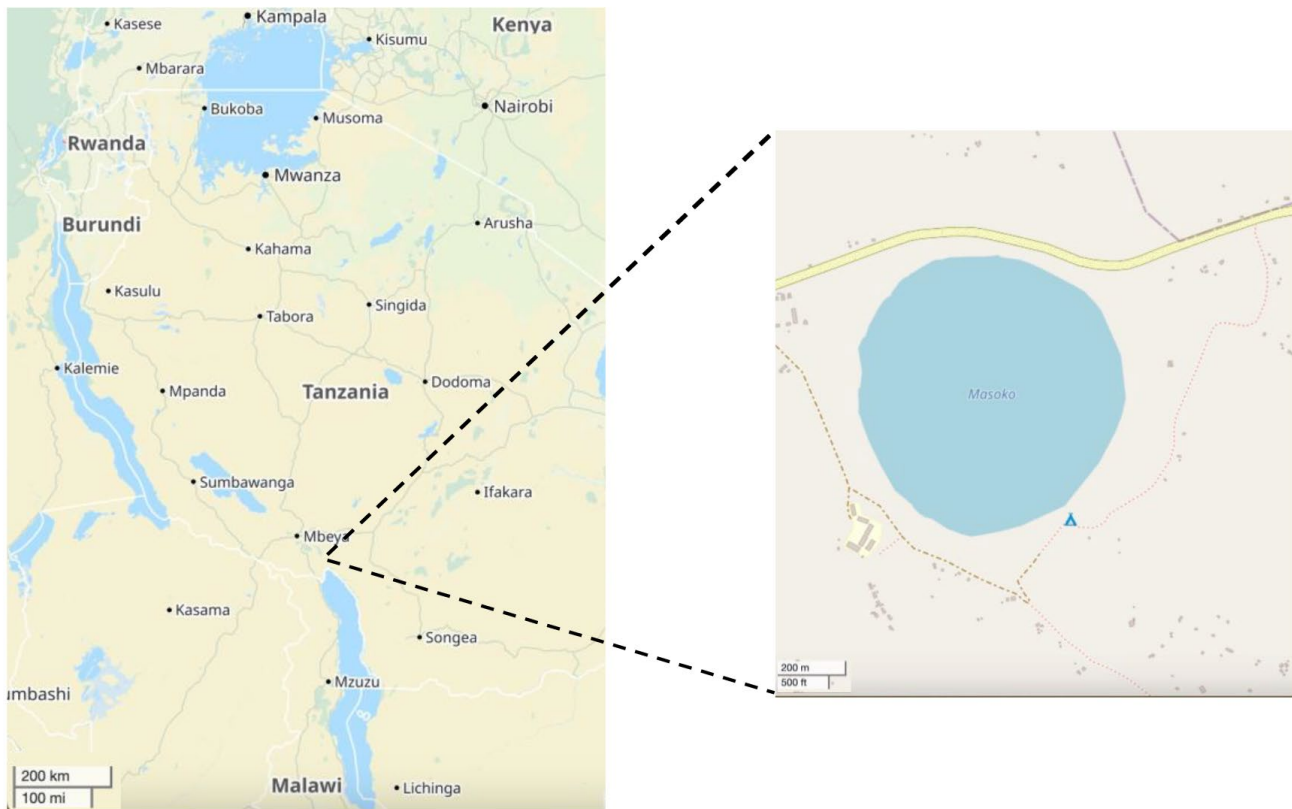
Dietary metabarcoding is able to identify prey species and has been used extensively to inform trophic positioning, food web dynamics (Carreon-Martinez and Heath 2010) and prey-predator interactions, subsequently informing management and conservation strategies (Roslin and Majaneva 2016). Investigating niche partitioning using metabarcoding has been conducted on both terrestrial and aquatic organisms that live in sympatry (Sato et al. 2018; Takahashi et al. 2020; Andriollo et al. 2021; Spence et al. 2022) including cichlids (Hata et al. 2014), but has not been used to investigate trophic specialization in an early stage of sympatric differentiation in cichlids. Based on previous findings of pharyngeal jaw morphology and stable isotope divergence (Malinsky et al. 2015; Carruthers et al. 2022), we expected the ecotypes to display distinct dietary composition, with benthic individuals primarily feeding on plankton and littoral individuals specializing in hard-shell invertebrates. The findings from this study will elucidate the significance of trophic specialization in the earliest stages of sympatric divergence.

2 | Materials and Methods

All fish specimen were collected between 2014 and 2016 (Table S1) using a variety of fishing methods and were euthanized by anesthetic overdose following European Union regulations for the protection of animals used for scientific research (DIRECTIVE 2010/63/EU). Following confirmation of death, the entire intestinal tract was removed and stored in absolute ethanol. Samples were transported back to the UK for molecular analysis. Stomach contents from individual fish were removed by careful longitudinal incision. Instruments were bleached (10%) and ethanol flamed for sterilization to prevent contamination between samples. Stomach contents were used rather than fecal material to avoid digestion induced degradation of DNA (Perry et al. 2023). A total of 166 samples were selected for molecular analysis comprising 74 benthic, 74 littoral and 18 intermediate/undetermined individuals. DNA was extracted from the isolated stomach contents using a modified ammonium acetate salt extraction method (Bruford et al. 1998; NEOF 2024).

Illumina MiSeq paired-end indexed amplicon libraries were prepared using a two-step PCR protocol at the NERC Environmental Omics Facility (NEOF) at The University of Sheffield (see Supporting Information). Two marker genes were amplified using universal primer pairs mCOIintF and jgHCO2198 (Geller et al. 2013) targeting the mitochondrial cytochrome c oxidase subunit I (COI) region and TAREuk454FWD1 and TAREukREV3r (Stoeck et al. 2010) in the 18S V4 region from the nuclear small subunit ribosomal DNA (Table S2). The COI region of the mitochondrial genome was chosen as it has high interspecific variability (Ward et al. 2009) and extensive databases are available (Ratnasingham and Hebert 2007; Leray et al. 2018). The specific COI primers chosen in this study had been successfully employed on invertebrate DNA from aquatic systems (Hajibabaei et al. 2019) and were found to be effective at recovering species-specific DNA from stomach contents (Leray et al. 2013). The V4 region of the ribosomal DNA-encoding gene

A



B



C



FIGURE 1 | Location of Lake Masoko (9°20.0'S, 33°45.3'E) and its resident ecotypes of *Astatotilapia calliptera*. (A) Map of the three great lakes in East Africa—Lake Victoria, Lake Tanganyika, and Lake Malawi. Map of Lake Masoko on the right in relation to the great lakes (OpenStreetMap contributors, 2024). (B) Photograph of Lake Masoko taken from the southern shore looking north. (C) Males of the littoral (yellow) and benthic (blue) ecotype of *A. calliptera* from Lake Masoko (Fish images credited to Ad Konings, figure editing credited to Callum Thorpe).

is known to be variable, and using the 18S primer set has the potential to target a wide diversity of eukaryotic taxonomic groups (Stoeck et al. 2010), although lacking the resolution to always resolve target sequences down to species level (Creer et al. 2016). PCR libraries were pooled at equimolar quantities and sequenced on an Illumina MiSeq V3 (2×300bp) kit (see [Supporting Information](#) for full protocol).

Data processing was carried out using the Super Computing Wales (SCW) platform and RStudio (v 4.1.3) (RStudio Team 2020). The sequences were denoised (sequence quality control; trimming, filtering and removal of chimeras) and merged using 'DADA2' package (v 1.16) (Callahan et al. 2016) in RStudio. Subsequently, the amplicon sequence variants (ASVs) produced were used for taxonomic assignment using BLASTn (Camacho et al. 2009)

against the National Centre for Biotechnology Information (NCBI) *nt* database with a 0.0001 e-value threshold and maximum of 20 hits retained. Because the expected eukaryotic diversity of the prey items of the Lake Masoko regions will be underrepresented in global reference databases, the prey item sequences were then parsed using a python script to assign generic taxonomic identity at 80% for phylum, 90% for family, and 97% for species (Sevigny 2020). Prior to statistical analysis host sequences were removed from the COI and 18S datasets which amounted to 7,257,511 and 7,402,895 reads, respectively (see [Supporting Information](#) for more details).

After taxonomic assignment downstream processing was carried out using the “phyloseq” package (McMurdie and Holmes 2013) in Rstudio. Rarefaction analysis was used to determine sufficient read depth and samples with < 100 sequences were excluded (Figure S2). The dataset was filtered to exclude clearly identifiable “non-target” cichlid dietary items (comprised of microorganisms likely to have been parasites or gut contents from the prey ingested) and negative controls were appropriately removed ([Supporting Information](#)). The proportion of reads per taxon per sample was then used as a measure of relative read abundance for downstream statistical analysis. Alpha diversity (Shannon Index) from each ecotype was calculated and plotted using “Vegan” package. A small number of individuals (18) could not be allocated with certainty to either ecotype, either because they appeared (potential hybrids) or were too small to show phenotype-diagnostic features. These were excluded from further analyses, but data collected has been archived with the rest of the dataset (Figure S3). Beta diversity (PERMANOVA) using Bray–Curtis dissimilarity matrix was applied to test for significant differences in dietary composition between the cichlid ecotypes and sampling groups. This was further visualized using a non-metric multidimensional scaling (nMDS) ordination (see [Supporting Information](#) for full details). Horn–Morisita method was used to calculate niche overlap using “bipartite” package (Dormann et al. 2008) in RStudio.

To understand how the diet composition of the stomach contents was structured, the effect of standard length and ecotype color were included in a single, two tailed generalized linear model using “manyglm” function in the package “mvabund” (Wang et al. 2012). The number of reads found in each sample was set as the multivariate response variable and predictor variables of standard length and ecotype were fit using a negative binomial distribution. The total number of reads per sample was included as an “offset” in the model in order to control for differences in the number of reads between samples.

3 | Results

A total of 25.25 million sequences were produced targeting the standard cytochrome c oxidase subunit I (COI) and the V4 region of the eukaryotic nuclear small subunit ribosomal DNA (18S) (BioProject accession PRJNA1145132). The number of raw reads per sample ranged from 235 to 363,660 with an average of $99,779 \pm 15,365$ (standard error, Figure S1).

After taxonomic assignment, 131 and 128 ASVs were retained for the COI and 18S datasets, respectively. Rarefaction curves

determined that samples with less than 100 reads should be removed due to insufficient read depth (47 and 58 samples removed within the COI and 18S dataset, respectively). After filtering, 72 benthic, 70 littoral, and 19 unassigned/intermediate specimens remained for the COI dataset, and in the 18S dataset, 41 benthic, 38 littoral, and 12 unassigned/intermediate individuals were retained.

A Kruskal–Wallis followed by Dunn’s test on Shannon diversity results showed that dietary diversity of both intermediate ($p=0.007$, z -statistic=3.05) and littoral ($p=0.045$, z -statistic=2.43) was higher than that of benthic. For the 18S dataset, only intermediates showed higher diversity than the benthic individuals ($p=0.020$, z -statistic=2.71; Figure 2) (refer to Table S3).

The beta diversity (PERMANOVA) of the stomach contents revealed significant ecotype differences for both markers (Table 1) with the primary distinction between the benthic and littoral ecotypes (Figure 3). Arthropoda were found to be the most abundant taxon within the COI dataset (70%), followed by Bacillariophyta (diatoms) (11%) and Cnidaria (7%) (none recorded in the lake, but perhaps could either be inconspicuous hydroids or parasitic myxozoa) (Figure 4A). For the 18S dataset, Arthropoda (26%) was also the most dominant taxon, followed by Nematoda (round worms) (23%), Annelida (segmented worms) (17%), and Chlorophyta (green algae) (12%) (Figure 5A) (Figure S5 for nMDS discrimination).

Niche overlap calculated using Horn–Morisita method (0.303 for COI and for 18S 0.331), indicating that the diets of the ecotypes overlap to some degree, but with some compositional differences. The diet of benthic individuals contained more Annelida and Bacillariophyta, whereas Lepidoptera (butterflies and moths), Ephemeroptera (mayflies), Ascomycota (fungi), Unionida (freshwater mussels), and Venerida (bivalves) (Figures 4B and 5B) were identified more frequently in the guts of littoral individuals (Table S5).

The specimens were collected on four different sampling trips that included two dry and two wet seasons. However, only a small number of samples were collected from the 2014 wet season ($n=14$) and unequal proportions of each ecotype were collected in the 2014 dry season (15 intermediate, 1 benthic, and 1 littoral), and to test for seasonal differences, a larger sample number from each season and ecotype was required. Therefore, beta diversity (PERMANOVA) was performed on the remaining samples from the 2015 dry and 2016 wet sampling groups, resulting in a significant difference for the COI marker only ($p=0.00840$, z -statistic=2.202). No difference was observed for the 18S marker ($p=0.05195$, z -statistic=1.2895). Post hoc analysis for the COI marker between benthic and littoral ecotypes for each sampling group (2015 dry and 2016 wet) resulted in a significant difference (Table S4). Because compositional differences in the stomach contents persisted between the ecotypes despite seasonal changes, they do not confound the dietary differences observed between the ecotypes.

The generalized linear model indicated that standard length and ecotype, and the interaction between these variables, were significant predictors of overall dietary composition (Table S6).

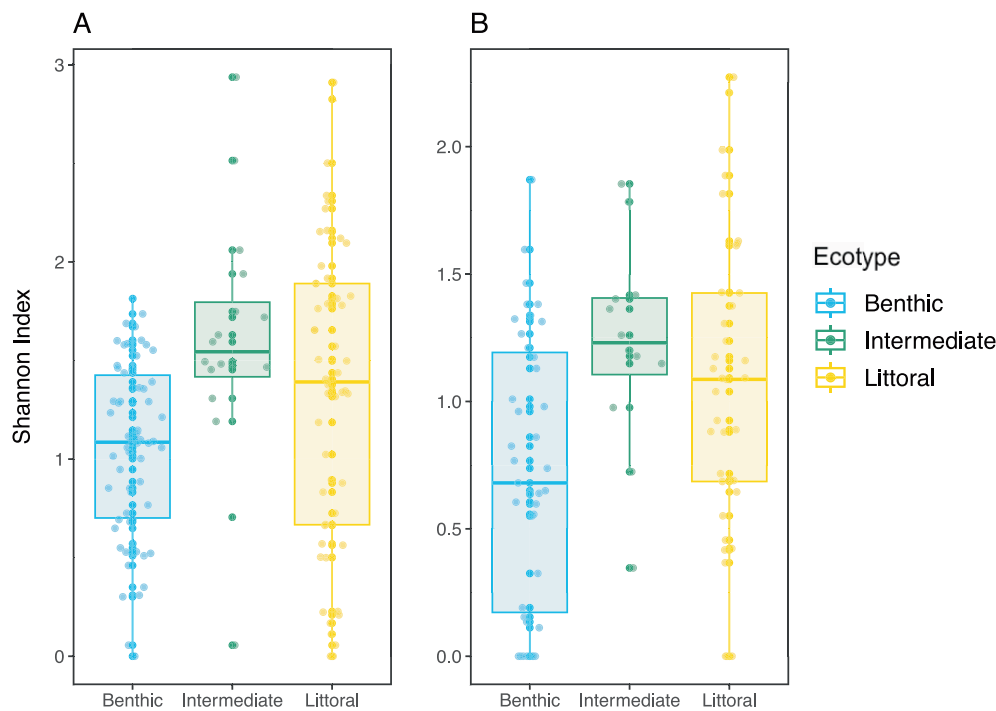


FIGURE 2 | Alpha diversity of stomach contents of three *Astatotilapia calliptera* ecotypes measured by Shannon indices of (A) mitochondrial cytochrome oxidase I (COI), significant differences observed between benthic and intermediate ($p=0.007$) and benthic and littoral ($p=0.045$). (B) 18S nuclear small subunit ribosomal DNA marker, diversity was significantly higher in intermediate individuals compared to benthic individuals ($p=0.021$). The boxes denote interquartile range with median as horizontal line in the middle and whiskers extending up to the most extreme points.

TABLE 1 | Results from pairwise PERMANOVA comparisons between ecotypes with Bonferroni-corrected p values for mitochondrial cytochrome oxidase I (COI) and 18S nuclear small subunit ribosomal DNA marker.

Marker	Pairwise comparison	F	df	Adjusted p value
COI	Benthic vs. littoral	4.59631	1	0.0003**
COI	Benthic vs. intermediate	1.643278	1	0.1
COI	Littoral vs. intermediate	1.885942	1	0.03*

Marker	Pairwise comparison	F	df	Adjusted p value
18S	Benthic vs. littoral	3.7672601	1	0.0003**
18S	Benthic vs. intermediate	1.6916892	1	0.01**
18S	Littoral vs. intermediate	0.9273412	1	1

*Significant at 95% confidence level.
**Significant at 99% confidence level.

4 | Discussion

The present study compared stomach content composition between benthic and littoral ecotypes of *A. calliptera* from an

isolated crater lake, Masoko, to investigate if dietary specialization following colonization of new habitats can contribute to local adaptation and continued divergence. We show that the benthic and littoral ecotypes share a proportion of their diet composition, although with some organisms that are disproportionately consumed by one ecotype over the other. Littoral ecotypes stomachs contained greater quantities of fungi, Lepidoptera, bivalve mollusks, and mayflies, while annelids and diatoms were better represented in the diet of benthic ecotypes. The variation observed in the dietary contents between the ecotypes indicates strong habitat fidelity where the presence of resource partitioning is driven by space partitioning, enabling the ecotypes to adapt to their respective environments.

Our results show that the diet of benthic individuals included an increased amount of arthropods and specifically by dipterans—*Chironomidae* (non-biting midges) (Figure S6). Even though benthic individuals have not been recorded at the surface of the water preying on these insects. Benthic individuals are not known to transverse great depths to forage on insects in the surface waters. Chironomids are abundant in profundal zones (i.e., deep) of freshwater lakes (Nyman and Korhola 2005; Adler and Courtney 2019; Gadawski et al. 2022) and high abundances of eggs (which sink to the bottom) and larvae (which hatch on the bottom and migrate to the surface) (Kranzfelder et al. 2015) are a potential food source for benthic foraging fish. The diet of closely related *Astatotilapia burtoni* (formerly *Haplochromis burtoni*) from Rwanda was found to be dominated by *Chironomidae* species (Janssens de Bisthoven et al. 1990). The zoobenthos of lakes consist mostly of dipteran larvae, oligochaetes, nematodes, microcrustaceans, rotifers,

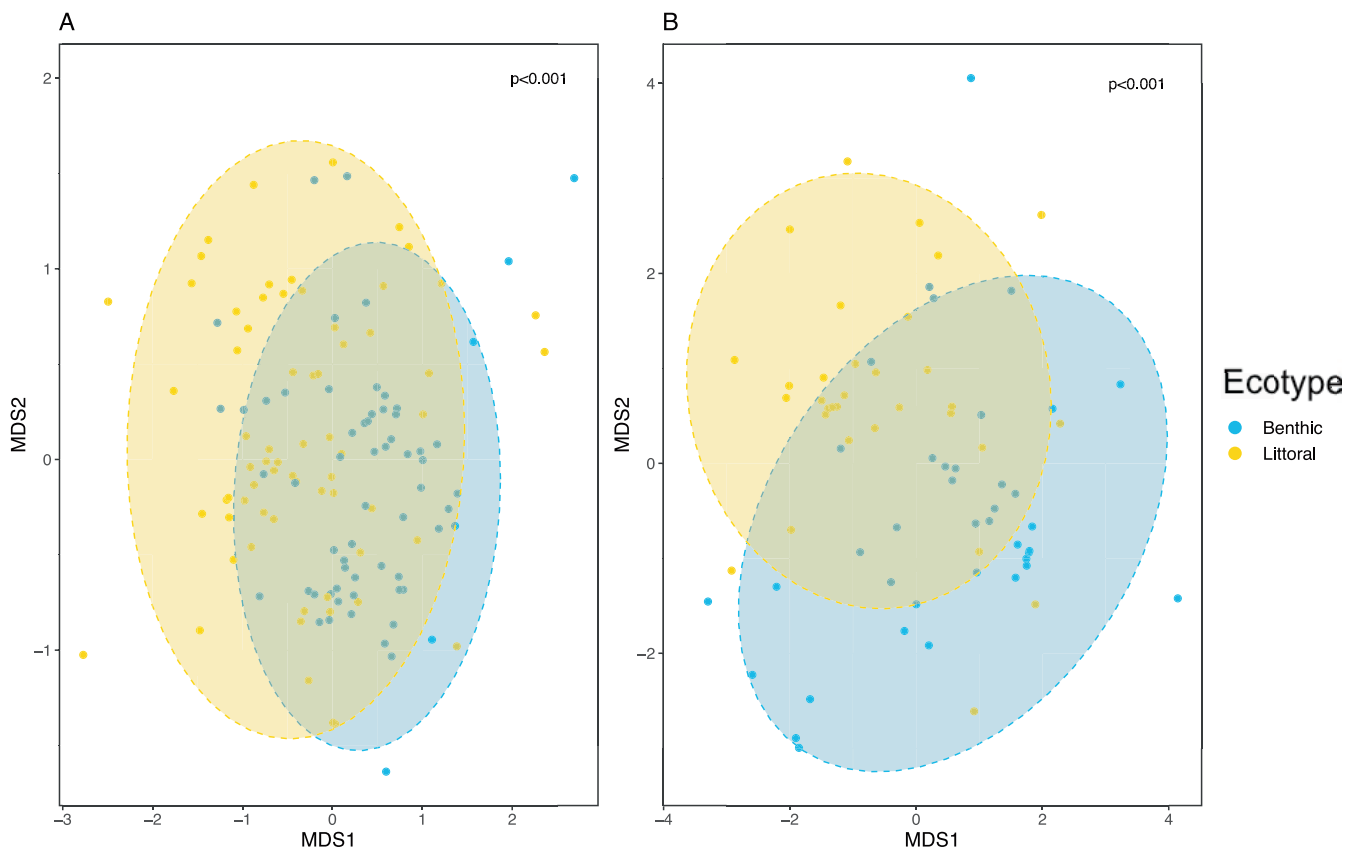


FIGURE 3 | Non-metric multidimensional (nMDS) ordination of diet from the two *A. calliptera* ecotypes derived from (A) mitochondrial cytochrome oxidase I (COI) (stress = 0.0842) and (B) 18S nuclear small subunit ribosomal (stress = 0.0386) DNA marker. Ellipses indicate 95% confidence. *p*-values refer to significant differences observed in beta-diversity analysis. Ordination plots for littoral, benthic, and intermediate/undetermined ecotypes are in Figure S4).

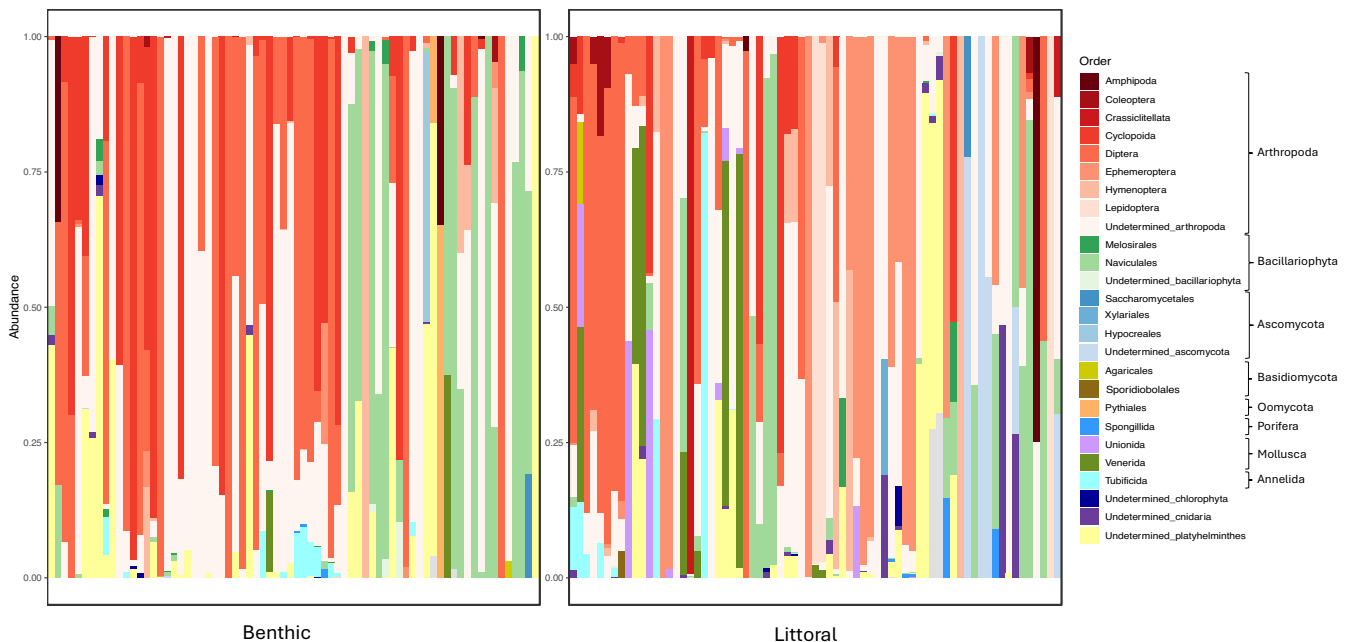


FIGURE 4 | Relative abundance of taxa identified from the stomach contents of the two *Astatotilapia calliptera* ecotypes using the mitochondrial cytochrome oxidase I (COI) DNA marker. Each bar represents overall taxonomic composition for a single individual.

and bivalves (Strayer 2009). The presence of nematodes, annelids, and arthropods found in benthic *A. calliptera* individuals suggests extensive foraging on zoobenthos. This does not necessarily point to targeted predation of all these taxa. The diet of

Archocentrus spilurus (jade-eyed cichlid) from Belize also contained dipteran individuals but this was reported to be the result of non-specific consumption when *A. spilurus* individuals consumed algal biofilms from rock surfaces (Cochran-Biederman

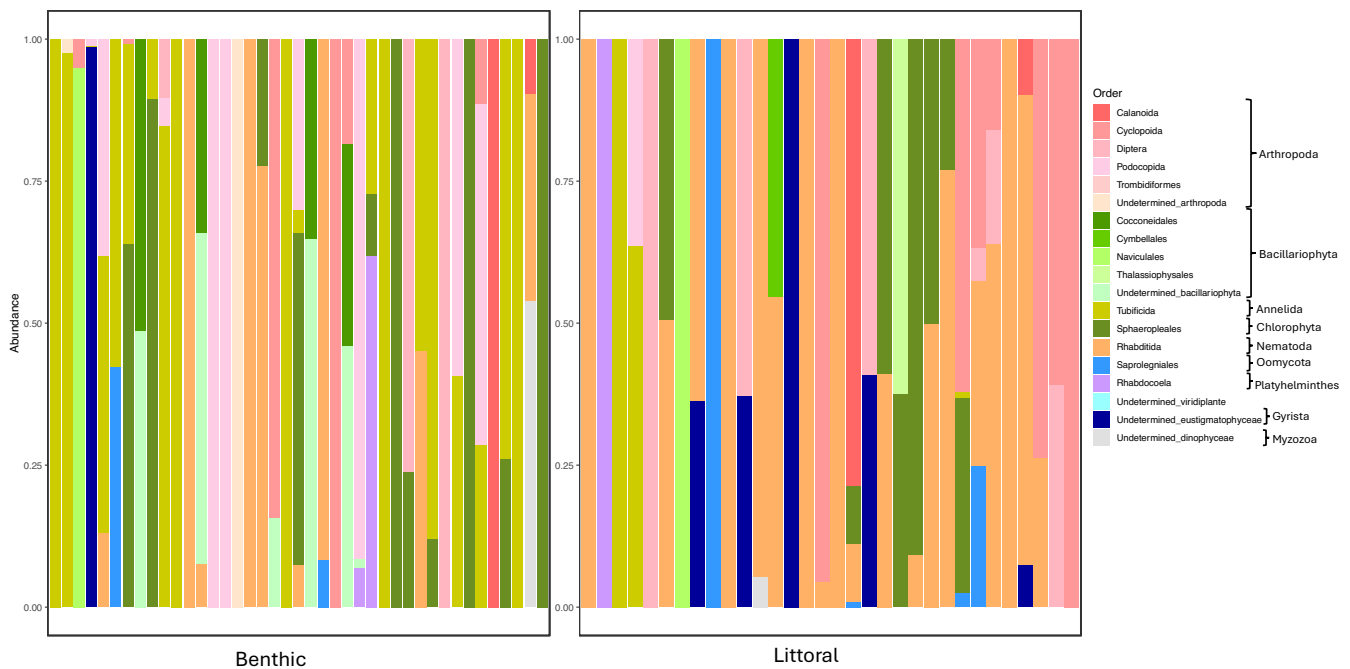


FIGURE 5 | Relative abundance of taxa identified from the stomach contents of the two *Astatotilapia calliptera* ecotypes using the 18S nuclear small subunit ribosomal DNA marker. Each bar represents overall taxonomic composition for a single individual.

and Winemiller 2010). Algal covered rocks in neighboring Lake Malawi host numerous arthropods, including dipteran larvae (Fryer 1959) and many Malawian cichlids are known periphyton feeders, consuming algae attached to submerged substrates (Fryer 1959; Ribbink et al. 1983; Rusuwa et al. 2006). There are many areas of rocks, gravel, and sunken wood on the bottom of the Lake Masoko (Turner et al. 2019). Hence, non-specific consumption could explain the presence of dipterans observed in benthic *A. calliptera* stomachs, which also contained abundant diatoms (Bacillariophyta), which are generally a significant component of periphyton in lentic systems (Goldsborough et al. 2005).

Hard substrates are scarce in the deep-benthic zone, which is dominated by a thick layer of ooze (Turner et al. 2019). Bacillariophytes (diatoms) in the diet of benthic ecotype fish are more likely to be the result of plankton or sediment feeding. Papilliform jaws (i.e., needle-like teeth from the lower pharyngeal jaws) are often associated with a plankton-rich diet (Streelman and Albertson 2006; Muschick et al. 2011), supporting the findings from Malinsky et al. (2015) and Carruthers et al. (2022) who observed stable isotope ratios indicating that benthic individuals had depleted ^{13}C commonly associated with a planktonic diet. However, this does not necessarily mean that the bulk of their diet is obtained by midwater feeding: the deep benthic habitat is dominated by a thick layer of sediment, likely to include deposited planktonic material. Notably, annelids were consistently abundant in the diet of benthic ecotypes, in both COI and 18S datasets. On closer inspection, the annelids belonged to family Naididae (Oligochaete worms), known to occupy depositional habitats, acting as decomposers by mixing and aerating organic matter through burrowing (Benbow 2009). The increased abundance of annelids and bacillariophytes in benthic compared to littoral individuals indicates dietary divergence between the ecotypes.

Unionid mollusks (freshwater mussels), were exclusively found in the diet of littoral individuals, *Coelatura* has been collected from Lake Masoko (Turner et al. 2019). Littoral individuals also showed a higher abundance of venerid bivalves (Figure 4A). The lower pharyngeal jaws of littoral individuals have molar-like (molariform) teeth, typically used for crushing shelled organisms. Like *A. calliptera*, populations of the central American cichlid *Herichthys minckleyi* (Minckley's cichlid), include individuals with both papilliform and molariform pharyngeal jaws. The molariform morph crushes snails and other harder food items while the papilliform morph primarily feeds on detritus, algae and soft-bodied invertebrates (Swanson et al. 2008). Swanson et al. (2003) suggested that divergent adaptations of pharyngeal jaw dentition facilitates resource partitioning resulting in reduced intraspecific competition. This suggest that the development of molariform littoral *A. calliptera* to consume mollusks and other hard bodied prey that are usually less accessible or favored food resources (Hulsey et al. 2006). In contrast, the lower pharyngeal jaws of benthic individuals are consistently papilliform: it is unclear whether this is directly beneficial for processing more locally abundant small, soft food items or because the production of molariform jaws is energetically more expensive. Haplochromine cichlid lower pharyngeal jaws are known to be phenotypically plastic under experimental treatment and this may facilitate specialization (Muschick et al. 2011).

A higher abundance of Ascomycota (fungi) (Figure 3) was observed in the stomach contents of the littoral *A. calliptera* individuals. Littoral zones in lakes experience a high abundance of plant litter from aquatic macrophytes that are subsequently colonized by fungi during decomposition (Gulis et al. 2006). Additionally, littoral individuals were observed to have consumed Lepidoptera and Hymenoptera (specifically Formicidae, ants) indicating consumption of terrestrial food sources, behavior which has been observed in freshwater fish from lotic

(Kawaguchi et al. 2003; Sullivan et al. 2012) and lentic systems (Masilya et al. 2011; Winant 2020). The terrestrial signature observed in littoral ecotypes indicates the presence of opportunistic feeding behaviors from *A. calliptera*. It has been suggested that most cichlids feed opportunistically when resources are abundant and consume whichever prey they encounter frequently (van Alphen et al. 2004). However, when resources are scarce, they exploit prey that they are morphologically adapted to, gaining a fitness advantage over their competitors (McKaye and Marsh 1983; Meyer 1990).

The overlap in diet of *A. calliptera* specifically in Arthropoda (Cyclopoida, Diptera) and Bacillariophyta (Figures 4 and 5) could be indicative of resource partitioning based on space where the ecotypes feed exclusively at benthic or littoral zones but the availability of some common resources, particularly planktonic or surface organism, at both habitats allows diet to overlap.

Bayesian isotope mixing models suggested that the largest contributor to benthic diet was zooplankton (depleted ^{13}C), whereas for littoral individuals, it was littoral arthropod macroinvertebrates (enriched ^{13}C) with a shared diet of algae, detritus, and terrestrial plant sources (Carruthers et al. 2022). The combination of the metabarcoding and stable isotope data here reflects the presence of partial/incomplete diet specialization between *A. calliptera* ecological morphs. Similar diet specialization has been observed in cichlid species from crater lake Barombi Mbo (Galvez et al. 2022) and *H. minckleyi* ecotypes (Robinson and Wilson 1998; Hulsey et al. 2006) where specialized resources are used to supplement their generalist diet, especially during periods of low resource abundance. Such feeding behaviors enable the fish to be optimal foragers by utilizing most-favored and less-favored resources while coexisting. Long-term feeding studies and monitoring of seasonal resource availability are required to further investigate optimal resource utilization of *A. calliptera* ecotypes from Lake Masoko.

The effect of standard length was observed to be a predictor of dietary composition (Table S6). Many teleost fishes including cichlids undergo ontogenetic dietary shifts where positive correlations between food size and body size have been documented in many species (Svanbäck and Eklöv 2002; Post 2003; Graham et al. 2007; Hellig et al. 2010; Burrell et al. 2013; Takahashi and Koblmüller 2011; Santos et al. 2023). To understand the effects of standard size and ontogenetic shifts on diet distribution additional morphological measurements (such as craniofacial morphology and intestine length) and age information is required. Nevertheless, the current dataset provides valuable insights into dietary partitioning during the earliest stages of sympatric divergence.

Research has shown that a combination of COI and 18S markers improves the detection of species (Zhang et al. 2018) and diversity estimates (Giebner et al. 2020). The COI primers used in the present study were designed to specifically amplify freshwater macroinvertebrates (Leray et al. 2013; Geller et al. 2013). However, the 18S primers provide greater amplification success across broad taxonomic groups (> 20 phyla) (including Nematoda, Platyhelminthes, Chlorophyta, Myxozoa, and Gyrista that were absent in COI dataset). Hence, using this multi-marker approach delivered enhanced taxonomic annotation power for macroinvertebrates (COI marker) down to the

family level while also allowing for amplification across a broad range of taxa (18S marker).

Molecular methods may have some shortcomings that affect the data produced. Firstly, quantification is subject to biases such as differential rates of DNA degradation (Murray et al. 2011), metabolism by consumers (Greenstone et al. 2014), and volume of prey consumed (Egenter et al. 2015). Additionally, PCR-based techniques introduce primer bias (Murray et al. 2011) and random sampling during sequencing makes the results difficult to quantify (Leray and Knowlton 2017). Quantifying metabarcoding data is also affected by the gene region chosen in the study, as some regions preferentially amplify certain taxa over others, subsequently introducing taxonomic bias and errors in the estimation of taxon abundance (Creer et al. 2016; Casey et al. 2021). Despite these drawbacks, we are able to use the data in a semi-quantitative way, estimating diet composition by transforming the data to relative read abundance as recommended by Deagle et al. (2018) and Littleford-Colquhoun et al. (2022).

In addition, dietary metabarcoding does not have the power to discriminate between scavenging, secondary predation, and accidental consumption (Tercel et al. 2021). Furthermore, metabarcoding neglects cannibalism, as it is unable to differentiate between consumer DNA and prey DNA (Cuff et al. 2022), so further observational data and morphological identification of stomach contents would be required.

The quality of taxonomic assignment largely relies on the quality of the reference databases being used, and gaps can lead to misassignments and false negatives (Keck et al. 2023). Extensive databases are available for COI and 18S markers (Ratnasingham and Hebert 2007; Leray et al. 2018; Quast et al. 2013), but some taxonomic groups and geographical areas are routinely under-represented. Species from less surveyed areas, like in our study (remote region of East Africa) are underrepresented in reference databases (Marques et al. 2021) and adding more taxa from this region would be beneficial.

5 | Conclusion

The findings from this study demonstrate clear divergence in the diets of the benthic and littoral ecotypes of *A. calliptera* from Lake Masoko. More robust understanding of the present role of trophic divergence in sympatric speciation would benefit from additional studies regarding the spatio-temporal variability in prey/forage availability and diet composition, in addition to feeding trials. Such studies would feature parallel seasonal trophic analyses with lentic eDNA, or traditional biodiversity analyses, complemented by dietary preference studies focused on heterogeneous littoral and benthic biota and the contrasting pharyngeal jaw morphologies of the ecotypes. Collectively, such studies would leverage a deeper synthesis of the role of trophic and microhabitat niche divergence in the ongoing sympatric speciation evident in *A. calliptera*.

Author Contributions

K.P. led methodology, project administration, data curation and visualization, writing and editing of the manuscript. G.T. and A.M.T. lead funding acquisition and conceptualisation. S.C. and G.T. supervised

this work, assisting with conceptualisation, method development in the laboratory, data analysis, study design and edited the manuscript. K.P., G.T., A.M.T. and M.A. contributed to data collection. E.G., W.P., G.B., D.A.D., K.H.M., and G.J.H. aided in method development in the laboratory and data analysis.

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Ethics Statement

All fish were ethically euthanised following the European Union regulations for the protection of animals used for scientific research (DIRECTIVE 2010/63/EU). Permits for collection of fish specimen was obtained from the Tanzanian Government.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw sequencing reads are deposited to GenBank under BioProject accession PRJNA1145132, <http://www.ncbi.nlm.nih.gov/bioproject/1145132>.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.