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Complementary uses of stable isotope and dietary metabarcoding analyses for trophic web determination in freshwater and estuarine tropical fishes

Pillay, Kirthana

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Complementary uses of stable isotope and dietary metabarcoding analyses for trophic web determination in freshwater and estuarine tropical fishes.

A thesis submitted to Bangor University by

G Kirthana Pillay
School of Natural Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW

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Supervised by:
Professor Simon Creer, Dr Nathalie Fenner, Dr Nigel Milner, Bangor University
Dr Michael Parsons, Dr Hidetoshi Urakawa, Florida Gulf Coast University

Declaration and consent

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

I confirm that I am submitting this work with the agreement of my Supervisor(s).'

'Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Rwy'n cadarnhau fy mod yn cyflwyno'r gwaith hwn gyda chytundeb fy Ngoruchwyliwr (Goruchwylwyr)

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Abstract

Understanding the network of interactions that support biological communities are vital for management and conservation of ecosystems. Characterising species interactions are vital for predicting ecosystem response to perturbation, however quantifying such interactions remains a challenge. Traditionally, behavioural observational data and morphological analysis of stomach contents have been used to identify prey-predator dynamics, but these methods are time consuming and introduce biases. Stable isotope analysis (SIA) of carbon and nitrogen have been extensively applied to trophic web studies to identify sources of carbon input and offer trophic level discrimination as well as provide long-term assimilation data. However, species level prey composition data is usually not attainable. DNA-based diet determination methods have been applied as an alternative to SIA as it produces data with high taxonomic resolution revealing species specific prey composition. Although, DNA-based methods also have limitations such as PCR and quantification biases. We propose that the complementary use of SIA and DNA-based techniques is necessary to obtain accurate representation of interactions within an ecosystem.

In this study we used a combination of carbon and nitrogen SIA techniques with dietary metabarcoding of intermediate trophic level fish from mangrove ecosystems. These fish are important conduits of energy transfer between basal organisms and top predators but have been rarely studied as they are not commercially important species. The SIA analysis reflected distinct carbon input between coastal and lagoon sites, where coastal fish were more enriched in ^{13}C. The results also unexpectedly reflected enriched ^{15}N signatures for species *Anchoa mitchili* (glass minnow/bay anchovy), a pelagic fish that usually occupies lower trophic levels. Due to the lack of prey SIA ratios, we were not able to confirm causes for elevated ^{15}N values observed in glass minnows, but dietary metabarcoding data revealed possibilities of ichthyoplankton ingestion. The mitochondrial cytochrome c oxidase subunit I (COI) and V4 region of the 18S ribosomal DNA-encoding gene were used to characterise prey diversity from the stomach contents.

Subsequently, the metabarcoding data was used to construct ecological networks which reflected that the intermediate trophic level fish had more specialised feeding preferences during wet season and generalised feeding patterns in mangrove habitats due to the greater

availability of resources. In addition, network models suggest that coastal networks are more resistant to potential extinction events.

Using similar metabarcoding techniques, we explored the role of diet and trophic niche divergence in the benthic and littoral ecomorphs of *Astatotilapia calliptera*. The stomach contents of benthic individuals consisted of nematodes, Bacillariophyta (diatoms) and copepods while the diet of littoral individuals comprised molluscs, annelids and fungi, supporting previous SIA data analysis. The dietary analysis reflected that the ecomorphs were likely generalist consumers but when resources are scarce they have the ability to expand their range and explore specialist prey.

Our findings demonstrate that the complementary use of SIA and dietary metabarcoding techniques can provide insights into interactions not offered when either technique is applied independently. The combination of these methods offer strong potential to develop a deeper understanding of feeding ecologies, that can be integrated into effective conservation and management strategies.

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Chapter 1: General introduction

An overview of trophic ecology is fundamental to understanding how biological communities are sustained as interactions between species underpin many processes and key ecological services (Ladd and Shantz 2020). Characterising species' interactions and structural mechanisms (such as stability, disturbance, ecosystem size, resource availability) are key to predicting ecosystem response to perturbation, however, quantifying such interactions remains a challenge (Clare 2014). Interactions between prey and predators structure communities through top-down and bottom-up controls. A classic example of top-down control is the regulation of sea urchin population by sea otters in Alaska, and the subsequent lack of urchin grazing enables kelp populations to flourish (Estes and Palmisano 1974). Plankton communities have shown to have bottom-up control in marine systems and this is especially true for cod recruitment in the North Sea. Beaugrand et al. (2003) demonstrated that as sea temperature rises, the abundance of calanoid copepods available for juvenile cod decreases, affecting cod growth rates and resulting in poor recruitment. Similar patterns have been observed in North Atlantic Salmon, where a decrease in zooplankton populations was correlated to a decrease in salmon numbers, as sea surface temperature increased (Beaugrand and Reid 2012). Bottom-up effects have also been experienced over multiple trophic levels, where sandeel biomass increased proportionally to plankton abundance, that subsequently had a positive effect on seabird breeding productivity (Frederiksen et al. 2006). However, the rate of human-driven changes in trophic interactions have been increasing due to overexploitation of natural resources (Salomon et al. 2010). To deal with this evolving network structure, conservation strategies and management policies need to be constantly reviewed and consider trophic interactions to optimise management practices (Perović et al. 2018).

1.1 Uses of trophic webs

Using the concept of trophic webs to explain energy flow, trophic cascades, species extinction and anthropogenic impacts on species interaction is one of the oldest in the field of ecology (Hussey et al. 2014). A typical trophic web comprises producers forming the base and consumers at discrete levels (e.g primary, secondary, tertiary etc) which provides a framework to understand energy flow within an ecosystem (Lindeman 1942). The nodes within food webs interact with one another, directly or indirectly (through intermediate species) (Woodward 2009a). Each link between prey and predator shows energy transfer from one individual to

another and are usually established by direct morphological identification of stomach or faecal contents. In addition, chemical analytics such as radio isotope labelling, stable isotopes, detection of prey using electrophoresis, isozymes and polyclonal antibodies have been used to quantify trophic links (Sheppard and Harwood 2005).

Trophic webs have also been used to understand ecosystem structure, dynamics, function and stability (Thompson et al. 2012). Theory has suggested that complexity (defined by high linkage density and/or species richness) together with distribution of linkage strength is vital for stability of food webs. Communities that have more stable food webs are less prone to trophic cascades and species extinction (Woodward and Hildrew 2002). Once a network is established, relationships such as species area, species distribution, body size rules and species range can be utilised to derive predictions on the ecological outcomes from environmental disturbance (Mestre et al. 2022).

1.1.1 Effects of body size on food web interaction

Aquatic food webs commonly comprise short-lived organisms that are small, abundant and diverse at the base (e.g., microalgae and zooplankton) followed by long-living larger, rare and less diverse organisms such as predatory fish. Due to differences in body-mass structures at different levels within the food web, effects of perturbation will affect organisms at varying rates with basal organisms reacting rapidly while responses from larger organisms will be slow and accumulative (Jackson et al. 2021).

Due to size-based feeding constraints, a single species can occupy several trophic levels as it grows during its lifetime (France et al. 1998; Romanuk et al. 2011). Size structure of organisms within food webs has an influence on energy flux between trophic levels. Metabolic theory (metabolism decreases with increasing size) coupled with body-size allometries also have an effect on overall food web stability (Yvon-Durocher et al. 2011a, Woodward et al. 2005) as turnover rates are allometrically related to body mass (Cohen et al. 2003). Metabolic effects have an impact on rate of consumption and digestion rates which in turn consequently determines interspecific interactions (Yvon-Durocher et al. 2011b). Organisms with larger body size are able to buffer against perturbations due to their low metabolic rates and the opposite applies for basal, small organisms that are able to react to perturbations quicker (Berlow et al. 2008).

1.1.2 Freshwater food webs

Freshwater food webs have been studied extensively because their taxonomy is well-resolved; feeding links of most species are well known and experimental manipulations are easy to conduct (Woodward 2009b). Studies of freshwater food webs have provided us with the fundamental concepts and theories of aquatic food webs as well as their limitations (Cohen et al. 2003; Petchey et al. 2004; Woodward, 2009; Woodward and Hildrew, 2002). However, these concepts are not always transferrable to marine food webs as they are intrinsically different from freshwater food webs due to differences in diversity and complexities that exist in marine environments, some of which include high rates of omnivory and high abundance of generalists (Cohen 1994; Link 2002; Dunne et al. 2004).

1.1.3 Marine food webs

Datasets on the feeding ecology and food webs of marine species are sparce and this is especially true for lower trophic level species (Rombouts et al. 2013). To date marine food web research has focussed mainly on commercially important species as intense economic pressure, overexploitation and the collapse of fisheries have been increasing (Dunne et al. 2004) due to unsustainable harvesting methods (Essington et al. 2006), thus focussing attention on such species. However, intermediate habitats such as estuaries that play a pivotal role in the life cycle of commercially important fish should not be neglected. Only a handful of studies have conducted ecosystem-based food web studies in estuarine environments (as reviewed by Bouillon et al. 2011) and more are required to predict the resilience of estuaries and evolution of ecosystem functioning due to human disturbance.

1.1.4 Estuarine trophic webs

Estuaries are uniquely situated between terrestrial and marine biomes experiencing high ecological productivity and anthropogenic pressures simultaneously (Chevillot et al. 2018). Estuarine trophic webs are extremely complex because they receive a variety of organic input from upstream freshwater to downstream oceanic waters. Moreover, other environmental factors, particularly salinity, which exerts a strong influence on fish distribution, and sediment supply are directly related to freshwater discharge. Fluctuating salinity levels has shown to affect distribution of macro zoobenthos, plankton, macroalgae, higher plants and fish species (Telesh et al. 2013; Teichert 2017). Hypersaline conditions resulting from the lack of freshwater

inputs decreases planktonic productivity and negatively impacts planktivorous fish (Whitfield 2005). In addition to this, lack of freshwater reduces olfactory cues that are exported to marine environments vital for recruitment of estuarine marine larvae (Strydom 2003). Conversely, major flooding events can lead to depletion of marine and estuarine fish species due to reduced food sources such as zoobenthos, zooplankton and flushing out of ichthyoplankton (Strydom et al. 2002). Producers within estuarine systems such as seagrass, microalgae, phytoplankton, and mangroves also contribute to organic matter production (Layman 2007; Bouillon et al. 2011). However, more quantitative research is required to determine interactions within estuarine ecosystems (Mestre et al. 2022).

1.2 Integration of trophic ecology in fisheries management

Ecosystem based management (EBM) is a more holistic approach to resource management that recognises the dynamic and heterogenous nature of ecosystems and uses ecological models to address complexity and connectivity at ecosystem scales (Curtin and Prellezo 2010; Thrush and Dayton 2010). Food webs reflect ecosystem dynamics that are important ecological indicators to EBM as they serve as proxies for ecological processes, represent the state of ecosystems (e.g resilience) and can be used to offer guidance for management actions (Tam et al. 2017). Accordingly, a multitude of examples exist, that demonstrate the use of interaction data to inform management practices of commercially important fish species. An example of feeding behaviour used to inform management has been demonstrated by Einoder (2009), where seabird diet has been used as a bioindicator for estimation of abundance, distribution, survival and recruitment of commercially exploited fish species. In addition, Scopel et al. (2018) used models from diet assessments of common tern (*Sterna hirundo*) colonies in the Gulf of Maine to demonstrate strong links to fishery data such as acoustic surveys, landings, recruitment and spawning stock biomass. Dietary, demographic and behavioural data from a colony of common guillemots (*Uria aalge*) revealed reduction in reproductive output, which was mainly driven by reduced energy content in sandeels, that are commonly preyed upon by the guillemots (Wanless et al. 2005). The authors suggested that the reduced energy levels observed in sandeels was attributed to changes in abundance or distribution of plankton communities and this in turn was used to inform sandeel fisheries in the North Sea (Wanless et al. 2005).

A study examining depredation behaviours of marine predators in the presence of fisheries observed that Crozet killer whales (*Orcinus orca*) in the Antarctic revealed over reliance on highly valuable toothfish (*Dissostichus eleginoides*) from longline fisheries (Tixier et al. 2019). The results indicated that toothfish play a crucial role as prey for top predators and assessing predator prey relationships are required to better fish stock management and conservation policies. In a review analysing the biotic and abiotic factors of the recruitment of commercially valuable Atlantic herring (*Clupea harengus*), the authors concluded that investigating diet preferences of herring is imperative for understanding growth and contribution to recruitment while stomach content analysis from potential predators and competitors will aid to identify interactions that influence survival and recruitment (Burbank et al. 2022). Dietary information has therefore aided fisheries' managers to develop a comprehensive understanding of the factors influencing changes in population abundances of Atlantic herring. In a recent study focussing on implementation of EBM techniques in Pacific salmon (*Oncorhynchus sp.*) fisheries stated that diet analysis of salmon individuals was used to inform hatchery management (mainly recruitment) (Wells et al. 2020). The study also suggested that models can be developed based on stomach content analysis or foraging behaviour against ecosystem attributes (or abiotic factors) and predator diets will reveal spatiotemporal distributions of potential predators. Dietary analysis has also been applied to investigate the level of microplastic ingestion in commercially exploited species *Chelon richardsonii*, the Southern mullet (McGregor and Strydom 2020). This species is primarily exploited for human consumption but also makes an important prey for other commercial species such as Dusky kob (*Argyrosomus japonicus*), Garrick (*Lichia amia*), Elf/Shad (*Pomatomus saltatrix*) and Bartail flathead (*Platycephalus indicus*). Morphological stomach content analysis of the mullet was found to ingest large amounts of microplastic fibres (40%) and fragments (5%) across all developmental stages, indicating poor water quality standards (McGregor and Strydom 2020). In addition, the authors concluded that the dietary analysis was imperative for understanding the cascading effects of ingested microplastics and associated chemicals into coastal food webs, which also includes humans. These examples re-iterate how interaction data from target and non-target species associated with commercially important fishing activities can be used in sustainable management of fisheries (McInnes et al. 2017).

1.3 Integration of trophic ecology in evolutionary studies

Feeding ecology studies also contribute to the understanding of a range of subjects such as resource partitioning, habitat preference, prey selection, competition, energy transfer (Braga et al. 2012) and ontogenetic dietary shifts (Kulatska et al. 2019; Sánchez-Hernández et al. 2019). Feeding ecology and food constraints influence morphological development and behaviour of organisms, helping them to adapt to their changing environment (Abrahamczyk and Kessler 2014). Studies have even shown that feeding preferences have led to the diversification of cranial morphology increasing adaptation to specialised diets (Christiansen and Wroe 2007). DNA-based methods have been used to understand evolutionary shifts in feeding guilds and dietary niche partitioning, as it is able to produce dietary data of high taxonomic resolution.

Several studies have applied DNA based techniques to reveal dietary partitioning between closely related species. Spence et al. (2022) used metabarcoding of hummingbird scat to analyse the extent to which dietary specialisation and niche partitioning was guided by beak morphological diversity. Furthermore, dietary metabarcoding provided evidence for adaptive diversification of diet that explain morphological differences observed in murid rodents (tribe Chrotomyini) from the Philippines (Petrosky et al. 2021). Hernández Macías et al. (2016) used metabarcoding to characterize dietary preferences of *Dysdera* in the Canary Islands and concluded that dietary data revealed presence of trophic segregation (via specialisation) among co-existing species as a primary driver of morphological diversification and speciation.

1.3.1 Trophic ecology of cichlids

Cichlids are one of the most species rich and ecologically diverse families of freshwater fishes worldwide (Vanhove et al. 2016). Due to their large variation in behaviour, ecology and morphology cichlids represent an ideal model in evolutionary research (Koblmüller et al. 2015). Cichlids naturally occur in Africa, Latin America, Madagascar and Asia, but most research has focussed on African cichlids due to their great adaptive radiation and importance as a food fish (Turner 2007). Previous research has used a range of morphological techniques, observational data and genomic analysis to uncover the mechanisms that drive diversification of these fish (Koblmüller et al. 2015). However, dietary metabarcoding has not been applied to investigate the role of dietary divergence in adaptive radiations of cichlid fish before.

1.4 Integration of trophic ecology in mangrove management

Mangroves occupy an interface between land and sea providing unique forested habitats for terrestrial and marine organisms (Sheaves 2009). Studies investigating the feeding ecology of both resident and migratory mangrove fish, invertebrates, birds and reptiles have recognised the importance of mangrove habitats as vital feeding and nesting grounds for a large number of species due to the high levels of resource availability in mangroves (Kathiresan and Bingham 2001). A study conducted by Tse et al. (2008), indicated that juvenile fish abundance was higher in mangrove habitats compared to adjacent estuarine habitats due to the higher prey abundance and rich organic detritus found in mangroves. Gut content analysis performed by Lugendo et al. (2006) on nine different mangrove fish species showed that they feed on a variety of organisms such as copepods, ostracods, crabs, shrimp, algae and detritus. Larger predatory fish are known to enter mangrove bays when tide and turbidity are in ideal conditions to feed on juvenile fish and prawns (Nagelkerken et al. 2008). Mangrove leaf litter and detritus are important sources of energy for Indian, Pacific and Tanzanian sesarmid crabs and Australian graspid crabs who have shown to preferentially consume these organic resources (Skov and Hartnoll 2002; Bui and Lee 2014; MacKenzie et al. 2020; Rani et al. 2023). Dietary studies have shown that prawns exclusively feed in mangrove forests when the tide is high, consuming a wide variety of organisms such as mangrove detritus, fish, insects, diatoms, crustaceans, bivalves, gastropods and polychaetes (Nagelkerken et al. 2008). Diets of avian species such as the broad billed flycatcher and mangrove robin primarily consume insects, whereas the red-headed and brown honeyeater consume nectar; coastal birds such as herons, kingfishers, egrets and cormorants tend to be piscivorous (Buelow and Sheaves 2015; De Dios Arcos et al. 2020). Mangroves also provide foraging grounds for a remarkable number of terrestrial vertebrates (Rog et al. 2017). *Procyon cancrivorus* (crab-eating racoon) is a carnivorous mammal endemic to south America and primarily feeds on arthropods and fruit produced in mangrove forests (Martinelli and Volpi 2010). Additionally, nectar feeding bats from Malaysian mangroves have demonstrated mutualistic relationships between feeding and pollination of *Sonneratia* mangrove trees (Nor Zalipah et al. 2021).

Despite the high level of contribution of mangrove resources to the survival of associated species, such interactions are usually classed under ‘non-market’ value when assessing the economic contribution of mangrove ecosystems, resulting in undervaluing mangroves that has led to unsustainable use (Macintosh and Ashton 2002). Therefore, quantifying these

interactions are vital for informing future policy making and resource management of mangrove ecosystems. In the eighteenth century, a shift from subsistence use to industrial exploitation, alongside colonisation where mangrove timber was harvested for European export, exacerbated the rate of mangrove deforestation (Friess et al. 2019). In the 1980s, large scale alteration of land use for aquaculture, agriculture, urban development and overextraction of forest products led to the loss of 35% of the world's mangrove forests. In recent years however, the rate of mangrove deforestation has reduced globally to between 0.16% and 0.39% per year (Hamilton and Casey 2016).

Conservation of mangrove forests is vital for achieving the United Nations Sustainable Development Goals (SDGs) 14 (Life Below Water), 2 (Zero hunger, through ecosystem services provided by coastal fisheries) and 13 (Climate Action via carbon sequestration and storage) (Friess et al. 2019). To achieve these goals and reduce the rate of deforestation, several management practices have been adopted. Integrated Coastal Zone Management has been implemented in many countries where natural resources and human activities have been managed together, and is mutually beneficial to the environment, coastal communities, industries, businesses and governments (Carter et al. 2015). Significant improvements in mangrove management were observed when a more holistic approach has been implemented through programmes such as the Ramsar convention (wetland conservation), Earth Summit, FAO Forestry department, International Tropical Timber Organisation (ITTO) (UN Initiative) (forestry management) and the International Society for Mangrove Ecosystems (ISME) (mangrove specific management), where there is a agreement on the crucial elements of mangrove management (Carter et al. 2015). Reducing emissions from Deforestation and Degradation (REDD+) a form of payments for ecosystem services (PES) scheme has also been suggested in mangrove forests as they are able to sequester five times more carbon than a typical terrestrial forest (Donato et al. 2011; Friess et al. 2019). Therefore, considering feeding ecology studies in management and economic evaluation of mangrove forests is crucial for informing management schemes and realising international goals. Researchers still depend on published information and expert knowledge (qualitative) to determine interactions (Mestre et al. 2022). The use of molecular methods such as DNA-based techniques (quantitative) and stable isotope analysis have been used to describe food-web structure, but these methods are still not widespread (Compson et al. 2019; Whitaker et al. 2019).

1.5 Application of dietary metabarcoding in trophic ecology studies

DNA-based diet determination has superseded many traditional methods such as morphological analysis of prey items from stomach contents and faeces, and direct observation of feeding behaviours (Elbrecht et al. 2017). Metabarcoding, high throughput sequencing coupled with DNA barcoding produces high volumes of data with high taxonomic resolution and is able to identify rare and cryptic species within highly degraded material (Nielsen et al. 2018; Taberlet et al. 2018). With the metabarcoding approach, universal primers are commonly used to amplify target taxa from bulk samples such as faeces or gut contents via the polymerase chain reaction (PCR) (Roslin et al. 2019; Bohmann et al. 2022). Metabarcoding has been successful at characterising diets from a range of animals including aquatic mammals such as otters and seals (Boyi et al. 2022), herbivorous reef fishes (Nalley et al. 2022), terrestrial mammals such as woodrats (Stapleton et al. 2022), elephants, zebras, dik diks, buffalos and cattle (Kartzinel et al. 2015), and insects such as dragonflies (Morrill et al. 2021) and damselflies (Kaunisto et al. 2017). Diet metabarcoding has enabled ecologists to detect trophic links between taxa efficiently and construct detailed interaction networks to evaluate impact of environmental change on ecosystem provision, structure and functions (Bascompte 2007; Ings et al. 2009; Cuff et al. 2022).

1.5.1 Benefits and limitations of dietary metabarcoding in network analysis

Ecological networks have been used to describe complex systems using interaction data and they provide an insight into ecosystem function through the calculation of network metrics (Guimarães 2020). Dietary metabarcoding has been integrated into network studies a handful of times as metabarcoding provides high taxonomic resolution that is ideal for constructing multilayer networks including interactions between and within trophic levels (Cuff et al. 2022). These DNA-based networks have been used to infer prey choice, evaluate ecological responses to perturbation, assess ecosystem health and design conservation schemes (Clare et al. 2019; Cuff et al. 2021; Mata et al. 2021). Unlike DNA-based networks, morphological and observational data is biased towards undigested prey items such as otoliths, mollusc shells, exoskeleton parts and underrepresents small prey items including soft-tissued prey and easily digested food that becomes undetected by visual analysis (de Sousa et al. 2019; Traugott et al. 2021). Furthermore, morphological and observational data is highly time-consuming, labour

intensive and places high reliance on the skills of taxonomic experts (Pompanon et al. 2012; de Sousa et al. 2019).

It is vital however to acknowledge that molecular methods have some shortcomings that affect the data produced. Firstly, quantification is not straight forward due to a range of biases such as differential rates of DNA degradation (Murray et al. 2011), metabolism by consumer (Greenstone et al. 2014) and volume of prey consumed (Egeter et al. 2015). Due to these biases, assuming that read counts equate to biomass will result in inaccurate interaction weightings within the network (Deagle et al. 2019a). Similarly, PCR based techniques introduce primer bias (Murray et al. 2011) and random sampling during sequencing make 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 estimation of taxon abundance (Creer et al. 2016; Casey et al. 2021).

Secondly, the lack of sampling completeness due to exclusion of temporal variabilities and rare taxa that are not captured through metabarcoding may result in construction of incomplete network interactions (Macgregor et al. 2017). In addition, dietary metabarcoding is not able to differentiate between scavenging from secondary predation or accidental consumption; this disproportionately inflates the trophic relevance of non-target taxa, introducing erroneous interactions (Tercel et al. 2021). Furthermore, metabarcoding does not consider the life stages of prey, disregarding ontogenetic shifts, altering network perspectives and the effects on taxa (Cuff et al. 2022). Despite these limitations, dietary metabarcoding is considered to be a superior method compared to observational data and morphological identification of consumed taxa from gut contents or faecal matter. Combining diet metabarcoding data with other complementary methods such as stable isotope analysis (SIA) can provide an accurate understanding of interactions as it provides long-term information on assimilated diet (Cordone et al. 2022).

1.6 Application of stable isotope analysis (SIA) in trophic ecology studies

Stable isotope analysis (SIA) of carbon and nitrogen have been traditionally used to identify sources of carbon input and provide trophic level discrimination. The carbon signature of primary producers is conserved throughout the food chain as it undergoes minimal

fractionation of only 1% per trophic level (Fry and Sherr 1989; Fredriksen 2003), and is subsequently used as a tracer of organic matter enabling consumers to reveal the distinct carbon source of their diet. Changes in carbon isotopic ratios can be used to determine original carbon source as ratios of carbon isotopes vary based on the photosynthetic pathway taken by primary producers (i.e., C_3 versus C_4 pathways in plants, whereby enzymes involved in each pathway discriminates carbon at different rates) (Layman et al. 2012). Conversely, the nitrogen isotope ratio ($\delta^{15}N$) is enriched by 3-4 % per trophic level and can be used to infer dietary preferences and trophic level positioning within a food web (Deniro & Epstein, 1981; Villamarín et al, 2018).

Unlike dietary metabarcoding, stable isotope analysis provides long-term feeding behaviour and assimilation data due to low turnover rate (Maloy et al. 2013). Trueman et al. (2012) suggested that in slow growing fish like sharks and deep-water fishes (that have slow metabolic rates) stable isotope ratios of muscle tissue samples can represent years. Thus, SIA is able to reflect temporal variabilities and depict structural changes in food webs over various timescales (Woodland et al. 2012). Traditionally mixing models have been applied to SIA results, as they are able to estimate the proportional prey contribution or diet composition of the target consumer (Phillips 2012). However, the stable isotope ratio of every prey/resource is required prior to applying mixing models. Given this shortcoming, metabarcoding is an excellent alternative to elucidate prey composition.

1.6.1 Uses of carbon in trophic webs

Primary producers in estuarine ecosystems have distinct carbon signatures (^{13}C) due to the utilisation of different processes to fix inorganic carbon during photosynthesis (DeNiro and Epstein 1978; Ehleringer et al. 1986; vander Zanden and Rasmussen 1999). Mangroves undergo the C_3 pathway of carbon fixation where inorganic carbon is in the form of free carbon dioxide and is fixed using the RuBP carboxylase (Rubisco) enzyme during photosynthesis (Abel 1984; Abrantes et al. 2015). The C_3 pathway of carbon fixation results in strong isotopic discrimination against ^{13}C, resulting in C_3 plants having lower levels of heavier carbon isotope (Hemminga and Mateo 1996). C_4 plants fix inorganic carbon (HCO_3^-) in aquatic environments such as marine waters using phosphoenolpyruvate carboxylase (PEP carboxylase) which discriminates ^{13}C to a limited extent (Hemminga and Mateo 1996) resulting in C_4 plants such as seagrass retaining larger amounts of ^{13}C. Recent research has shown that seagrasses are an

intermediate of C_3 – C_4 plants as Rubisco and PEP carboxylase appear to play different roles in specific conditions. Touchette and Burkholder, (2000), suggest that variation in seagrass ^{13}C values may be attributed exposure to light intensity leading to photorespiration and depletion of ^{13}C. Similarly, marine phytoplankton use a mixture of CO_2 and HCO_3^- as sources of inorganic carbon due to limitations of light intensity and utilise both the C_3 and C_4 carbon fixation pathway (Riebesell 2004). Between phytoplankton and benthic algae, benthic algae are more enriched in ^{13}C because they experience reduced water turbulence. The boundary layer surrounding phytoplankton is defined as the zone of little or no flow and movement is able to break down/reduce this layer leading to accelerated ^{13}C depletion through diffusion (France 1995). As a result of different carbon fixation and fractionation processes, the carbon isotope ratio is a useful tool to distinguish between primary producers at the base of trophic webs.

1.6.2 Application of carbon and nitrogen stable isotope ratios in trophic web studies

The distinctive carbon signature of primary producers is conserved throughout the food chain as it undergoes minimal fractionation of only 1% per trophic level (Fry and Sherr 1989; Fredriksen 2003), enabling consumers to reveal the photosynthetic carbon source of their diet. Unlike carbon, the nitrogen isotope ratio (^{15}N) is enriched by 3-4 % per trophic level which can be used to infer predator prey relationships and feeding dynamics (Deniro and Epstein 1981). Nitrogen ratios have also been used to identify changes in feeding preferences during different life stages of an organism (Jarman et al. 1996). In addition to revealing trophic dynamics, carbon and nitrogen isotope ratios have been used to understand the impact of human induced changes on fish communities. Nitrogen isotope ratios have been used to reveal that biomass of larger fish decrease in areas vulnerable to fisheries exploitation compared to non-exploited marine environments (Jennings et al. 2008). Carbon and nitrogen isotopes have also been used to infer heavy metal contamination in marine and macroinvertebrate food webs (Jarman et al. 1996; Watanabe et al. 2008) because heavy metals like mercury have direct correlations to ^{13}C and ^{15}N isotope ratios (Watanabe et al. 2008). Other elemental ratios such as oxygen in fish otoliths ($^{18}O_{oto}$) can be used to reflect migration of fish over different temperature and salinity gradients (Trueman et al. 2012) as surface water temperature rises and migratory fish move through different trophic zones.

1.7 Benefits of using dietary metabarcoding and stable isotope analysis (SIA) complementarily

The first study to combine DNA based methods and stable isotope analysis to resolve trophic interactions was published in 2010. Hardy et al. (2010) set out to resolve carbon flows through a complex riverine food web based in southern Australia using a combination of carbon and nitrogen natural abundances and dietary metabarcoding from five different fish species. The results showed good correlation on available food sources between stable isotope ratios and genetic data and the study concluded that use of both methods are essential to understand trophic webs and their relationships with nutrient and energy fluxes. Subsequent research following this study have applied a combination of DNA based methods and stable isotope techniques to examine dietary patterns in a range of terrestrial organisms such as *Blackburnia hawaiiensis* (carabid beetle) (Roy et al. 2021), wolves and black bears in Canada (Bonin et al. 2020), arctic small rodents (Soininen et al. 2014) and large African herbivores (Kartzinel et al. 2015). Application of these combined techniques have also been used in marine organisms including *Mytilus* spp. (mussels) (Maloy et al. 2013), key fishery species *Plectropomus* spp. (grouper) (Matley et al. 2018) and invasive species such as *Carcinus maenus* and *C. aestuarii* (European green crabs) (Cordone et al. 2022).

Despite the ability to provide complementary information on trophic ecology, only one study to date has used SIA and dietary metabarcoding in mangrove ecosystems. This study analysed the diet of the critically endangered *Pristis pectinata* (smalltooth sawfish) from southwest Florida to investigate the extent of resource partitioning with sympatric elasmobranch species (Poulakis et al. 2017). The carbon ratios from SIA showed that *P. pectinata* exhibited resource partitioning as juveniles when compared against bull sharks while nitrogen ratios indicated consumption of higher trophic level species regardless of life stage. The metabarcoding data indicated that *P. pectinata* diet mainly comprised teleost and elasmobranch fishes, confirming the SIA results. The study concluded that the combination of SIA and dietary metabarcoding uncovered detailed differences between the sympatric elasmobranch species and the resultant data has the potential to provide species-specific management strategies. Therefore, the use of SIA and dietary metabarcoding in network interaction studies is necessary to obtain accurate representation of ecosystem structure and functions.

1.8 Objectives and hypotheses of this thesis

Extensive research conducted in freshwater systems has led to highly resolved trophic webs (Thompson et al. 2012) and their response to environmental perturbation has been simulated through mesocosm experiments as they act as proxies of natural ecosystems (Brown et al. 2011; Ledger et al. 2011). Such freshwater network studies have provided us with theories and fundamental understanding of aquatic food webs along with their limitations (Woodward and Hildrew 2002; Cohen et al. 2003; Petchey et al. 2004; Woodward 2009a). However, these concepts are not always directly applicable to marine and estuarine food webs due to the complexity of systems (Dunne et al. 2004). Mangrove forests especially with their unique positioning in the interface between land and sea, are influenced by myriad of abiotic and biotic factors such as tides, salinity, forest composition and runoff (Buelow and Sheaves 2015). Current literature on marine and estuarine trophic web research have focussed largely on invertebrate fauna (Peterson et al. 1985; Abrantes and Sheaves 2009; Abeels et al. 2012; Bernardino et al. 2018; Jung et al. 2019) and economically important fisheries species (Essington et al, 2006; Lugendo et al, 2006; Jinks et al, 2020; Chapman et al, 2020). However, non-economically important species from intermediate trophic levels, that form important links between benthic invertebrates, plankton communities and higher trophic levels that contribute to commercially important species, have rarely been studied even though they have a substantial influence on the interactions within food webs (Pauly et al. 1998; Hall 1999; Cury et al. 2003).

In this thesis we used a combination of dietary metabarcoding and SIA of estuarine fish from intermediate trophic levels to quantify interactions between different taxa. In addition, we applied dietary metabarcoding techniques to investigate the diet differentiation between sympatric cichlid ecomorphs, *Astatotilapia calliptera*, from crater Lake Masoko in Tanzania. The outcomes of this research will provide more evidence to understand the extent to which dietary metabarcoding and stable isotope analysis are complementary techniques that can be used in feeding ecology studies. Specifically, this thesis aims to:

1. Use a combination of carbon and nitrogen stable isotope ratios to identify ultimate carbon sources and trophic dynamics in fish communities from intermediate trophic levels. In addition, we test if intermediate trophic level fish display significant intraspecific and interspecific differences in resource use between habitats, seasons and salinity zones. With reference to the SIA results we aim to develop a simple conceptual

network, recreating trophic linkages amongst mangrove dwelling organisms. Here, we focussed only on estuarine fish from the Estero Bay Aquatic Preserve located in southwest Florida. Estero Bay is Florida's first aquatic preserve and consists of mangrove forests, seagrass and algal beds, blackwater streams, beach dunes, coastal berms, sponge beds, salt marshes and mollusc reefs. It is also supplied with freshwater from several small rivers and creeks. Due to its complex habitat composition the preserve has been described to be a moderate to highly productive system (Florida Department of Environmental protection (3) 2015), thus ideal for investigating trophic interaction in a heterogenous ecosystem. We predict that the fish will have ^{13}C values reflective of the primary producers consumed at the base of the food web (Duarte et al. 2018), while a linear enrichment in ^{15}N is expected as trophic positioning and size of organism increases (Deniro and Epstein 1981). *(Chapter 2: Using stable isotope analysis (C and N) to determine ultimate carbon source and trophic positioning of intermediate trophic level fish from mangrove ecosystem)*

2. Use dietary metabarcoding to evaluate and compare the diets of intermediate trophic level fish from Estero Bay Aquatic Preserve. In addition, we assess the ability of DNA metabarcoding to obtain species level interaction data from stomach contents of fish to complement the stable isotope analysis results. We aim to construct ecological networks from the resultant metabarcoding data and use network metrics (such as robustness, nestedness, extinction slope) to investigate if changes in feeding interactions are driven by salinity, season and habitat assemblage. We predict that metabarcoding will elucidate feeding interactions via species level taxonomic resolution and expect the intermediate trophic level fish to be generalist consumers. Furthermore, we predict that changes in network interactions are determined by several interlinked factors such as salinity, seasonal changes and habitat composition. *(Chapter 3: Application of DNA metabarcoding to elucidate diets and ecological networks of intermediate trophic level fish from mangrove ecosystem).*

3. Apply dietary metabarcoding techniques to investigate trophic specialisation of *Astatotilapia calliptera* in the early stages of adaptive divergence. *A. calliptera* from crater Lake Masoko in southern Tanzania consist of littoral (shallow-water) and benthic (deep-water) ecomorphs. Previous genomic research coupled with morphological analysis of body form and mate choice experiments have suggested that *A. calliptera*

have diverged sympatrically (Tyers 2013; Malinsky et al. 2015). Stable isotope analysis of carbon and nitrogen performed has also indicated potential diet segregation where benthic individuals preferred a planktivorous diet compared to littoral yellow individuals who consumed molluscs (Carruthers et al. 2022). As stable isotope analysis only classifies diets into broad functional groups and does not elucidate species level prey composition, dietary metabarcoding is required to provide a clearer understanding of diet diversification between the ecomorphs and provide evidence for the role of dietary divergence in sympatric speciation of *A. calliptera.* Based on the results from Malinsky et al. (2015) and Carruthers et al. (2022), we expect the benthic ecomorph to primarily consume zooplankton and phytoplankton while littoral individuals to specialise in hard bodied macroinvertebrates. *(Chapter 4: Investigating diet differentiation among sympatric ecomorphs of the cichlid fish Astatotilapia calliptera from Lake Masoko (Kisiba), Tanzania).*

Chapter 2: Using stable isotope analysis (C and N) to determine ultimate carbon source and trophic positioning of intermediate trophic level fish from mangrove ecosystem.

2.1 Abstract

Estuarine ecosystems around the world form intermediate environments between marine and freshwater systems that make them highly productive environments. Tropical estuarine systems are highly complex environments because they comprise unique habitats such as mangrove forests, seagrass meadows, oyster and coral reefs and salt marshes within close proximity to each other. Consequently, there is limited understanding of the whole ecosystem effect on ichthyofaunal diversity and productivity. Here we use a combination of carbon and nitrogen stable isotope ratios to identify ultimate carbon input and trophic positioning of omnivorous fish from a heterogeneous estuarine ecosystem (Estero Bay Aquatic Preserve, Florida, USA). Our results showed distinct differences in carbon input between coastal and lagoon dwelling fish despite similarities in habitat composition between the different locations. Coastal sites showed enriched $\delta^{13}C$ values indicating influence of marine autotrophs while lagoon fish had depleted $\delta^{13}C$ signatures representing contribution from mangrove detritus. $\delta^{15}N$ was used to postulate a four-tier trophic network and additionally revealed enriched signatures for species *Anchoa mitchili* (glass minnow/bay anchovy), a pelagic fish that usually occupies lower trophic levels. Our analyses show that the role of photosynthetic marine organisms are magnified when proximity and frequency of mixing with seawater is increased in estuarine habitats. In addition, debates on contribution of mangroves to fisheries should be reconsidered as results from this study reveal mangrove derived carbon sources significantly influences the carbon ratios of organisms occupying lagoon mangrove fringes.

2.2 Introduction

Coastal and freshwater ecosystems around the world are intricately linked to each other through the transport of nutrients and movement of organisms across the salinity gradient. Tropical estuarine ecosystems are useful models for investigating the mechanisms involved in ecosystem connectivity because they feature a mosaic of highly productive habitats such as mangrove forests and seagrass meadows (Igulu et al. 2013). Mangroves provide a variety of ecosystem services such as protection against shoreline erosion and subsistence in the form of firewood and building material for local communities (Walters et al. 2008). Mangrove forests are one of the world's most productive ecosystems and are estimated to be worth US$ 194 000 per hectare per year (Costanza et al. 2014). Despite their importance to the global ecosystem, mangrove forests have undergone extensive deforestation (Fry and Ewel 2003) and are currently facing additional anthropogenic stresses such as aquaculture, agriculture and housing (Lewis et al. 2016).

2.2.1 Contribution of mangrove habitats to fisheries

Mangrove systems and coastal fisheries have strong trophic linkages, however, the extent to which mangroves support fishery production and catch has been heavily disputed (Nagelkerken et al. 2008). Some studies have shown that the relative abundance or area of mangrove correlates with fishery catch (MacNae 1974; Martusubroto and Naamin 1977) and this is especially true for shrimps (Paw and Chua 1989; Staples et al. 1984; Turner 1977). Some studies support the importance of mangroves to fisheries by claiming that mangrove forests play an important part in the juvenile stage of organisms providing them with resources and other benefits (Fry and Ewel 2003).

However, several studies have stated that the use of mangrove habitats by juveniles are species specific (Dorenbosch et al. 2006a, 2006b; Mumby et al. 2004; Nagelkerken et al. 2002) and movement of juveniles between estuarine mangroves and other adjacent habitats such as seagrass, saltmarshes and coral reefs should be accounted for (Nagelkerkan et al. 2008). Some adult fish such as *Epinephelus itajara* (goliath grouper) (Koenig et al. 2007) and *Scarus guacamaia* (rainbow parrotfish) (Machemer et al. 2012) spawn at sea where the eggs are dispersed within the water column. The planktonic larvae developed from these eggs migrate to inshore estuarine waters through swimming or passive tidal support, using the mangroves for habitat and forage before moving offshore again upon maturity (Baran and Hambrey 1998).

Similar behaviour is observed in commercially valuable species *Mugil cephalus* (striped mullet), *Cynoscion nebulosus* (spotted seatrout), *Sciaenops ocellatus* (red drum) and *Lutjanus griseus* (grey snapper) (Heald and Odum 1970). Conversely, some species remain in the mangroves throughout the year due to their euryhaline nature such as *Centropomus undecimalis* (snook), *Callinectes sapidus* (blue crab) and *Crassostrea virginica* (American oyster) and they are recreationally and commercially significant (Hutchison et al. 2014).

Traditionally, assessing commercial fish catch related to estuarine habitats has employed a single-habitat approach, instead of an ecosystem approach, that neglects the strong links existing between mangroves, seagrass meadows, salt marshes and the surrounding habitats (Sheridan and Hays, 2003; Meynecke et al. 2007). The number of studies investigating the contribution of independent seagrass and mangrove habitats to fishery activities has been on the rise (Hutchison et al. 2014; Herrera et al. 2022) but ecosystem level studies are absent. It is vital to acknowledge that the inputs derived to sustain mangroves as nursery habitats can be a consequence of nutrient exchange between mangroves and neighbouring habitats, including freshwater and terrestrial environments (Sheaves et al. 2015). Many factors influence the productivity of mangroves besides just mangrove cover, including the extent of shallow seas, intertidal areas, tidal creeks, organic material, and length of coastline (Baran and Hambrey 1998). There is also a lack of studies investigating the food sources of mangrove-dependent animals (Bouillon et al. 2008). To achieve a deeper understanding of the impact of mangrove forests on fishery production and catch, it is important for research to account for the interaction between habitats (such as seagrass meadows, oyster reefs, salt marshes and mangrove forests) within an estuarine system.

2.2.2 Prevalence of mangroves in Florida

Globally, there are 14.8 million hectares of mangroves forests remaining (FAO 2020). The third most mangrove abundant region is situated in North America with 1.2 million hectares of mangrove forests, of which 20% is in Florida and primarily located in the Southwest region (Florida Department of Environmental Protection (1), 2022), on the Gulf coast. Mangroves are native to Florida's landscape and are vital in supporting recreational and commercial fisheries. Florida is comprised of 67 counties of which 37 counties generate significant income from commercial marine fishing. In 2021, Lee County in Southwest Florida generated US$ 20 million from commercial fisheries landings for food and bait (Florida Fish and Wildlife

Conservation Commission 2022). Lee County is home to five aquatic reserves and is a popular area for recreational fishing, which also generates substantial revenue through license and tackle sales, local employment and support services (Florida Fish and Wildlife Conservation Commission 2021). Many species are exploited in sport fisheries, including Centropomidae *undecimalis* (snook), *Sciaenops ocellatus* (redfish), *Cynoscion sp.* (trout), *Epinmephelus sp*. (grouper), *Equetus sp*. (drum), *Archosargus probatocephalus* (sheepshead), *Caranx* sp. (jacks), *Trachinotus carolinus* (Florida pompano) and *Lutjanus* sp. (snappers)(Florida Department of Environmental Protection (2) 2022).

2.2.2.1 Estero Bay Aquatic Preserve

Estero Bay Aquatic Preserve located in Fort Myers, Lee County (Figure 2.1), was given the accolade as Florida's first aquatic preserve in 1966 when the threat of continued coastal developments on fisheries, tourism, and the habitat itself was recognised (Florida Department of Environmental protection (3) 2015). The preserve lies within the Coastal and Heartland National Estuary Partnership (formerly known as Charlotte Harbour National Estuary Program (CHNEP)) established in 1987 which aims to improve the quality of estuaries with national significance. Estero Bay also benefits from the Outstanding Florida Water (OFW) designation which prevents direct pollution discharges into its watershed. Estero Bay is 17.7 km long and has variable width of 2.8 to 11 km. The total watershed area is 758.5 km^2 (Abeels et al. 2012) and is supplied with freshwater from several small rivers and creeks. Much of the coastline is comprised of mangrove swamps with extensive areas covered in seagrass beds. Other natural communities include algal beds, blackwater streams, beach dunes, coastal berms, sponge beds, salt marshes and mollusc reefs. Due to its complex habitat composition the bay has been described to be moderate to highly productive providing home to a wide range of birds, aquatic vertebrates and invertebrates, and mammals (Florida Department of Environmental protection (3) 2015).

Figure 2.1. Map of Estero Bay Aquatic Preserve, including freshwater rivers and passes (Google Earth 2022).

The Caloosahatchee River Estuary (Figure 2.1) north of Estero Bay, present a transition from the Gulf of Mexico marine ecosystem to the vast freshwater Lake Okeechobee (10.76 km^2) and associated catchment area. Since the 1800s, the Caloosahatchee River Estuary has been subject to severe hydrological alterations of a meandering river ecosystem to a series of canalised

waterways (Mitra et al. 2011). The river has been impounded by several control structures to regulate the release of freshwater from Lake Okeechobee to prevent it from flooding (Tolley et al. 2005). Okeechobee watershed is used for beef cattle pasture (32%), cultivation of crop plants (18%) and residential purposes (10%). A large amount (5554 tonnes) of nitrogen is deposited into the lake every year and more than half of the deposits are exported (2986 tonnes per year) through the Caloosahatchee River (Havens et al. 2001). The nitrogen rich water from Caloosahatchee River travels into Estero Bay through Matanzas Pass and Mullock Creek via the Tenmile Canal (Figure 2.1) (Thomas and Rumbold 2006). Several measures have been implemented to control the inflow of freshwater and maintain salinity throughout the year and these are currently being observed through long-term water quality monitoring programmes (Thomas and Rumbold 2006).

Several studies have been conducted in Estero Bay with a large focus on understanding hydrodynamics and impacts of anthropogenic activities such as fishing and boating on surrounding habitats and fauna (Byrne and Gabaldon 2007; Mitra et al. 2011; Hotaling-Hagan et al. 2017). Only two published studies have investigated the feeding dynamics of organisms that inhabit this reserve. These studies have examined trophic transfer from oyster reefs to primary consumers and predatory fish (Abeels et al. 2012; Wasno et al. 2020). Relatively little is known about groups of fish that occupy intermediate trophic levels connecting primary consumers to predatory fish. Due to the juxtaposition of distinct habitats in Estero Bay it is an ideal ecosystem to study the dynamics of trophic energy flow of fish communities derived from mangrove, seagrass, oyster and coastal habitats.

2.2.3 Aims and objectives

In this study we used a combination of carbon and nitrogen stable isotope ratios to investigate trophic dynamics and identify ultimate carbon sources in fish communities from lower trophic levels, occupying heterogeneous habitats (mangrove, seagrass and coastal) and salinities in a productive, subtropical estuarine ecosystem of Estero Bay Aquatic Preserve. Specifically, we test if small pelagic fish display significant interspecific differences in trophic resource use between habitats and salinity zones, with reference to stable isotope signatures from invertebrates, benthic microalgae, mangrove leaves and organic matter. Subsequently, we develop a simple conceptual network, recreating subtropical estuarine trophic linkages amongst mangrove dwelling organisms across salinity gradients.

We focus on intermediate trophic level fish (or forage fish) as they are generally numerically dominant in most marine ecosystems and have substantial impact on fishery catch and diets of predatory fish (Hall 1999). Intermediate trophic level fish can exert both bottom-up control on predatory fish and top-down control on zooplankton (Cury et al. 2003), thus forming important links between basal producers and consumers at the top of the food chain. Since several intermediate trophic level fish from Estero Bay Aquatic Preserve are not directly exploited by fisheries, they have not been included in trophic ecology studies. We predict that the intermediate trophic level fish will have ^{13}C values reflective of the primary producers they have consumed directly, or that underpin the food chain of their prey species (Duarte et al. 2018) and this will be used to determine the main driver of their productivity. Furthermore, we should observe a linear enrichment in ^{15}N as trophic positioning and size of organism increases (Deniro and Epstein 1981).

2.3 Methods

2.3.1 Study location

Four locations: Vester Field station (26 19 52 N, 81 50 14 W), Big Hickory (26 21 40 N, 81 51 28 W), Lover's Key (26 23 50 N, 81 52 00 W) and Hendry Creek (26 26 50 N, 81 51 33 W), were chosen as they represent distinct mangrove and seagrass habitats as well as experiencing varying levels of freshwater input (Figures 2.2 and 2.4). All four sites are characterised by mangrove forest; primarily red mangrove (*Rhizophora mangle*) with its prominent prop roots and to a lesser extent, black mangrove (*Avicennia germinans*) that grows close to shorelines, whilst white mangrove (*Laguncularia racemose*) and buttonwood (*Conocarpus erectus*) inhabit lagoon areas (Thomas and Rumbold 2006). Vester Field station and Big Hickory sites constituted mainly of mangrove fringe with scattered clusters of American oysters (*Crassostrea virginica*). Lover's Key and Hendry Creek sites were abundant in seagrass (Figure 2.3). All sampling locations were visited twice, once in November 2018 and once in May 2019. Estero Bay experiences seasonal rains from May to September reducing salinities in parts of the estuary and hypersaline conditions for the remainder of the year (Tolley et al. 2005) (Figure 2.2). Seasonality has an influence on the decapod and fish composition and abundance of oyster reefs. High salinity promotes susceptibility of parasite *Perkinsus marinus* while continued exposure to freshwater inhibits oyster growth, consequently having an impact on communities that reside and feed on oyster reefs (Tolley et al. 2005).

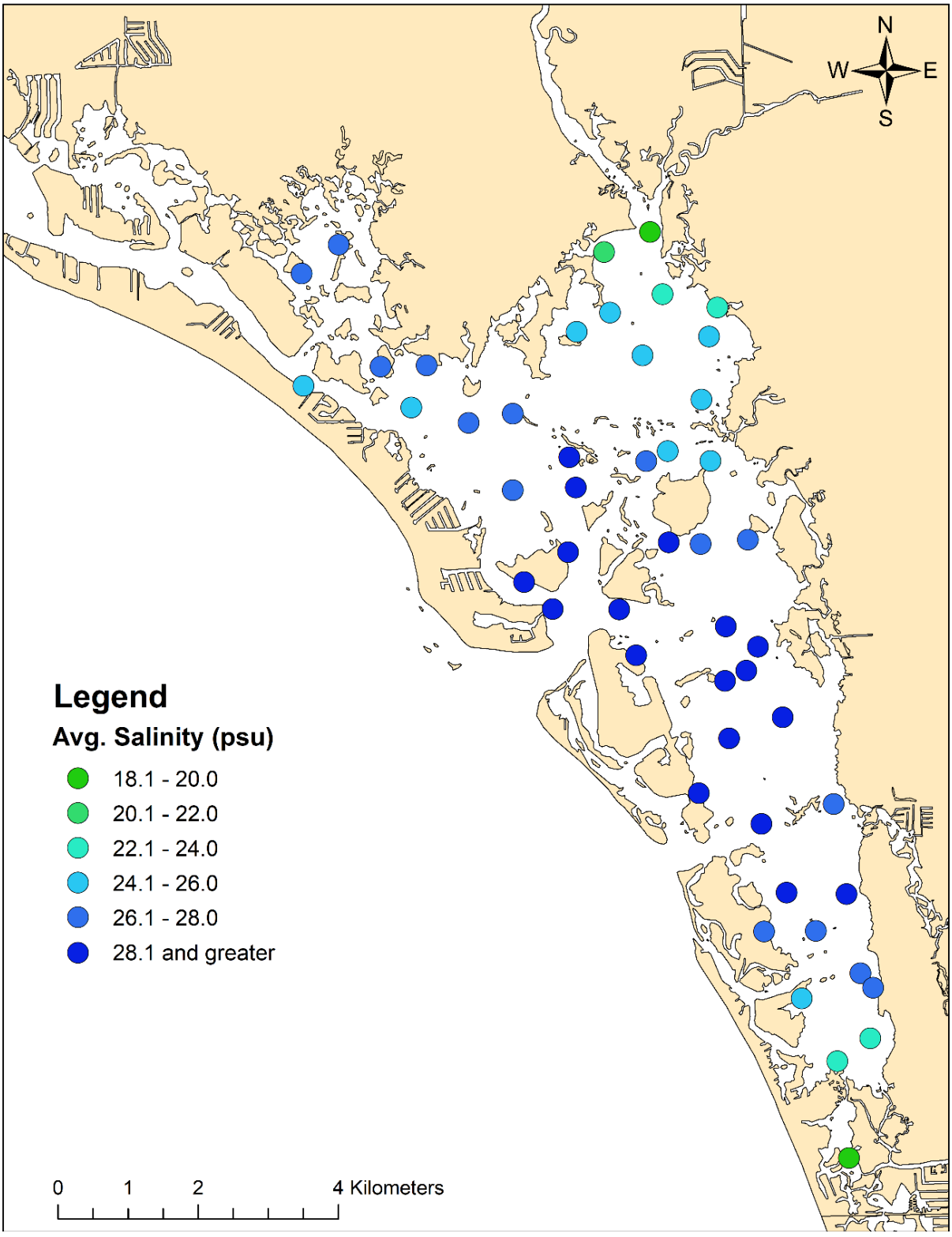


Figure 2.2. Map of Estero Bay with salinity values from May 2017 to April 2018. Sampling locations with acronyms denoting the names of each site. V - Vester, BH - Big Hickory, LK – Lover's Key and HC – Hendry Creek. (Map credit: Lisa Rickards)

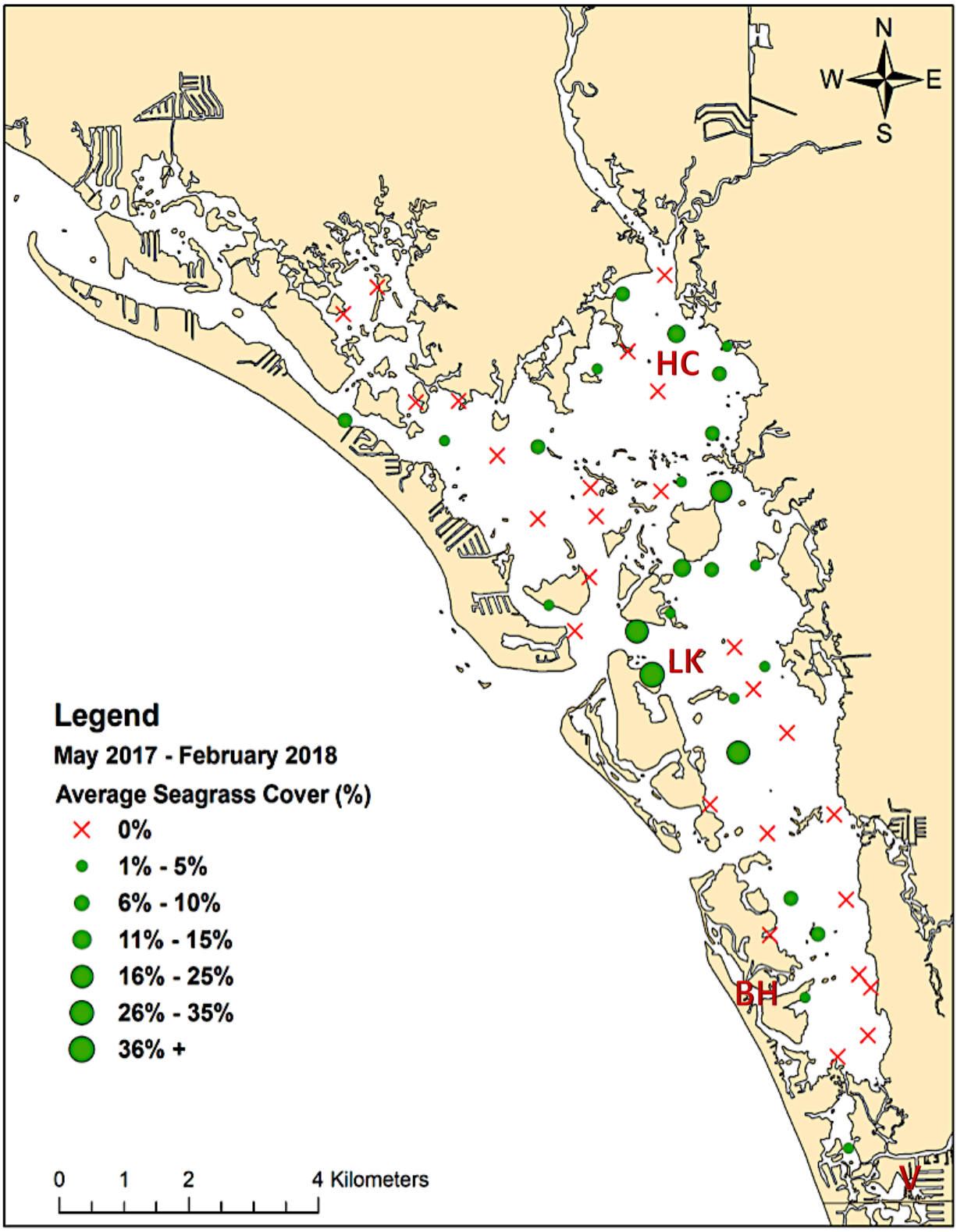


Figure 2.3. Map of Estero Bay with percentage seagrass cover from May 2017 to April 2018. Sampling locations with acronyms denoting the names of each site. V - Vester, BH - Big Hickory, LK – Lover's Key and HC – Hendry Creek. (Map credit: Lisa Rickards)

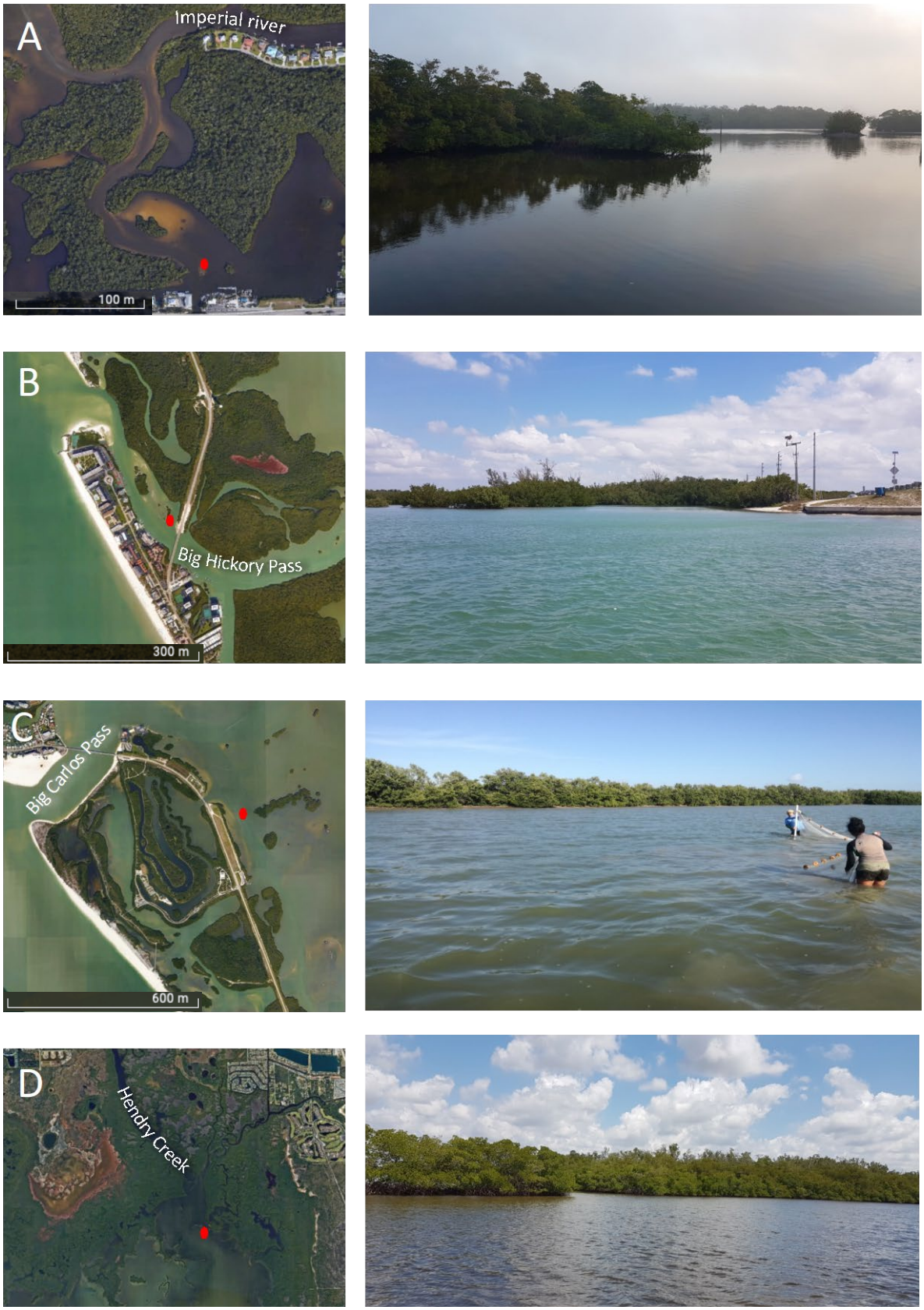


Figure 2.4. Google Earth images of 4 locations with images taken in the field (Google Earth 2022) Red dot (●) indicates sampling site. A – Vester Field Station, B – Big Hickory, C – Lover's key and D – Hendry Creek.

2.3.2 Sampling protocol

Seine nets with an approximate area of 4.6 m^2 with a 0.6 m^2 cod-end and 4mm stretched mesh were used to capture fish less than 20 cm in total length and invertebrates such as crabs and shrimps. Otter trawls and angling methods were trialled to collect individuals larger than 20cm, but these methods yielded very low catch numbers and thus seine nets were consistently used in both seasons. An average of 20 individuals per species was captured at each location. Salinity, pH, turbidity, dissolved oxygen, depth, and water temperature was collected at each location using a YSI ProDSS probe.

The fish were collected (euthanised by emersion in ice bath, according to the American Veterinary Medical Association, 2020) and transported on ice back to the Vester field station where individuals were identified to the lowest possible taxonomic level. Total length (millimetre) and weight (grams) of individual fish were recorded followed by removal of 1cm^3 of muscle tissue from the flank, under the dorsal fin above the lateral line, midway between the ventral limits, with epidermis removed. The tissue was placed in a 1.5ml Eppendorf or 2ml screw cap tube and placed in a drying oven at 60ºC for 48 hours until the fish tissue was completely dried. Unlike the fish, invertebrates were dried whole in tubes at 60ºC for 48 hours.

2.3.3 Sample processing

A subset of three individuals per fish species within their median length range were chosen for carbon and nitrogen elemental analysis. Median length was chosen to minimise length induced variation within each species per site. All dried tissue were ground using a porcelain mortar and pestle until a fine sand consistency was achieved. The porcelain mortar and pestles were baked at 550ºC for 4 hours (to sterilize) before they were used. The mortar and pestle were rinsed with deionised water, wiped with Kimwipe tissues, then rinsed in 10% hydrochloric acid (HCl) followed by deionized water between each sample (Abeels et al. 2012). A new set of sterilized mortar and pestles were utilised for each sampling location. Between 1 and 1.5mg of the ground tissue was weighed in acetone cleaned tin capsules (8 x 5 mm) before they were enclosed and placed in a sealed 96-well plate.

A subset of ground invertebrate tissue underwent lipid extraction before encapsulation. The samples were treated in a 2:1 ratio of chloroform to methanol solution and placed in a water bath at 30ºC for 24 hours (Lees and Stanley 1956). After 24 hours, the samples were centrifuged

to remove supernatant and another 1.9ml of the chloroform-methanol solution was added. The samples were centrifuged again, and supernatant was discarded. Samples were left to dry in the fume hood for 24 hours before they were ground again using sterile porcelain mortar and pestles. Once the tissue was ground, they were weighed (between 1 – 1.5mg) in acetone cleaned tin capsules, encapsulated, and placed in a sealed 96-well plate.

The encapsulated samples were sent to the University of California Davies (UC Davies) Stable Isotope Facility for dual ^{13}C and ^{15}N analysis. Tissues were analysed using PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass Spectrometer (Sercon Ltd, Cheshire, UK) (UC Davies Stable Isotope Facility, 2022).

Additional ^{13}C and ^{15}N values of sinking particulate organic matter (POM), suspended POM, benthic microalgae, sediment, worms, amphipods and *Crassostrea virginica* (American oyster) from sampling seasons May and November 2008 were extracted from Abeels et al. 2012. Stable isotope composition (C and N) of mangrove leaves collected from Estero Bay close to the Vester field station sampling location were obtained from Dr Nathalie Fenner (Bangor University). As with the vertebrate and invertebrate tissues, the plant tissues were dried at 60ºC for 48h, pulverised, encapsuled in tin capsules, sealed in a 96-well plate and sent to UC Davies for analysis.

2.3.4 Data analysis

Post's (2002) correction equation was applied to the resultant carbon values of fish tissues to account for lipid composition. However, the application of the correction equation did not result in any changes to the overall values. Post et al. (2007) and Papiol et al. (2017) suggest that lipid correction equation to be only applied when C:N bulk ratio is more than 3.5. In this study the C:N bulk ratio did not exceed 3.4 and hence a change in the values after application of correction equation was not observed. Therefore, only the uncorrected values have been presented here and used in downstream analysis.

All data analysis was performed using Rstudio (v 4.1.3) (R Core Team 2022). The 'Vegan' package was used to conduct one-way ANOVA followed by Tukey's test (v 2.6-2) (Oksanen et al. 2022). One-way ANOVA was performed to test for significant differences in the mean delta C and N within and between species groups and sites. Prior to ANOVA, Shapiro-Wilk test was

applied to test for normality. Akaike Information Criterion (AIC) was applied using 'AICcmodavg' package (v 2.3-1) (Mazerolle 2020) to determine the main variable underpinning the differences observed between species and sampling sites. An AIC test simultaneously compares multiple competing models and estimates which best approximates the true biological pattern that is observed (Symonds and Moussalli 2011). AIC is calculated using the number of fitted parameters, and either maximum likelihood or the residual sum of squares of the model. Five single variables (species, length, season, site, and salinity) and four combinations of variables (site-salinity-season, site-salinity, species-length and salinity-season) were determined as independent factors. The combination of factors was based on a priori knowledge on fundamental variables that affect trophic interactions and resource availability (Deniro and Epstein 1981; Duarte et al. 2018). A modified version of AIC was applied here which accounts for small sample sizes (AICc) (Symonds and Moussalli 2011). Linear regression models were applied to investigate the presence of relationship between $\delta^{15}N$ and length using 'stats' package in R (v 4.1.3) (R core Team, 2022). To ensure regression models have adequate power, a power analysis was performed using the 'WebPower' (v 0.8.6) (Zhang et al. 2022) package in R. Effect size was calculated using R^2 values (from 'lm' function used for calculating linear regression) in equation $f^2 = R^2/(1 - R^2)$, default power score of 0.8 was applied that is often used as a baseline to ensure good statistical power (Cohen 1992) and number of predictors was set to 1 (length is the only variable we are testing). All figures were plotted using 'ggplot2' (v 3.3.6) (Wickham 2016) and 'ggpubr' (v 0.4.0) packages (Kassambara 2020).

2.4 Results

A subset of 93 fish samples comprising 12 different species representative of each site and season was used in analysing carbon and nitrogen stable isotope ratios. The number of species at each site per sampling season is shown in Table 2.1 (refer to Appendix A for total number of individuals caught and species composition). *Eucinostomus argenteus* (Spotfin mojarra) was found at all sites in both seasons and *Lagondon rhomboides* (Pinfish) was found at all locations in May'19.

Table 2.1. Number of vertebrate species included in stable isotope analysis for each season and the location of where it was captured.

Common name	Species	Vester		Big Hickory		Lover's Key		Hendry Creek	
		Nov 18	May 19	Nov 18	May 19	Nov 18	May 19	Nov 18	May 19
Goby	*Gobiidae spp.*	-	-	-	-	-	3	-	-
Glass minnow	*Anchoa mitchilli*	-	-	-	3	-	3	-	3
Gulf killifish	*Fundulus grandis*	-	-	3	-	-	-	3	-
Hogchoker	*Trinectus maculatus*	-	-	-	-	-	-	3	-
Inshore lizard fish	*Synodus foetens*	-	-	-	-	3	3	3	-
Pipefish	*Syngnathus scovelli*	-	-	-	-	-	3	-	-
Pinfish	*Lagodon rhomboides*	-	3	-	3	-	3	-	3
Rough silverside	*Membras martinica*	3	3	-	-	3	-	-	-
Sheepshead minnow	*Cyprinodon variegatus*	-	-	3	-	-	-	-	-
Silver jenny	*Eucinostomus gula*	3	-	-	-	-	-	-	-
Snapper	*Lutjanus griseus*	3	-	3	-	3	-	-	-
Spotfin mojarra	*Eucinostomus argenteus*	3	3	3	3	3	3	3	3
Total (93)		12	9	12	9	12	18	12	9

2.4.1 $\delta^{13}C$ stable isotope ratio

Fish from Big Hickory and Lover's Key sites were more enriched in $\delta^{13}C$ with average carbon signatures ranging from -14.2 to -19.4 (Table 2.2). In contrast, individuals from Hendry Creek and Vester sites were more depleted, with $\delta^{13}C$ values ranging from -19.4 to -24.5 (Table 2.2). Substantial overlap was observed in $\delta^{13}C$ values between individuals from coastal sites of Big Hickory and Lover's Key (Figures 2.5, 2.6 and 2.7). Overlap in $\delta^{13}C$ values was also present in individuals from lagoon locations of Vester Field station and Hendry Creek (Figures 2.5. 2.6 and 2.7). Shapiro-Wilk test reflected that the data was normally distributed for ^{13}C ($p = 0.0825$), however an outlier for $\delta^{15}N$ data affected normality thus it was removed and all downstream analysis was performed with the corrected data. A one-way ANOVA followed by post hoc Tukey test supports overlaps observed and shows that the pair of sites; Big Hickory and Lover's Key have $\delta^{13}C$ values that were significantly higher ($p<0.001$) compared to individuals from Hendry Creek and Vester (Table 2.3).

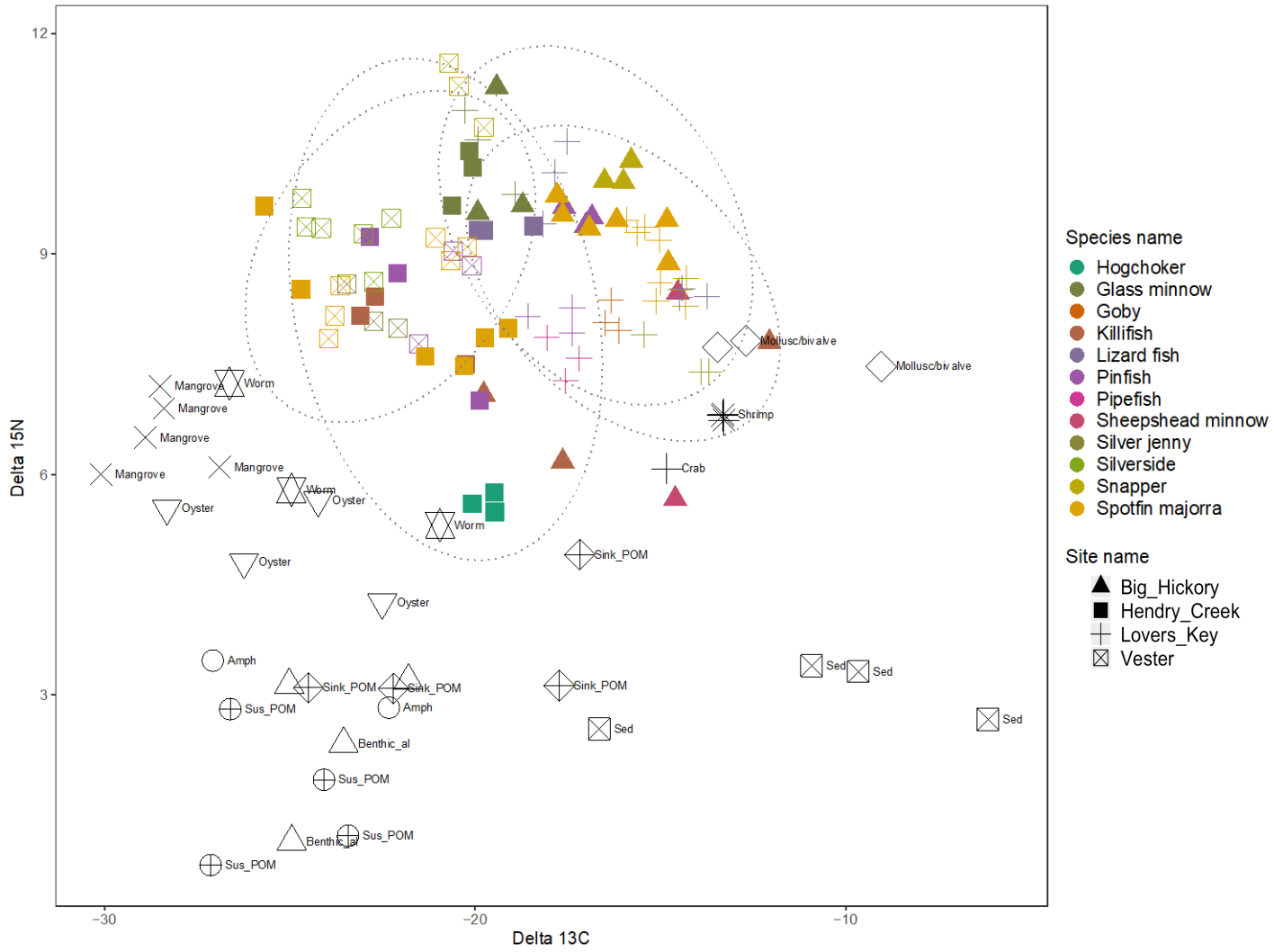


Figure 2.5. Stable isotope values of $\delta^{13}C$ and $\delta^{15}N$ from vertebrates and invertebrates collected within the Estero Bay Aquatic Preserve. Reference data points in black included are labelled with acronyms; Amph: amphipods, Benthic_al: benthic microalgae, Crab: juvenile crab, Mangrove: mangrove leaves, Mollusc/bivalve: bivalve attached to mangrove root, Oyster: American oyster, Shrimp: juvenile pink shrimp, Sed: sediment, Sink_POM: sinking particulate organic matter, Sus_POM: suspended particulate organic matter, Worm: worm. Ellipses are drawn based on default parameters (function: stat_ellipse) in RStudio and is drawn to visualise grouping driven by site variable.

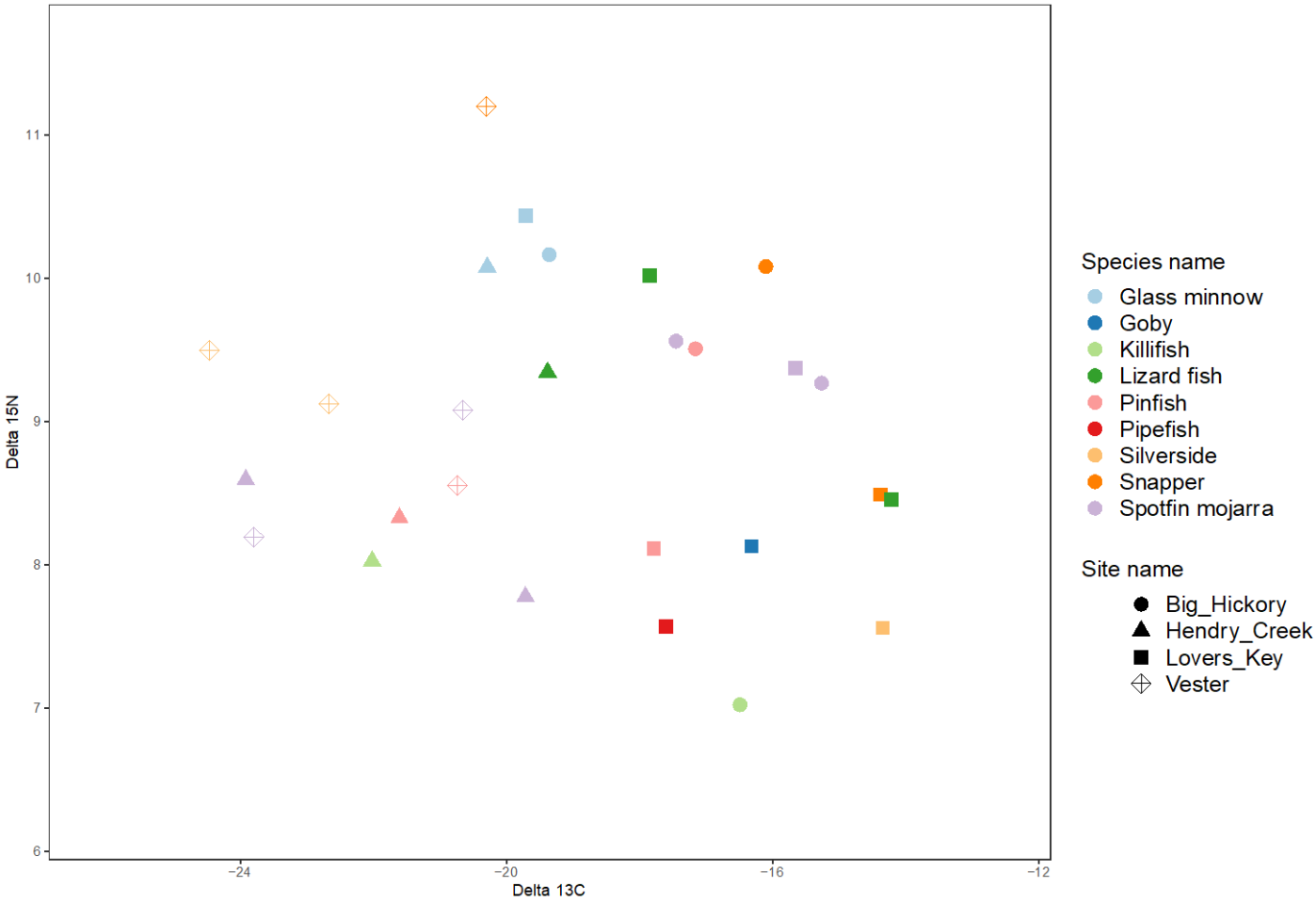


Figure 2.6. Carbon and nitrogen biplot representing stable isotope average values of $\delta^{13}C$ and $\delta^{15}N$ from vertebrates and invertebrates collected within the Estero Bay Aquatic Preserve. Error bars represent standard deviation. Polygons included represent grouping of data points by sample sites.

Table 2.2. Mean $\delta^{13}C$ and $\delta^{15}N$ stable isotope values of fish samples from each sampling location from Estero Bay Aquatic Preserve. Mean values are provided with standard error and dates included reflect sampling season.

Common name	Species	$\delta^{13}C$								$\delta^{15}N$							
		Vester		Big Hickory		Lover's Key		Hendry Creek		Vester		Big Hickory		Lover's Key		Hendry Creek	
		Nov 18	May 19	Nov 18	May 19	Nov 18	May 19	Nov 18	May 19	Nov 18	May 19	Nov 18	May 19	Nov 18	May 19	Nov 18	May 19
Goby	*Gobiidae spp.*	-	-	-	-	-	-16.3 ± 0.107	-	-	-	-	-	-	-	8.13 ± 0.123	-	-
Glass minnow	*Anchoa mitchilli*	-	-	-	-19.4 ± 0.351	-	-19.7 ± 0.408	-	-20.3 ± 0.174	-	-	-	10.2 ± 0.554	-	10.4 ± 0.334	-	10.1 ± 0.219
Gulf killifish	*Fundulus grandis*	-	-	-16.5 ± 2.30	-	-	-	-22.0 ± 0.891	-	-	-	7.02 ± 0.469	-	-	-	8.03 ± 0.274	-
Hogchoker	*Trinectus maculatus*	-	-	-	-	-	-	-19.68 ± 0.200	-	-	-	-	-	-	-	5.62 ± 0.0784	-
Inshore lizard fish	*Synodus foetens*	-	-	-	-	-14.2 ± 0.234	-17.8 ± 0.188	-19.4 ± 0.472	-	-	-	-	-	8.45 ± 0.0384	10.0 ± 0.327	9.34 ± 0.0186	-
Pipefish	*Syngnathus scovelli*	-	-	-	-	-	-17.6 ± 0.252	-	-	-	-	-	-	-	7.57 ± 0.170	-	-
Pinfish	*Lagodon rhomboides*	-	-20.74 ± 0.422	-	-17.2 ±0.227	-	-17.8 ± 0.398	-	-21.6 ± 0.891	-	8.55 ± 0.391	-	9.51 ± 0.0751	-	8.11 ± 0.102	-	8.32 ± 0.673
Rough silverside	*Membras martinica*	-24.5 ± 0.160	-22.7 ± 0.219	-	-	-14.3 ± 0.552	-	-	-	9.49 ± 0.133	9.12 ± 0.259	-	-	7.56 ± 0.17	-	-	-
Sheepshead minnow	*Cyprinodon variegatus*	-	-	-14.6 ± 0.762	-	-	-	-	-	-	-	7.07 ± 1.68	-	-	-	-	-
Silver jenny	*Eucinostomus gula*	-22.8 ± 0.396	-	-	-	-	-	-	-	8.22 ± 0.187	-	-	-	-	-	-	-
Snapper	*Lutjanus griseus*	-20.3 ± 0.283	-	-16.1 ± 0.214	-	-14.4 ± 0.0536	-	-	-	11.2 ± 0.255	-	10.1 ± 0.095	-	8.49 ± 0.110	-	-	-
Spotfin mojarra	*Eucinostomus argenteus*	-23.8 ± 0.0895	-20.7 ± 0.246	-15.3 ± 0.458	-17.5 ± 0.269	-15.7 ± 0.145	-15.1 ± 0.0338	-19.7 ± 0.345	-23.9 ± 1.32	8.19 ± 0.209	9.08 ± 0.0902	9.27 ± 0.193	9.56 ± 0.131	9.37 ± 0.0493	8.72 ± 0.246	7.78 ± 0.153	8.59 ± 0.590

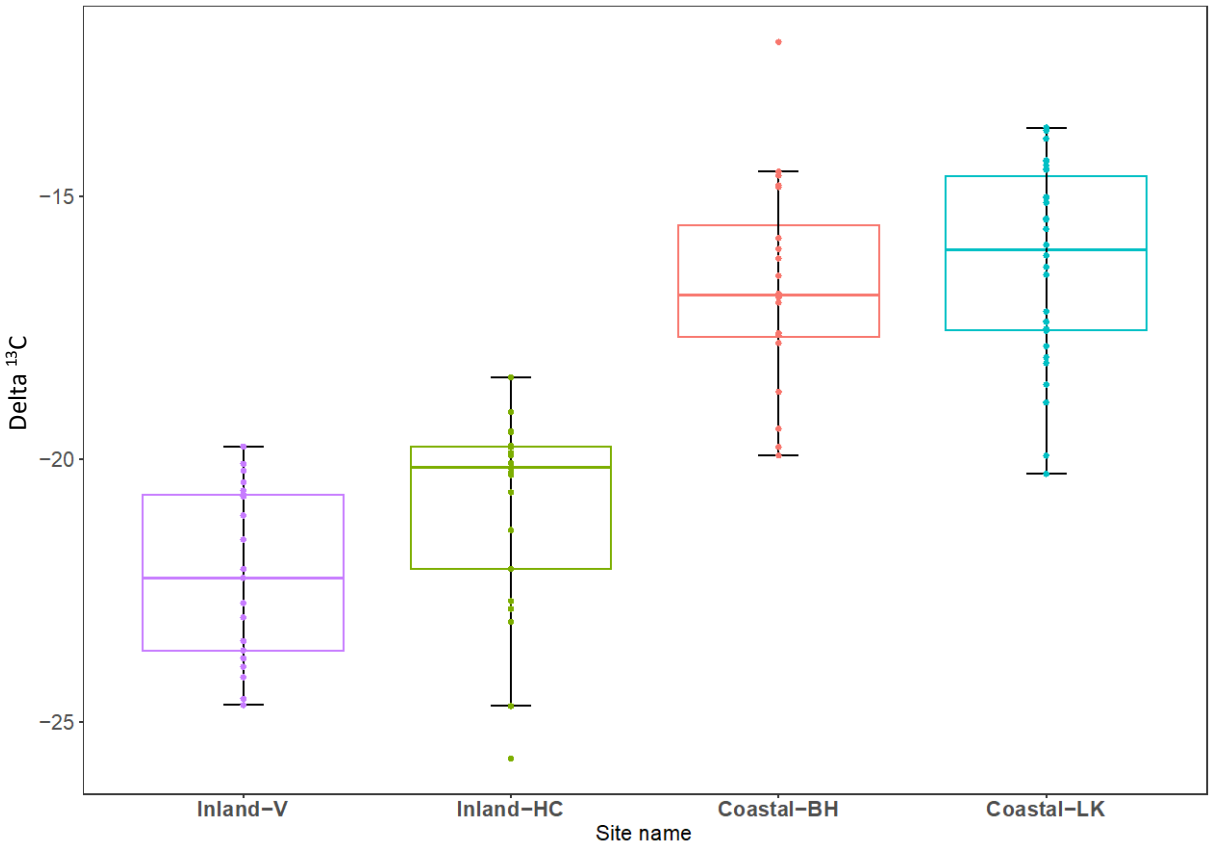


Figure 2.7. Boxplot of $\delta^{13}C$ stable isotope values of fish from four locations in Estero Bay Aquatic Preserve. V - Vester, HC – Hendry Creek, BH - Big Hickory and LK – Lover's Key.

Table 2.3. Results from one-way ANOVA with post hoc Tukey test on $\delta^{13}C$ values from each site sampled in Estero Bay Aquatic Preserve. (*) signifies where $p<0.05$. V - Vester, HC – Hendry Creek, BH - Big Hickory and LK – Lover's Key.

Site name comparison	Standard error	Adjusted p value
Lagoon (HC) – Coastal (BH)	0.5875	<0.001 *
Coastal (LK) – Coastal (BH)	0.5348	0.758
Lagoon (V) – Coastal (BH)	0.5875	<0.001 *
Coastal (LK) – Lagoon (HC)	0.5791	<0.001 *
Lagoon (V) – Lagoon (HC)	0.6281	0.195
Lagoon (V) – Coastal (LK)	0.5791	<0.001 *

The carbon isotope overlap between sites was also observed when a within-species comparison was performed on two species that were consistently abundant across all four sampling locations. *Eucinostomus argentus* (spotfin mojarra, represented in orange in Figure 2.5) from Big Hickory and Lover's Key (average $\delta^{13}C$ of -15.9 ± SE 0.308) had significantly ($p<0.05$) higher $\delta^{13}C$ values compared to spotfin mojarra individuals from Vester and Hendry Creek (average $\delta^{13}C$ of -22.0 ± SE 0.635) (Table 2.4). Similarly, *Lagondon rhomboides* (pinfish, represented in dark purple in Figure 2.5) from Big Hickory and Lover's Key had significantly ($p<0.05$) higher $\delta^{13}C$ values with an average of -17.5 (SE ± 0.248) compared to individuals from Vester and Hendry Creek with an average of -21.2 (SE ± 0.481).

Table 2.4. Results from one way ANOVA of *Eucinostomus argenteus* (Spotfin mojarra) and *Lagondon rhomboides* (Pinfish) of $\delta^{13}C$ values from each sampling site. (*) signifies where $p<0.05$.

Site name	**Standard error**	**Adjusted p-value**	**Standard error**	**Adjusted p-value**
	Eucinostomos argenteus (Spotfin mojarra)		*Lagondon rhomboides* (Pinfish)	
Hendry Creek – Big Hickory	1.0193	0.00166 *	0.7686	0.00172 *
Lover's Key – Big Hickory	1.0193	0.759870	0.7686	0.84571
Vester – Big Hickory	1.0193	<0.0001 *	0.7686	0.00704 *
Lover's Key – Hendry Creek	1.0193	<0.0001 *	0.7686	0.00477 *
Vester – Hendry Creek	1.0193	0.977639	0.7686	0.68417
Vester – Lovers Key	1.0193	<0.0001 *	0.7686	0.02029 *

Results from the Akaike Information Criterion (AIC) analysis performed indicated that the combination of site, salinity and season gave the highest AICc weight of 99% (Table 2.5); a model with high percentage or the lowest AICc score best fits the variation in the data observed.

Table 2.5. Akaike Information Criterion (AICc) values of the different models used to explain $\delta^{13}C$ variation observed in fish species from Estero Bay Aquatic Preserve. Analysis was performed with the test corrected to small sample size.

Parameter	K	AICc	AICcWt	CumWt
Site-Salinity-Season	5	438.5188	9.9994×10^{-1}	0.999943
Site-Salinity	4	458.1355	5.4986×10^{-5}	0.999998
Site	3	464.7310	2.0326×10^{-6}	1
Species-Length	14	476.3111	6.2156×10^{-9}	1
Length	3	478.0997	2.5415×10^{-9}	1
Salinity-Season	4	497.2376	1.7756×10^{-13}	1
Salinity	3	510.6106	2.2152×10^{-16}	1
Species	16	516.0109	1.4886×10^{-17}	1
Season	3	528.2577	3.2615×10^{-20}	1

K – number of parameters in the model (default is 2)
AICc – information score of the model (the lower, the better fit the model is)
AICcWt – AICc weight which explains the total amount of predictive power within that given model
CumWt – sum of AICc weights

2.4.2 δ15N stable isotope ratio

Predatory species such as *Lutjanus griseus* (grey snapper) and *Synodus foetens* (inshore lizard fish) were more enriched in the heavier nitrogen isotope with high $\delta^{15}N$ averages of 11.2 and 10.0 respectively (Table 2.2 and Figure 2.8). Conversely, prey species such as *Trinectus maculatus* (hogchoker) and *Cyprinodon variegatus* (sheepshead minnow) had low $\delta^{15}N$ averages of 5.62 and 7.07 respectively. However, *Anchoa mitchilli* (glass minnow), a species known to occupy lower trophic levels had high $\delta^{15}N$ levels (10.4) comparable to predatory species *Lutjanus griseus* and *Synodus foetens* (Table 2.2 and Figure 2.8). Shapiro-Wilk test reflected that the data was normally distributed after the removal of a single outlier ($p = 0.0636$), downstream analysis was conducted with the corrected data. A one-way ANOVA followed by a post hoc Tukey test was performed to identify species that had significantly different $\delta^{15}N$ values (Figure 2.9 and refer to Appendix B for detailed results from one-way ANOVA).

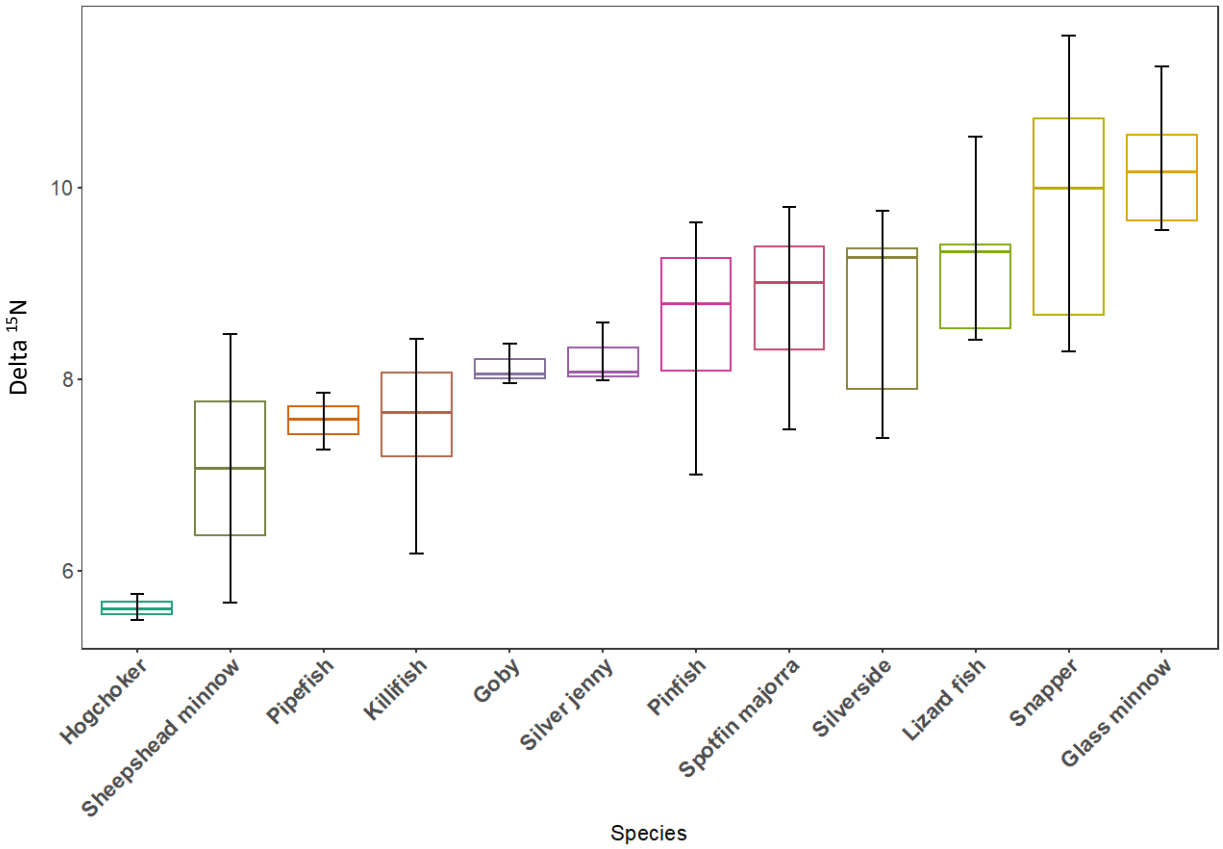


Figure 2.8. Boxplot of $\delta^{15}N$ stable isotope values of each fish species (x-axis) from all four sampling locations in Estero Bay Aquatic Preserve.

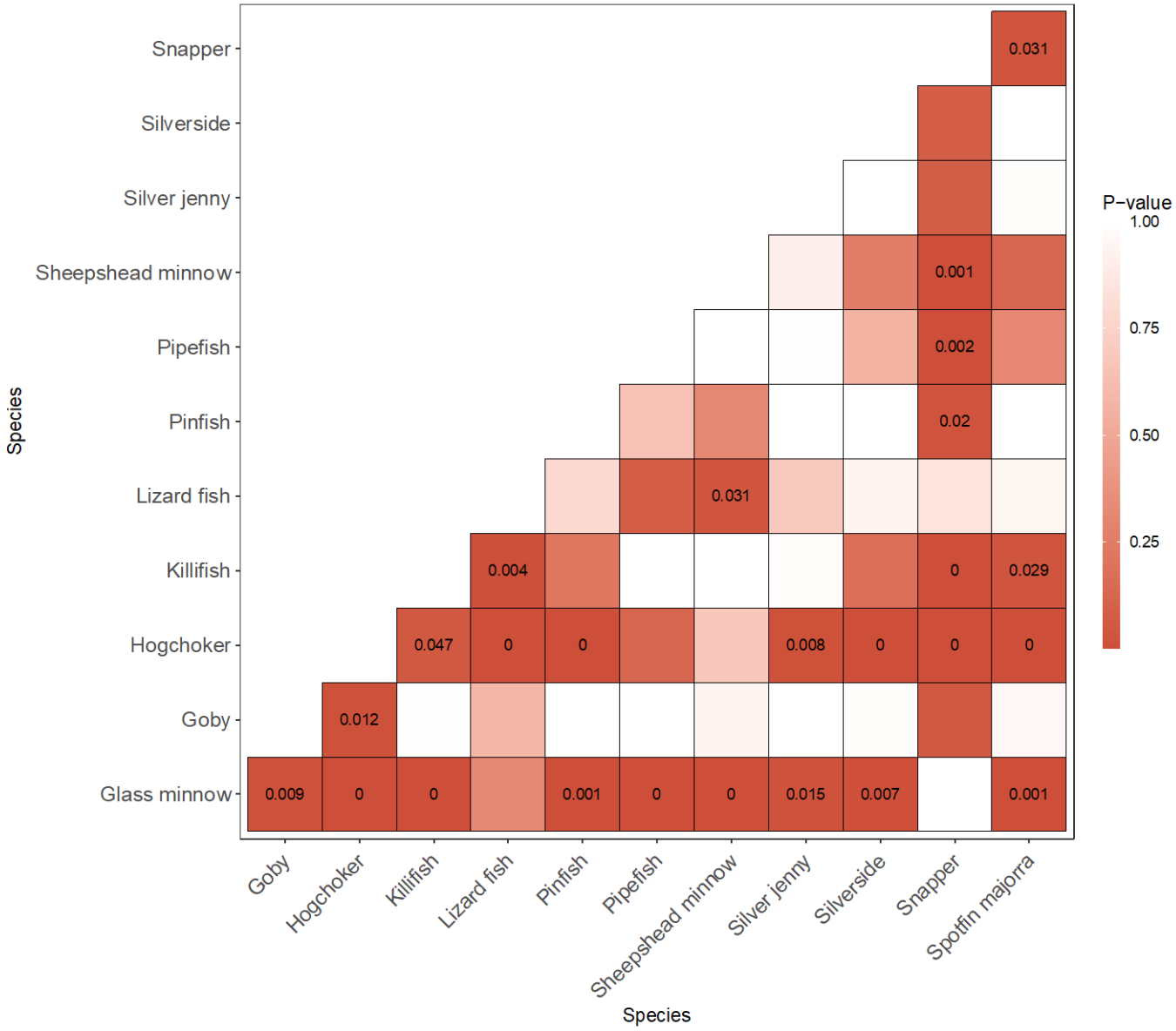


Figure 2.9. Results of one-way ANOVA with post-hoc Tukey test comparing the means of $\delta^{15}N$. P-values are only represented for significantly (<0.05) different groups.

Akaike Information Criterion was performed to identify the main variable/s that best fit the differences in $\delta^{15}N$ observed between the species. Species-length combination had the highest AICc weight of 99% (Table 2.6). Hence, the combination of species and length variables are the main factors influencing differences observed in $\delta^{15}N$ values between the species.

Table 2.6. Akaike Information Criterion (AICc) values of the different models used to explain $\delta^{15}N$ variation observed between fish species from Estero Bay Aquatic Preserve. Analysis was performed with the test corrected to small sample size.

Parameter	K	AICc	AICcWt	CumWt
Species – length	14	228.0176	9.9990×10^{-1}	0.9999
Species	16	256.0316	8.2570×10^{-7}	1
Length	3	287.2620	1.36533×10^{-13}	1
Salinity-Season	4	322.3788	3.23367×10^{-21}	1
Season	3	322.8331	2.57683×10^{-21}	1
Site-Salinity-Season	5	324.3265	1.22119×10^{-21}	1
Salinity	3	326.7595	3.61802×10^{-22}	1
Site – salinity	4	328.4762	1.53352×10^{-22}	1
Site	3	333.5220	1.23032×10^{-23}	1

K – number of parameters in the model (default is 2)
AICc – information score of the model (the lower, the better fit the model is)
AICcWt – AICc weight which explains the total amount of predictive power within that given model
CumWt – sum of AICc weights

Linear regression models were applied to understand the relationship between $\delta^{15}N$ and length further. A significant p value ($p<0.001$) was obtained when a regression curve was plotted with all values in the dataset, however, there were three large snapper individuals presenting to be outliers (Figure 2.10a). A second regression model was applied where the large snapper samples were removed (Figure 2.10b) and a significant relationship was not obtained ($p=0.0661$). Regression models were applied at species level for individual species, however as median size of each species was selected, we did not detect significant relationship between $\delta^{15}N$ and length. In addition, the power analysis showed weak statistical power (power < 0.2) for all regression models applied at species level. The predicted sample number calculated to achieve power of 0.8 is considerably larger than the sample sizes included in this study (Table 2.7).

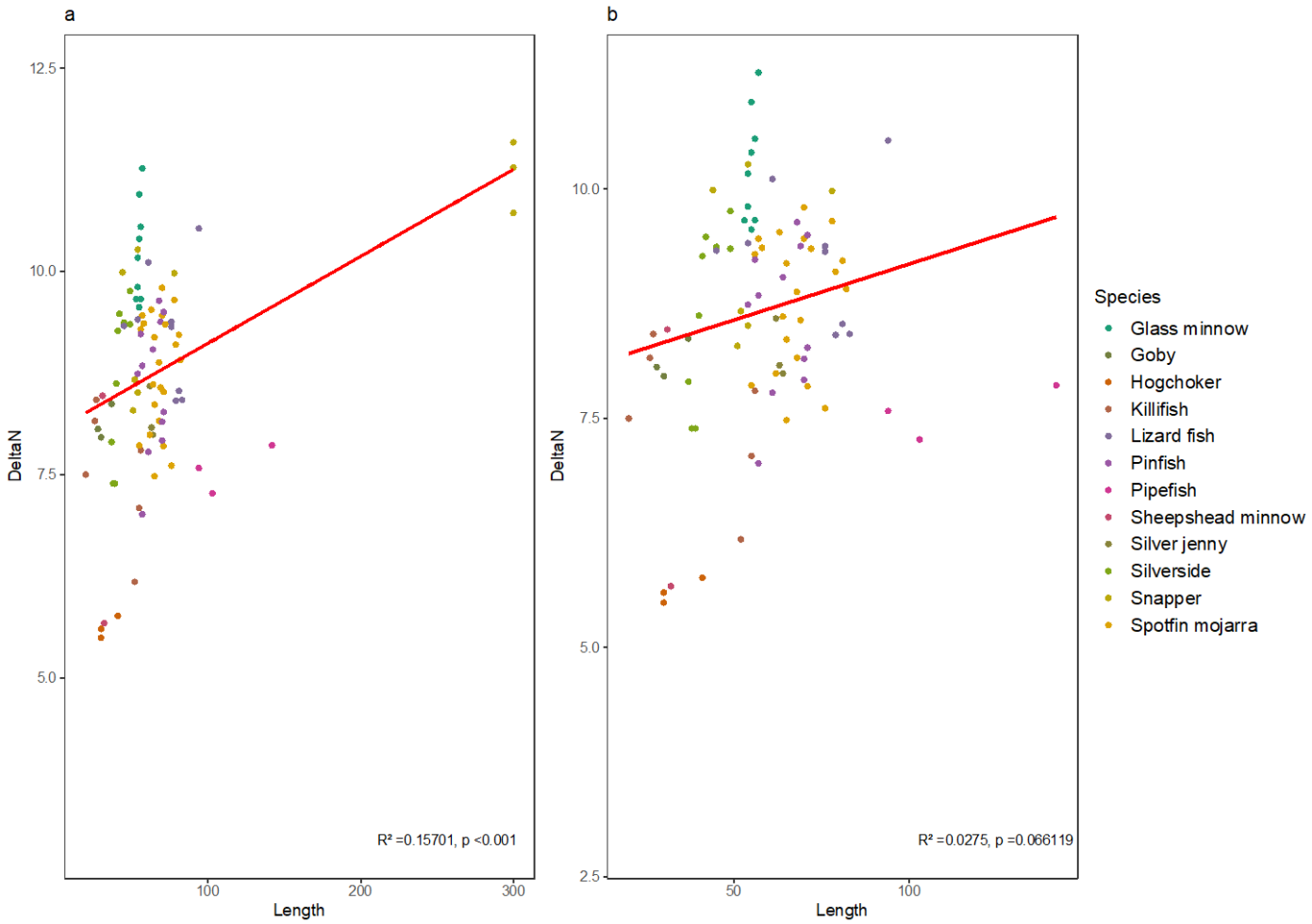


Figure 2.10. Regression lines describing relationship between length and $\delta^{15}N$ of fish tissues from Estero Bay Aquatic Preserve. a) includes three large snapper (Lutjanus griseus) individuals while b) is a subset that excludes these the snapper specimens. R^2 coefficient and p values are provided.

Table 2.7. Results from power analysis. R^2 values obtained from linear regression models and used to calculate f^2 values using formula $f^2 = R^2/(1 - R^2)$. Power score was set to 0.8 when calculating predicted sample sizes and alpha value was always set to 0.05.

	Sample size (n)	R^2	f^2 (effect size)	power	Predicted sample size
Mixed species	90	0.0459	0.0481	0.539	165
Spotfin mojarra	24	0.00877	0.00885	0.0726	889
Pinfish	12	0.0223	0.0228	0.0762	346
Rough silverside	9	0.165	0.198	0.212	42
Glass minnow	9	0.286	0.401	0.375	22
Killifish	6	0.0276	0.0284	0.0621	278
Lizard fish	9	0.00363	0.00364	0.0529	2156
Snapper	6	0.0205	0.0209	0.0589	377

2.5 Discussion

The objective of this study was to uncover the trophic dynamics and identify the ultimate carbon sources of intermediate trophic level fish from a heterogeneous estuarine habitat with varying salinity levels using stable isotope signatures of carbon and nitrogen. We found evidence that individuals occupying coastal and lagoon sites have distinct $\delta^{13}C$ values despite the similarities in composition of vegetation available at each site. Even though coastal site Lover's Key and lagoon site Hendry Creek were abundant in seagrass, the coastal sites were enriched (-16.3 SE ± 0.339) in $\delta^{13}C$ compared to the lagoon sites (-20.9 SE ± 0.413). Big Hickory and Vester both featured oyster reefs close to the mangrove fringe, yet the coastal Big Hickory site was enriched in $\delta^{13}C$ (-16.7 SE ± 0.422). Conversely, the lagoon Vester sampling location showed depleted $\delta^{13}C$ (-22.2 SE ± 0.353) signatures. The $\delta^{15}N$ values observed provided an insight into the trophic positioning of the 12 species, enabling us to develop a conceptual network displaying linkages from primary producers to predatory fish. Regression analysis applied to test the relationship between total length of fish and $\delta^{15}N$ signature revealed that significant associations were true in selected species only.

2.5.1 Distinct $\delta^{13}C$ values in coastal and lagoon locations

Due to spatial and temporal variables affecting ^{13}C, dissolved inorganic carbon affects the ^{13}C of aquatic producers (Abrantes et al. 2015). As a result of the assimilation of distinctive carbon

sources and different photosynthetic pathways utilised by various primary producers, we expected that locations abundant in mangroves would display depleted $\delta^{13}C$ values, whereas seagrass dominated habitats would be more enriched in $\delta^{13}C$ (Vaslet et al. 2012; Duarte et al. 2018). However, the predicted differences in $\delta^{13}C$ values between mangrove and seagrass habitats were not apparent. Fish from Vester Field Station (mangrove dominated) and Hendry Creek (seagrass dominated) were depleted in $\delta^{13}C$ with values ranging from -25 to -18. Conversely, fish from Big Hickory (mangrove dominated) and Lover's Key (seagrass dominated) were more enriched in $\delta^{13}C$ with values ranging from -20 to -12. Heavier $\delta^{13}C$ signatures are commonly attributed to seagrass and macroalgae whereas more depleted isotopic ratios are found in phytoplankton and mangroves (Keough et al. 1998; Abeels et al. 2012; Duarte et al. 2018).

Davias et al. (2014), showed that Atlantic silverside (*Menidia menidia*) and White perch (*Morone americana*) from different habitats such as bulkhead (vertical structure installed parallel to the water), riprap (rocky material placed along shorelines), beach and marsh reflected a positive relationship between $\delta^{13}C$ signature and salinity. Enhanced salinity levels due to increased frequency of mixing with seawater, result in high levels of exchange with marine organic matter (Hunt 1966). Marine organic matter is more enriched in $\delta^{13}C$ and is assimilated by phytoplankton (Fry 2002) that enter the marine food chain and is eventually reflected in fish tissue collected from coastline habitats. The positive relationship between salinity and $\delta^{13}C$ is also reflected in other estuarine ecosystems (Nashima et al. 2020). As Big Hickory and Lover's Key locations are close to outlets leading to the Gulf of Mexico, the frequency of mixing with seawater is higher compared to lagoon areas (as reflected in salinity values observed in Figure 2.4). The enriched $\delta^{13}C$ values are reflected in the tissues of fish caught from Big Hickory and Lover's Key as phytoplankton are key primary producers in marine environments (Jennings et al. 2008). Specifically, the spotfin mojarra and pinfish collected in Big Hickory and Lover's Key locations were significantly enriched in $\delta^{13}C$ compared to individuals from Vester and Hendry Creek that were depleted in $\delta^{13}C$. Therefore, the individual species analyses further supports the hypothesis of a higher rate of marine phytoplankton assimilation at coastal locations compared to lagoon sites that are less abundant in marine phytoplankton.

Mangrove plants typically have a lighter $\delta^{13}C$ signature (Duarte et al. 2018) and tend to exhibit $\delta^{13}C$ variation along the salinity gradient (Wei et al. 2008). Mangrove detritus is mineralised

forming inorganic carbon that is depleted in $\delta^{13}C$ and is assimilated by other photosynthetic organisms such as seagrass in the tidal waters surrounding mangrove forests (Hemminga and Mateo 1996). Typically, primary producers undergo minimal fractionation ($\delta^{13}C$ signature) of only 1% per trophic level, and their distinct carbon signature is conserved throughout the food chain (Fredriksen 2003; Fry and Sherr 1989). Based on this theory, Lee (2005) suggested that mangroves do not support estuarine food webs due to large trophic fractionation (+ 5%) observed between mangroves and consumers. Conversely, Post (2002) stated that the fractionation observed is a result of the presence of fungi in the detrital food-web. The ^{13}C of mangrove detritus is fractionated by fungi during decomposition and carboxylation (Ehleringer et al. 2000) and as a result, the $\delta^{13}C$ of mangrove detritus is lower than its consumers (Bouillon et al. 2008). Abrantes et al. (2015) stated that carbon from both high and lower abundance mangrove forests were the main source of nutrients supporting surrounding food webs. The depleted $\delta^{13}C$ signature of fish tissue (ranging from -25 to -18) from Vester and Hendry Creek locations could therefore be a result of nutrient input into the food web from mangrove detritus. This is similar to the findings from Abeels et al. (2012) who concluded that $\delta^{13}C$ signatures from Hendry Creek are derived from C_3 plant sources such as mangroves. Because mixing with coastal waters at Vester and Hendry Creek sites is infrequent, primary producers such as seagrass and algae are heavily influenced by depleted ^{13}C mangrove detritus resulting in more depleted $\delta^{13}C$ tissues than primary producers from Big Hickory and Lover's Key locations (Bouillon et al. 2008; Abrantes and Sheaves 2009).

2.5.2 Feeding dynamics of fish

Fundulus grandis (Gulf killifish) and *Cyprindon variegatus* (sheepshead minnow) are known to eat small crustaceans, with sheepshead minnow additionally supplementing its diet with detritus and algae (Rozas and Lasalle 1990; Shepta et al. 2021). The two sheepshead minnows included in this study ranged from -14.6 and -14.53 in $\delta^{13}C$ values. However, a larger sample number would be required to elucidate sheepshead minnow diet better.

Lophogobius sp. (goby sp.) are known opportunistic feeders as they consume a varied diet of algae, detritus, amphipods, copepods, molluscs, bivalves, chironomid larvae, small crabs and barnacles (Darcy 1981; Yeager and Layman 2011). However, this opportunistic feeding behaviour was not observed in this study as $\delta^{13}C$ value only ranged from -18.58 to -16.34 and $\delta^{15}N$ values between the 3 samples differed by a small margin of 0.45.

Both species from the Gerridae family; *Eucinostomus gula* (silver jenny) and *Eucinostomus argenteus* (spotfin mojarra) are omnivores with similar feeding adaptation of strong protrusible jaws (Gilmore and Greenfield 2002). Morphological analysis of stomach contents conducted by (Vasconcellos et al. 2018), showed that several silver jenny individuals had consumed large amounts of algae. This divergence in feeding pattern of silver jenny has been attributed to trophic niche partitioning so that both *Eucinostomus* species are able to co-exist. Here, *Eucinostomus argenteus* from coastal habitats had elevated $\delta^{13}C$ values ranging from -17.79 to -14.79, while individuals from inshore habitats ranged from -25.7 to -19.74. The wide range of $\delta^{13}C$ signature observed within each habitat can be attributed to opportunistic feeding behaviours of *Eucinostomus argenteus* species. To understand *Eucinostomus gula* feeding behaviours and investigate the level of niche partitioning, additional samples are required.

Predatory fish such as the *Lutjanus griseus* (gray or mangrove snapper) and *Synodus foetens* (inshore lizard fish) have displayed dietary preference for crabs, shrimp and fish (Croker 1962; Yeager and Layman 2011; Murdy and Musick 2013). The variability observed within snapper individuals based on $\delta^{15}N$ values reflect that some individuals sampled were adults (mean $\delta^{15}N$ = 11.2) whereas the remaining were juveniles ($\delta^{15}N$ = 9.29) (length of individuals ranged from 44mm to 300mm). Yeager and Layman (2011) found that juvenile snappers displayed a preference for polychaetes, which may be an explanation for the lower $\delta^{15}N$ values observed in this study.

Unexpectedly, *Anchoa mitchilli* (glass minnow also known as bay anchovy) had some of the highest $\delta^{15}N$ values in this study. Glass minnows, a fast-swimming pelagic species, primarily feed on amphipods, isopods, mysids, insect larvae, shrimp and fishes (Sheridan 1978) and are not considered to occupy trophic levels dominated by predatory fish. However, the elevated $\delta^{15}N$ values for glass minnows were concordant with a previous study reporting $\delta^{15}N$ values of 13.1 ± 0.5 (Olsen et al. 2014). It is noteworthy that glass minnows have selective preferences for large zooplankton such as *Uca megalopae*, shrimp zoeae and amphipods especially during high tide (Johnson 1990). Embryos make up the zooplankton (ichthyoplankton) community and tend to have elevated $\delta^{15}N$ signatures because they inherit nitrogen from their parents through endogenous feeding from the yolk sac (Vander Zanden et al. 1998). The elevated $\delta^{15}N$ signatures observed in zooplankton have an enrichment effect on the stable isotope signatures of predators; in this case, reflected in the glass minnows (Olsen et al. 2014; Giménez et al. 2018). When prey with high $\delta^{15}N$ is consumed, enrichment effects are the strongest in smaller

fish where the freshly acquired nitrogen would be less diluted by pre-existing nitrogen tissue which is considerably lower compared to prey (Fry et al. 1999). To fully understand these dynamics further, ^{15}N analysis of the prey tissue (ichthyoplankton) is required.

2.5.3 Length as a predictor of $\delta^{15}N$

The literature indicates that tissues become more enriched in ^{15}N as individuals grow and have the ability to capture and ingest larger prey, increasing trophic position with age and size (France et al. 1998; Currin et al. 2003; Pereira et al. 2010; Romanuk et al. 2011). This positive relationship has been determined to be true for a variety of fish species including *Salmo salar* (Atlantic salmon) (Wankowski, 1979), *Esox lucius* (northern pike) (Beaudoin et al. 1999), *Perca fluviatilis* (Eurasian perch) (Persson and Hansson 1999), *Microstomus pacificus* (Pacific Dover sole) (Rau et al. 1981.; Spies et al. 1989), *Micropterus salmoides* (largemouth bass) (Gu et al. 1996), *Lepisostues platyrhincus* (Florida gar) and *Fundulus heteroclitus* (mummichog) (Currin et al. 2003; Davias et al. 2014). However, the positive relationship between ^{15}N enrichment and size does not apply to all species. No relationship between $\delta^{15}N$ and length was found for *Salvelinus namaycush* (lake trout) (vander Zanden et al., 2000) and a negative relationship was observed in *Menidia menidia* (Atlantic silverside) (Davias et al. 2014).

In this study, median lengths of fish were chosen for stable isotope analysis as explained in the methods section (2.3.3), thus preventing length to be a predictor for $\delta\ ^{15}N$. Random sampling and increased sample sizes of each species, as suggested by the power analysis, could have achieved higher statistical power and a greater probability of observing a linear relationship (Cohen 1992; Wilson Van Voorhis and Morgan 2007). However, it is key to address that the sample size predicted by the power analysis (Table 2.7) for some species such as spotfin mojarra (n=889) and lizard fish (n=2156) are incredibly large, and it would not have been feasible to analyse such large datasets due to time, ethical and financial limitations.

2.5.4 Effects of nitrogen input into Estero Bay from the Caloosahatchee estuary

Excess freshwater inflow into the Estero Bay Aquatic Preserve is common during the summer months (July to October) and this freshwater input is largely derived from tributaries such as Estero River and Tenmile Canal that subsequently flows into Mullock Creek (Thomas and Rumbold 2006) (Figure 2.1). The Tenmile Canal, that covers an area of 13 square miles, is located within the Caloosahatchee River Estuary that is connected to Lake Okeechobee, the

largest single body of freshwater in Florida, surrounded by land that is primarily used for agriculture and housing (Mitra et al. 2020). Due to intensive farming practices, the main source of nitrate run-off from Lake Okeechobee is derived from the application of ammonium fertilizers (Ma et al. 2020). Mitra et al. (2010) found relatively high levels of contaminants such as nitrogen derived organic pesticides (triazine and carbamates) and hydrocarbons in *Crassostrea virginica* (American oyster) from Estero Bay. The results indicated that a considerable amount of nutrients/pollutants from the Lake Okeechobee catchment enter, and are accumulated, in Estero Bay biota. Furthermore, due to the nitrogen enrichment of the Caloosahatchee (resulting from run-off from Lake Okeechobee) toxic red tide (*Karenia brevis*) blooms have been a frequent seasonal occurrence (Medina et al. 2022). Assimilation of nitrogen compounds from ammonium inputs affect the $\delta^{15}N$ signature of soil and surface water (Ma et al. 2020). To understand if nitrogen from fertiliser run-off affects $\delta\ ^{15}N$ values and trophic positioning of ichthyofauna in Estero Bay, an in-depth analysis comparing organisms from across the trophic webs of Estero Bay, the Caloosahatchee Estuary and Lake Okeechobee is required.

2.6 Conclusion

Overall, the results revealed that fish species from coastal locations of Big Hickory and Lover's Key assimilate marine organic matter, most likely underpinned by marine phytoplankton and integration into marine food webs, due to the high frequency of mixing with seawater. Conversely, at Vester Field Station and Hendy Creek sites, which are situated lagoon and away from the influence of outlets leading to the Gulf of Mexico, the main carbon source is likely derived from mangrove detritus. The detritus in turn is assimilated by detritivores and by surrounding autotrophic organisms as their source of organic carbon for photosynthetic processes. To support this observation further, additional samples from primary producers such as seagrass, seagrass epiphytes, phytoplankton and detritus from each sampling location and upstream sites will be required to provide a deeper understanding of carbon sources available and to trace their pathways through the trophic webs.

The use of carbon and nitrogen stable isotope ratios have made it possible to infer the probable trophic pathway from autotrophs to consumers that occupy lower trophic levels. Based on $\delta^{15}N$ values we can postulate that a four-tier trophic network is present with phytoplankton, mangrove detritus, amphipods, benthic algae, suspended POM and sinking POM forming the

base of the trophic web (Figure 2.11). The base layer of the trophic web is likely followed by primary consumers; zooplankton, shrimp, hogchokers (*Trinectus maculatus*), crabs, oysters (*Crassostrea virginica*) and polychaetes. Glass minnows (*Anchoa mitchilli*), *Eucinostomus* species (silver jenny and spotfin majorra), gulf pipefish (*Syngnathus scovelli*), gobies (Gobiidae sp.), pinfish (*Lagodon rhomboides*), rough silversides (*Membras martinica*) and juvenile snappers comprise the secondary consumer level. Predatory species such as lizard fish (*Synodus foetens)*, and adult snappers (*Lutjanus griseus*) constitute the tertiary level.

Complex food webs typically range between 6 and 9 trophic levels. Results from this study indicate the presence of at least 4 trophic levels but has not included vital linkages within the microbial loop as well as with phytoplankton, zooplankton, mammal, avian and reptile communities (Fry et al. 1999). With the inclusion of these communities, complexity of this food web will be greatly increased indicating the presence of a stable and resilient food web that is less susceptible to trophic cascades and species extinction (Woodward and Hildrew 2002; Narwani and Mazumder 2012; Lynam et al. 2017).

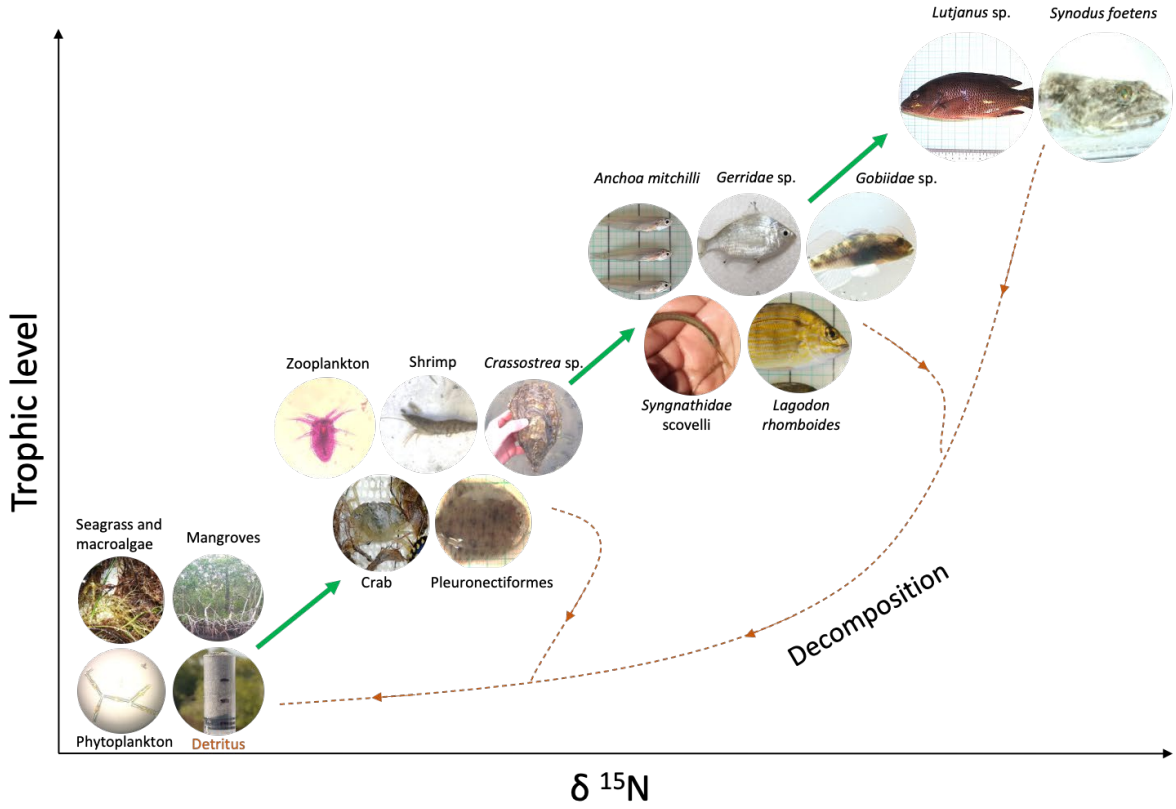


Figure 2.11. Simplified food web of lower trophic level fish from Estero Bay Aquatic Preserve based on $\delta^{15}N$ values and broad trophic levels.

Despite the ability for stable isotope analysis to provide a broad overview of the linkages present within Estero Bay, it is vital to address that there are additional interactions yet to be identified as stable isotope analysis only provides information for part of the food web (Compson et al. 2019). The presence of overlap observed along the $\delta^{13}C$ scale (Figure 2.5) between fish species suggests that combination of different food webs is possible (Abarntes and Sheaves 2009) and the reflected $\delta^{13}C$ values may be an average of these combined food webs. In addition, trophic levels are discrete and are usually defined with an integer value, but this is not true when omnivory is present. Omnivory refers to more than just consumers feeding on animal and plant tissue; it also includes organisms that feed on prey items at different trophic levels (Kratina et al. 2012). Due to the lack of specificity in stable isotopes, additional analysis is required to provide species specific details to elucidate the overlap of food webs and validate the presence of omnivory. In the next chapter we will be using dietary metabarcoding to obtain species level taxonomic information of ingested prey.

Appendix A

Table including the total number of individuals from Estero Bay Aquatic Preserve and predatory fish from the Gulf of Mexico. Nov 18 and May 19 refer to the sampling season.

Common name	Species	Vester		Big Hickory		Lover's Key		Hendry Creek	
		Nov 18	May 19	Nov 18	May 19	Nov 18	May 19	Nov 18	May 19
Goby	*Gobiidae spp.*	1	7	-	1	11	20	16	8
Glass minnow	*Anchoa mitchilli*	-	-	-	30	-	20	-	25
Gulf killifish	*Fundulus grandis*	-	-	24	-	-	-	14	-
Hogchoker	*Trinectus maculatus*	-	-	-	-	-	-	7	-
Inshore lizard fish	*Synodus foetens*	1	1	1	2	20	6	6	2
Pipefish	*Syngnathus scovelli*	-	-	-	2	-	19	2	5
Pinfish	*Lagodon rhomboides*	-	23	-	30	-	16	-	30
Rough silverside	*Membras martinica*	20	20	-	-	20	-	-	-
Sheepshead minnow	*Cyprinodon variegatus*	-	-	18	-	-	-	-	-
Silver jenny	*Eucinostomus gula*	7	-	-	-	-	-	-	-
Snapper	*Lutjanus griseus*	-	2	4	-	21	-	-	-
Spotfin mojarra	*Eucinostomus argenteus*	70	30	98	19	23	12	7	18
Total (739)		**99**	**83**	**145**	**84**	**95**	**93**	**52**	**88**

Predatory fish from the Gulf of Mexico		
Catfish	*Ariopsis felis / Bagre marinus*	4
King mackerel	*Scomberomorus cavalla*	5
Lady fish	*Elops saurus*	1
Little tunny	*Euthynnus alletteratus*	3
Mangrove snapper	*Lutjanus griseus*	4
Pompano	*Trachinotus carolinus*	1
Red grouper	*Epinephelus morio*	3
Sheepshead	*Archosargus probatocephalus*	6
Triple tail	*Lobotes surinamensis*	5
Total		***32***

Appendix B

Results of one-way ANOVA with post-hoc Tukey test comparing the means of $\delta^{15}N$. Species comparisons indicated with * signify that there is a significant difference in mean $\delta^{15}N$ value between the species.

Species	Comparison species	Std. error	Adjusted p-value
Hogchoker	Glass minnow*	0.59773	<0.01
	Gobiidae sp*	0.73207	0.0373
	Gulf killifish*	0.57026	0.0468
	Lizard fish*	0.59773	<0.01
	Pinfish*	0.57875	<0.01
	Pipefish	0.73207	0.2422
	Sheepshead minnow	0.73207	1
	Silver jenny*	0.65848	<0.01
	Rough silverside*	0.59773	<0.01
	Snapper*	0.59773	<0.01
	Spotfin mojarra*	0.5905	<0.01
Glass minnow	Gobiidae sp*	0.59773	0.0304
	Gulf killifish*	0.47255	<0.01
	Lizard fish	0.42266	0.4889
	Pinfish*	0.39536	<0.01
	Pipefish*	0.59773	<0.01
	Sheepshead minnow*	0.63044	<0.01
	Silver jenny*	0.59773	0.0463
	Rough silverside*	0.42266	0.0266
	Snapper	0.42266	0.9999
	Spotfin mojarra*	0.35045	<0.01
Gobiidae sp	Gulf killifish	0.63399	0.9979
	Lizard fish	0.59773	0.7292
	Pinfish	0.57875	0.9992
	Pipefish	0.73207	0.9997
	Sheepshead minnow	0.73620	0.9443
	Silver jenny	0.73207	1.0000
	Rough silverside	0.59773	0.9969
	Snapper*	0.53764	0.0486
	Spotfin mojarra	0.54905	0.9794
Gulf killifish	Lizard fish*	0.47255	<0.01
	Pinfish	0.44830	0.3596
	Pipefish	0.63399	1.0000
	Sheepshead minnow	0.63399	0.1036
	Silver jenny	0.63399	0.9930
	Rough silverside	0.47255	0.3080
	Snapper*	0.47255	<0.01
	Spotfin mojarra*	0.36810	0.0286

Lizard fish	Pinfish	0.39536	0.8781
	Pipefish	0.59773	0.1665
	Sheepshead minnow*	0.60344	0.0306
	Silver jenny	0.59773	0.8183
	Rough silverside	0.4226	0.9745
	Snapper	0.4226	0.9145
	Spotfin mojarra	0.35045	0.9750
Pinfish	Pipefish	0.57875	0.7819
	Sheepshead minnow	0.73620	0.999
	Silver jenny	0.57875	0.999
	Rough silverside	0.39536	1.000
	Snapper*	0.35562	0.0198
	Spotfin mojarra	0.31699	1.000
Gulf Pipefish	Sheepshead minnow	0.73207	0.2267
	Silver jenny	0.73207	0.9989
	Rough silverside	0.59773	0.7135
	Snapper*	0.59773	<0.01
	Spotfin mojarra	0.5905	0.4765
Sheepshead minnow	Silver jenny*	0.73207	0.0239
	Rough silverside*	0.59773	<0.01
	Snapper*	0.63044	<0.01
	Spotfin mojarra	0.59354	0.1308
Silver jenny	Rough silverside	0.59773	0.9993
	Snapper	0.59773	0.1649
	Spotfin mojarra	0.54905	0.9993
Rough silverside	Snapper	0.42266	0.1713
	Spotfin mojarra	0.35045	1.0000
Snapper	Spotfin mojarra*	0.31522	0.0306

Chapter 3: Application of DNA metabarcoding to elucidate diets and ecological networks of intermediate trophic level fish from mangrove ecosystem

3.1 Abstract

Two thirds of the human population live in or near coastal areas, which has caused extensive damage to coastal ecosystems. In particular, mangrove ecosystems have undergone extensive damage due to anthropogenic pressure. Mangrove forests play an integral role for the sustenance of commercial fisheries and fuel such economies. Previous research has focussed mainly on the effects of abiotic fluctuations on mangrove communities. However, relatively little is known about the nuanced interactions between mangrove ecosystems and associated organisms. Trophic interaction networks are well established in freshwater, marine and terrestrial environments but estuarine networks are understudied. Advances in DNA based molecular methods has revolutionised the field of molecular analysis for trophic interactions providing exceptional resolution in dietary assessments. Here, we used dietary metabarcoding of mangrove fish to construct ecological networks that identified changes in interactions across seasons, salinity gradients and habitat compositions within a subtropical estuary. The interaction networks constructed from dietary metabarcoding data revealed that fish species have more specialised feeding preferences during the wet season and generalised feeding patterns in mangrove habitats due to the greater availability of resources. In addition, network models suggest that coastal networks are more resistant to extinctions. We also demonstrate that dietary metabarcoding is complimentary to stable isotope data and is able to elucidate prey composition to lower taxonomic levels, where stable isotope analysis (SIA) could not. Our findings demonstrate that the application of multiple molecular based techniques can provide insights into interactions not offered when either technique is used independently. Furthermore, we demonstrate that dietary metabarcoding can be successfully utilised to construct ecological networks. The combination of metabarcoding and SIA offer strong potential to evaluate the effects of perturbation on species interactions and detect subsequent shifts in ecosystem function.

3.2 Introduction

Analysing feeding patterns is vital for understanding trophic and population dynamics (Amundsen and Sánchez-Hernández 2019) that in turn can be used inform conservation and management practices (Murray et al. 2011; Careddu et al. 2020). Dietary information can additionally be used in network ecology that aims to characterise organismal interactions within and between complex ecosystems (Bascompte 2007). Conventional methods such as observation of interactions in the field and laboratory (Cuff et al. 2022) and morphological identification of dietary remains have dominated trophic ecology studies. Morphological and observational data has been used to create highly resolved and well quantified ecological networks, especially in insect pollinator systems (Chacoff et al. 2012; Pocock et al. 2012; Gonzalez and Loiselle 2016). In fish communities, the use of morphological identification of diet from stomach contents and faecal samples have accurately revealed feeding patterns and trophic structure (Vander Zanden and Vadeboncoeur 2002; Araújo et al. 2008). A large benefit of visual methods is that they do not require any consumables and they are the only approach that can reliably identify different life-stages of prey (Nielsen et al. 2018). However, it has been acknowledged that morphological data derived from gut content analyses is biased towards undigested prey items. Otoliths, mollusc shells and exoskeleton parts are frequently encountered while small prey items, soft tissues of prey and easily digested food remain undetected by visual analysis (de Sousa et al. 2019; Traugott et al. 2021). Furthermore, morphological and observational data is highly time-consuming, labour intensive to gather and places high reliance on the skills of taxonomic experts (de Sousa et al. 2019; Pompanon et al. 2012).

Stable isotope analysis (SIA) is often used as a less labour-intensive alternative to observational/morphological data, providing information on dietary breadth and trophic positioning of individuals (Newsome et al. 2007). However, species-specific prey composition is hard to derive from SIA as ecologically similar, but phylogenetically distinct prey cannot be differentiated and prior knowledge on prey composition is required (Hoenig et al. 2022). In the previous chapter, SIA provided in-depth information on primary carbon sources and trophic positioning of different fish species, though, it was not able to discern the dietary overlap observed along the carbon isotopic axis. DNA based methods and specifically dietary metabarcoding has been proven successful in differentiating prey species and obtaining

species-specific prey composition data, addressing the shortcomings associated with observational/morphological and SIA datasets (Nielsen et al. 2018).

Molecular methods have the ability to provide high taxonomic resolution of prey items and identify rare and cryptic species within highly degraded material (Neilson et al. 2018). Metabarcoding specifically analyses a broader diversity of taxa within a mixed sample, targeting the whole dietary breadth within a food web (Pompanon et al. 2012). Using metabarcoding data derived from ingested components of stomach or faecal material has recently been merged into a limited number of network ecology studies. Clare et al. (2019) used metabarcoding data from faecal contents of insectivorous bats to create a network of networks that included plant-bat, parasite-bat and arthropod-bat interactions demonstrating that molecular approaches can be used as a powerful tool to construct complex network interactions with a high degree of taxonomic detail. Molecular based ecological networks have also been successful for informing biocontrol measures as stated in Mata et al. (2021) where faecal samples from bats were used to identify the role of bats in pest control and estimate effects of bat extinction on pest populations. Parimuchová et al. (2021) used ecological network theories to provide an insight into feeding behaviours of subterranean arthropods and how the lack of primary producers drives variability in prey preferences and level of interaction between different trophic guilds.

Ecological networks have been used to describe complex systems using interaction data and they provide an insight into ecosystem function through the calculation of network metrics (Guimarães 2020). Some metrics that can be explored in the context of ecological network analysis include nestedness (the extent to which organisms are generalists (Nielsen and Bascompte 2007)), robustness (the tolerance of network to species extinction (Dunne et al. 2002; Memmott et al. 2004) and extinction slopes (the possibility of extinction following species removal (Dormann et al. 2009)). Ecological networks are vital for estimating resilience and mitigating impacts of environmental and anthropogenic perturbation (Ings et al. 2009; Trifonova et al. 2015). In the context of fisheries, ecological and food-web networks are crucial for informing impacts on biomass and harvest levels (Dame and Christian 2006). Ecological networks that reflect ecosystem structure and dynamics are important indicators for Ecosystem Based Management (EBM) and have been used to inform management strategies (Tam et al. 2017). Ecological networks have also been integrated with economic dynamics to understand the anthropogenic impacts on single species and open-access fisheries. Simulations of

ecological networks demonstrated that profit driven growth in fishing effort can threaten long-term economic and ecological sustainability with impacts cascading to non-harvested species resulting in increased strength of disturbances (Glaum et al. 2020).

Notwithstanding the synergistic opportunities between DNA metabarcoding and network analyses, it is still crucial to acknowledge some challenges associated with amplicon sequencing. Firstly, biases arise during DNA isolation, amplification and sequencing but these can be mitigated through careful selection of primers and integration of PCR-free approaches (Bennett et al. 2019). A large concern that has been addressed extensively is the use of incomplete and uneven reference databases that can lead to inaccurate and higher taxonomic assignments resulting in exclusion of nodes and interactions (Compson et al. 2020). However, comparing barcodes via *in-silico* analysis against reference databases prior to choosing primers will prevent the use of non-suitable databases and the use of multiple target gene regions can improve taxonomic coverage and increase probability of identification (Pompanon et al. 2012; Nielsen et al. 2018). Finally, quantifying PCR-based metabarcoding data is highly challenging and the alternative of using binary presence/absence data may inaccurately represent true interaction events and ultimately impact network weighting (Clare 2014; Cuff et al. 2022). Deagle et al. (2019) proposed the use of weighted percent occurrence data from normalised frequency of occurrence data to produce accurate representations of network weightings and suggests that it is more appropriate when comparing against observational networks. Despite the challenges associated with molecular methods, dietary metabarcoding has been able to resolve feeding interactions to a higher degree compared to morphological methods due to its ability to provide unprecedented level of taxonomic resolution (Taguchi et al. 2014; Berry et al. 2015; Nielsen et al. 2018).

Mangrove forests are globally important for their ability to store carbon as they are capable of sequestering about eight times more carbon than a typical terrestrial forest (Donato et al. 2011). Mangroves also provide a range of ecosystem services that benefit human welfare by providing protection against coastal erosions and tsunami events, building material and firewood, and improvement of water quality by filtering pollutants (zu Ermgassen et al. 2020). Mangrove habitats create sheltered environments producing ideal habitats for farming crustaceans such as crabs and shrimp, consequently benefitting local economies (Huxham et al. 2017). Furthermore, mangrove forests lie in the transition zone between land and sea, moderating

flows from freshwater streams and marine waters, creating a highly productive environment that harbours distinctive groups of aquatic and terrestrial biodiversity (O'Connell et al. 2022).

Despite the ecological and socio-economic benefits of mangrove forests, they are still being degraded to facilitate aquaculture and agricultural crops such as rice and oil palm (Richards and Friess 2016). Urbanization of coastal habitats via land reclamation and development of shorelines to accommodate the rising human population has driven mangrove forests further into a critically vulnerable state (Lai et al. 2015). Research has shown that the rate of mangrove loss has been declining due to a combination of reduction in deforestation rates and increase in reforestation, but mangrove forests remain as threatened ecosystems because the potential gains have not yet balanced out a legacy of anthropogenic losses (Feller et al. 2017). The current literature is inundated by studies on mangrove biology, hydrology, provisioning and ecosystem services that provide a basic understanding on the biological requirements necessary for mangrove expansion (Feller et al. 2017), but more studies are required to understand the drivers of ecological change (such as salinity, tidal fluctuations) and interactions with neighbouring estuarine environments.

Mangrove forests function synergistically with neighbouring habitats such as coral reefs, seagrass meadows and saltmarshes to facilitate various ecosystem services via trophic and hydrological connectivity (Lee et al. 2014). However, there is a lack of studies analysing the connectivity and trophic interactions that underpin ecological processes and ecosystem function in mangrove forests.

3.2.1 Objectives and hypothesis

In this study, we use metabarcoding data from dietary analysis of fish to evaluate and compare the structure of mangrove fish species interaction networks across four sites located in Estero Bay Aquatic Preserve, Florida. We evaluate the utility of DNA metabarcoding to obtain species level interaction data from stomach contents of fish and use ecological networks to compare network structure between estuarine habitats and salinities. Since our dataset is dominated by forage fish from intermediate trophic levels, we expect them to be generalist consumers while larger, predatory fish to be specialists. We also use ecological network indices (nestedness, robustness and extinction slopes) to evaluate the influence of habitat and abiotic drivers on trophic interactions of fish that occupy intermediate trophic levels associated with

commercially and recreationally important species. We predict that feeding preferences of fish will be influenced by resource availability driven by habitat composition, salinity and trophic position while network indices will differ based on salinity only (inferred from SIA data).

3.3 Methods

3.3.1 Sample collection and processing

Samples were collected using seine nets from all four locations as described in Chapter 2. The dataset was supplemented with large predatory fish samples from the Gulf of Mexico caught by local fishing guides (refer to Appendix A for total number of samples and list of species used in metabarcoding analysis). Larger fish were included in the analysis to create a multitrophic interaction network and assess the level of predation/reliance on mangrove dwelling forage fish. Stomach contents were extracted from each fish carefully with an initial insertion from the anal opening towards the mouth. The entire digestive tract was removed and placed on a piece of sterilised aluminium foil. The dissection apparatus was then bleached (10%) and flamed (70% ethanol) before the intestinal tract was dissected. A longitudinal dissection was made along the tract and the contents were picked out (instead of scraping) to minimise contamination of host DNA in downstream analysis. Between each sample, the dissection instruments were sterilized as above.

3.3.2 Inhibition testing

Prior to DNA extraction an inhibition test was performed to determine the best DNA extraction method of fish stomach contents. Four methods: salt (Aljanabi and Martinez 1997), phenol-chloroform (Urakawa et al. 2010), ammonium acetate (Bruford et al. 1998) and DNeasy Blood and Tissue kit (Qiagen) were tested. DNA was extracted from the stomach contents of a *Scomber scombrus* (European mackerel) sample and spiked with *Lolium perenne* (perennial Ryegrass) DNA (1µl). Quantitative polymerase chain reaction (qPCR), targeting *L. perenne* was performed on each sample with 10µl as final reaction volume using a QuantStudio 6 Flex Real-Time qPCR machine (ThermoFisher Scientific). Each 10µL reaction contained 1 x PrecisionPLUS qPCR Master Mix, with ROX at a lower level (PPLUS-LR, Primer Design, UK), 6 µmol/L species specific probe and forward and reverse primer (refer to Rowney et al. 2021), 4µl of the DNA template mix at 1:3 ratio of grass template to mackerel stomach content template and 0.5 µl of nuclease-free water. Thermocycling began with an initial 95ºC for 2 min followed by 50 cycles of 10s at 95ºC and 1 min at 60ºC, as per the manufacturer's instructions.

Negative controls were run under the same conditions, but nuclease-free water replaced grass DNA in these reactions. The resultant amplification plots were analysed to reveal level of amplification of grass pollen. We concluded that inhibitors were present in plots where no or poor amplification of grass pollen was observed. The Ammonium acetate, DNeasy Blood and tissue extraction kit and phenol chloroform extraction methods produced good amplification curves. Since the ammonium acetate extraction method was the most cost and time efficient method of the three, this was used to extract DNA from the isolated stomach contents. Prior to PCR, all DNA extracts were cleaned using a Zymo OneStep PCR inhibitor Removal Kit (Zymo Research) to remove PCR contaminants.

3.3.3 Sediment sample collection and processing

Environmental DNA was extracted from sediment and water samples to characterize the biodiversity of each sampling site. Four sediment samples were collected from each sampling location using a core and only the top two centimeters of the core was transferred into a clean falcon tube. Each sediment sample was collected using a new sterilized core. All sediment samples were transported in a cool box until freezer storage at -20°C. A ZR Fecal DNA Miniprep Kit (ver 1.1.2, Zymo Research) was used to isolate DNA from sediment samples according to the manufacturer's protocol. Prior to extraction, all four sediment samples collected from each location were mixed manually and two samples from each pool (approximately 0.260g) were used in the final extraction.

3.3.4 Water sample collection and processing

Three one-liter water samples were collected at each site using a sterile bottle and filtered with 0.22µm polyethersulfone membrane Sterivex filters (Merck Millipore) using a sterile syringe. All filters were stored in sterile Longmire's solution (approx. 1.5ml) (Longmire et al. 1997)via the inlet valve and kept in the fridge (4ºC). DNA extraction from the water samples were performed in a PCR-free clean laboratory and followed the SX$_{\text{CAPSULE}}$ method stated in Spens et al. (2017).

3.3.5 PCR and sequencing

Prior to PCR, *in-silico* testing of primer suitability was performed using the 'Biostrings' package (v 2.66.0) (Pagès et al. 2022) in RStudio (RStudio Team 2020). Database for *in-silico*

testing was curated by extracting a subset of the recorded fauna and flora information from Estero Bay Aquatic Preserve Management Plan (Florida Department of Environmental Protection (3), 2015). Sequences of the target species was downloaded from the NCBI database (Schoch et al. 2020) and aligned in 'AliView' (Larsson 2014) prior to analysis in RStudio.

Illumina MiSeq paired-end indexed amplicon libraries were prepared using a two PCR step protocol and sequenced over two runs. Two marker genes were amplified using universal primer pairs, the first being mlCOIintF (Leray et al. 2013) and jgHCO2198 (Geller et al. 2013) targeting the mitochondrial cytochrome c oxidase subunit I (COI) region, recognised for amplifying invertebrates to the lowest taxonomic level. TAReuk454FWD1 and TAReukREV3r (Stoeck et al. 2010) were used to target eukaryotic diversity (Table 3.1) from the 18S V4 region of the nuclear small subunit ribosomal DNA (18S). A 5' universal tail was added to both forward and reverse primers, and a 6N sequence was added between the forward universal tail and the template specific primer. The 6N addition is known to improve clustering and cluster detection on Illumina Miseq sequencing platforms (Miya et al. 2015).

First round PCR was conducted in triplicate using the Qiagen Multiplex PCR Kit to a final volume of 15 μl, which comprised 0.2 μM of forward and reverse primers, 2x Qiagen Multiplex PCR Master Mix (containing HotStarTaq DNA Polymerase, multiplex PCR buffer and dNTP mix) and 0.6μl of DNA template. The thermal cycling conditions for amplification of the COI region were an initial activation step at 95ºC for 15 mins; 35 cycles at 94ºC for 30 seconds, annealing at 54ºC for 90 seconds and extension at 72ºC for 60 seconds and a final extension at 60ºC for 30 minutes. The 18S rDNA PCR amplification differed only at the annealing stage where temperature was set to 55ºC. Each 96-well PCR plate contained two PCR1 and PCR2 negative controls where water was substituted for DNA templates and two positive controls that were made up of DNA extractions from nine freshwater invertebrate species endemic to the UK. Products from the first PCR were purified using Agencourt AMPure XP beads (Beckman Coulter), at a 1:0.7 ratio of PCR product : AMPure XP beads.

Table 3.1. Primer name and sequences used in library preparation. Round 1 primer sequences contain forward and reverse template primers, the forward primer sequence contained 6N's to improve clustering and cluster detection on Illumina Miseq sequencing platforms. Round 1 primers include mitochondrial cytochrome c oxidase subunit I (COI) (Leray et al. 2013; Geller et al. 2013) and V4 region of the 18S ribosomal DNA-encoding gene (Stoeck et al. 2010). Round 1 and round 2 sequences contain complementary universal tails. Round 2 PCR primer sequence contained the P5 and P7 Illumina adapters (Integrated DNA Technologies) and an 8bp unique index both in forward and reverse primers used for demultiplexing samples.

Round 1 PCR
Forward Universal Tail – NNNNNN – Template specific primer ***mlCOIintF***   [ACACTCTTTCCCTACACGACGCTCTTCCGATCT]-[NNNNNN]-   [GGWACWGGWTGAACWGTWTAYCCYCC]
Reverse Universal Tail – Template specific primer ***jgHCO2198***   [GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT]-[TAIACYTCIGGRTGICCRAARAAYCA]
Forward Universal Tail – NNNNNN – Template specific primer ***TAReuk454FWD1***   [ACACTCTTTCCCTACACGACGCTCTTCCGATCT]-[NNNNNN]-[CCAGCASCYGCGGTAATTCC]
Reverse Universal Tail – Template specific primer ***jgHCO2198***   [GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT]-[ACTTTCGTTCTTGATYRA]
Round 2 PCR
P5 Illumina adapter – **i5 index** – Forward Universal Tail   [AATGATACGGCGACCACCGAGATCTACAC]-[**i5 index**]-   [TCTACACGTTCAGAGTTCTACAGTCCGACGATC]
P7 Illumina adapter – **i7 index** – Reverse Universal Tail   [CAAGCAGAAGACGGCATACGAGAT]-[**i7 index**]-   [GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT]

Before the second round PCR was conducted, PCR 1 product from each plate was pooled at 5ul per sample, resulting in 15µl of pooled PCR 1 product in each well. Second round PCR was performed at a final volume of 15 µl, 3 µl of purified template from PCR 1, 2x Qiagen Multiplex PCR Master Mix and 2 µM of the unique identical i5 and i7 indexes and the P5 and P7 Illumina adapters. Thermal cycling condition were 95 ºC for 15 minutes, followed by 10 cycles of 98 ºC for 10 seconds, 65 ºC for 30 seconds and 72 ºC for 30 seconds and a final extension at 72 ºC for 5 minutes. The amplified libraries were cleaned for a second time using Agencourt AMPure XP beads at a 1:0.7 ratio of DNA library : AMPure XP beads. Two Illumina runs were performed in this study. In the first run, the libraries were quantified using a Qubit broad range kit (ThermoFisher Scientific) and pooled at equimolar concentrations. Prior to the second run the libraries were pooled into two groups, one consisted low concentration (< 1ng/ul) and the other high concentration (> 1ng/ul) libraries and a final pool of 3:7 ratio of low concentration to high concentration libraries was submitted for sequencing. A trial run of this pooling method was performed using an Illumina MiSeq Nano Kit (v2, 500 cycles). The results from the Nano kit demonstrated homogeneous sequencing of the pooled proportions. Prior to sequencing, the final pooled library was purified using a Pippin Prep (Sage Scientific) according to the manufacturer's protocol. All sequencing runs were performed in Bangor University (CEB Lab) using a 2x250 Illumina MiSeq Kit (v2).

3.3.6 Bioinformatic analysis

Bioinformatic analysis was performed on the Supercomputing Wales cluster and RStudio (v 4.1.3) (RStudio Team 2020). FastQC (v 0.11.8) (Andrews 2010) was used to analyse the quality of raw sequences. Sequences were filtered using Cutadapt (v 3.7) (Martin 2011) to only include those that contained forward and reverse primer sequences across both read pairs for each fragment and primer regions were identified removed from each fragment for the remaining sequences. A total of 6 362 868 and 5 353 875 paired reads were recovered from COI and 18S markers respectively, collectively over the two sequencing runs. The sequences were denoised (sequence quality control; trimming, filtering and removal of chimeras) using the DADA2 pipeline (v 1.16) (Callahan et al. 2016) in Rstudio. Default parameters of DADA2 were used unless otherwise stated. After manual examination of the read quality profile from FastQC results, forward reads of COI and sequences were trimmed at 240 bp using the 'filterAndTrim' function in the 'DADA2' package, while the reverse reads were trimmed at 220bp. No trimming was performed for 18S as it is a variable marker but a minimum length of 150bp was

imposed using the 'filterAndTrim' function. After denoising an amplicon sequence variant (ASV) by sample table was produced. The denoised ASVs were used for taxonomic assignment using the 'blastn' command in BLAST+ (Camacho et al. 2009) against the MIDORI (v GB 241) (Leray et al. 2018) and SILVA (v 138.1) (Quast et al. 2013) databases for COI and 18S markers respectively. The COI and 18S ASVs were assigned to taxonomy at 97% sequence similarity threshold to species level as an objective, yet general measure of species dissimilarity using the COI and 18S markers (Drake et al. 2022). In some analysis order and family levels (at 97% identity match) were used for visual grouping. E-value was set at <0.00001 and only ASVs above 313 and 390 base pairs were retained for the COI and 18S regions respectively.

3.3.7 Statistical analysis

Rarefaction curves were computed and visualised using 'vegan' (v 2.6-2) (Oksanen et al 2022) and 'ggplot2' (v 3.3.6) (Wickham 2016) packages. Samples with read counts less than 100 were not included in downstream analysis. ASV tables were rarefied 100 times (Weiss et al. 2017) using 'phyloseq' (v 1.38.0) (McMurdie and Holmes 2013). Subsets based on species (field assignment) were created for the COI dataset and host species based on family assignment were removed throughout each subset (i.e in the glass minnow subset all ASVs assigned to *Engraulidae* was removed). All ASVs reflecting phylum *Chordata* were removed from 18S dataset as species specific assignment was not possible with 18S marker. ASV counts were transformed to proportional data and used in downstream analysis. Permutational multivariate analysis (PERMANOVA) from the vegan package (using 'adonis2' function) was used to assess differences in beta diversity based on Bray-Curtis dissimilarities. Pairwise differences were identified with 'pairwise.adonis' function in 'pairwiseAdonis' package (v 0.4) (Martinez Arbizu 2020) and Bonferroni correction was applied to the resulting p-values to control for false positives. Non-metric multidimensional scaling (nMDS) ordination was calculated in 'vegan' using 'metaMDS' function.

Sampling effort directly affects the interactions observed within ecological networks, compromising the accuracy of the results produced (Rivera-Hutinel et al. 2012; Costa et al. 2016). By quantifying the proportion of interactions present in the sampled system, we are able to understand if the sampling undertaken is complete enough to confirm the validity of the networks produced (Macgregor et al. 2017). A test for sampling completeness was conducted based on the Chao2 method proposed by Macgregor et al. (2017). This method is a modification

of the previous method proposed in Traveset et al. (2015), where equal weight is placed on each interaction instead of species, reducing biased estimation towards specialist species. 'BipartiteD3' (v 0.3.0) (Terry 2018) and 'circlize' (v 0.4.16) (Gu et al. 2014) packages were used for visualisation of trophic interactions and calculation of network metrics. 'Bipartite' (v 2.18) (Dormann 2022) package was used to calculate a number of ecological indices. Each are presented below, followed by an explanation of how the metrics can be interpreted in the context of network ecology. (a.) Number of nodes where each node represents a set of species), (b.) nestedness that measures the extent to which organisms are generalists (Nielsen and Bascompte 2007) (higher values reflect more nested species only interacting with a limited number of generalists, while lower values indicate the presence of generalists who interact with many species (Corso et al. 2011)). (c.) Extinction slope measures the possibility of extinction given the species it is interacting with is lost (Dormann et al. 2009) (higher values reflect less vulnerability to extinction) and (d.) robustness quantifies impact of loss (species extinction) of one set of species on the other (Dunne et al. 2002; Memmott et al. 2004) (higher values indicate that the network is more resilient to species loss). The Input data for network analysis was proportional data from ASV tables aggregated at family (COI) and order (18S) levels and subsequently converted to binary (presence/absence) form. The null model was computed via 'shuffle.web' method using 'nullmodel' function in bipartite and set to generate 1000 null models. 'Ggplot2' and 'fantaxtic' (v 0.2.0) (Teunisse 2022) were used to generate bar plots.

3.4 Results

A total of 739 fish (less than 20 cm in total length) comprising 12 different species were caught. DNA was isolated from stomach contents of each individual specimen and every three extractions were pooled (based on species and site) to make a single sample for metabarcoding making up 290 stomach content samples. This pooling method was the most efficient manner to capture the full range of diet consumed and a sustainable way to include all fish individuals caught while keeping within costs of the research budget. A total of 32 large predatory fish were also included in the dataset that were caught by a professional angler/fish guide. Soil (n=26) and water (n=17) extractions, along with the 32 stomach contents from the large fish were sequenced individually. In addition, the *in-silico* test performed prior to PCR indicated that chosen COI and 18S primers would be successful at amplifying a broad range of taxa in found in Estero Bay Aquatic Preserve.

3.4.1 Sequencing results

A total 11 716 743 reads were produced targeting the cytochrome c oxidase subunit I (COI) and the V4 region of the eukaryotic nuclear small subunit ribosomal DNA (18S). The number of raw reads per sample averaged at 36 742 ± 5 283 (standard error). Number of reads per ASV found in the negative controls and extraction blanks were deducted from the entire dataset. Since the negative control samples showed low level of cross-contamination it did not affect the overall distribution of reads (Taberlet et al. 2018).

3.4.2 COI dataset results (6 362 868 reads)

The sediment and water extracts collected were aimed at characterising biodiversity at each sampling location (Figure 3.1). Across all sites a large proportion of the ASVs detected in sediment came from phyla Annelida (segmented worms), Arthropoda (invertebrates) and Chordata (vertebrates). PERMANOVA results, for sediment samples, calculated from Bray-Curtis distance showed significant differences in beta diversity were only present between coastal sites Big hickory and Lover's key (df=1, F=1.85104, p=0.014985). The most dominant taxa from Big hickory were chordates and arthropods whereas in Lover's key annelids were most abundant (Figure 3.1). An overall difference in diversity between coastal and lagoon sites was not observed. Sediment samples from Vester site were excluded from analysis as only one sample was retained after denoising and it reflected only one taxa (Annelida).

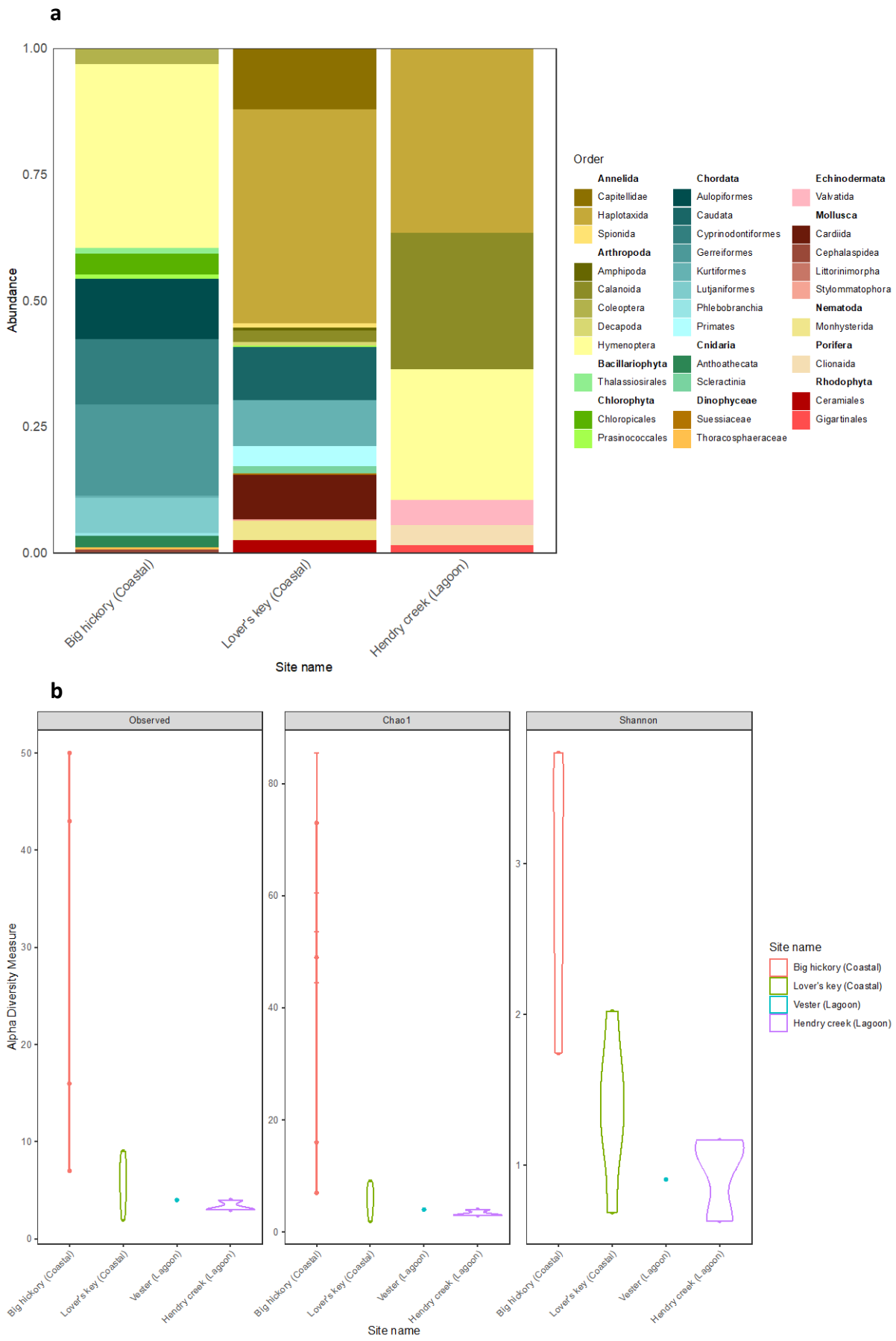


Figure 3.1. Mitochondrial cytochrome c oxidase subunit I (COI) ASVs from major sediment biota groups from each sampling location. a) Stacked bar plot reflecting proportional abundance b) violin plots depicting alpha diversity estimates.

Water samples from coastal sites were abundant in chlorophyta (36%), chordata (32%) and synurophyceae (green algae) (11%) whereas lagoon sites were dominant in chordata (49%), arthropoda (23%) and synurophyceae (8%) (Figure 3.2). PERMANOVA performed resulted in an overall difference between coastal and lagoon diversity (df=1, F.model = 3.472, p-value = 0.000999).

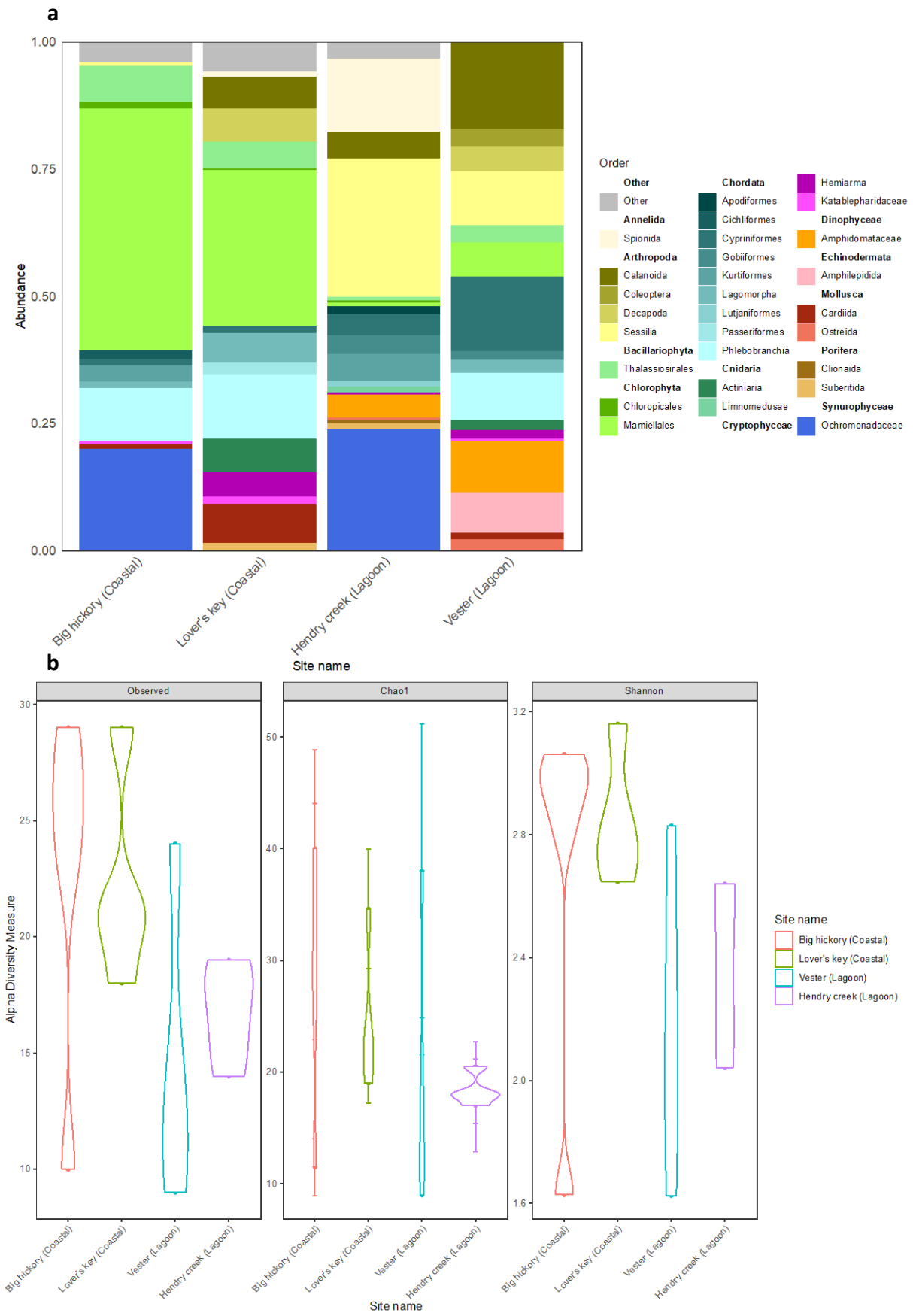


Figure 3.2. Mitochondrial cytochrome c oxidase subunit I (COI) ASVs from major water biota groups from each sampling location. a) Stacked bar plot reflecting proportional abundance b) violin plots depicting alpha diversity estimates.

After taxonomic assignment 2150 amplicon sequence variants (ASVs) were returned from the fish stomach content samples. When host ASVs were removed from each sample 668 ASVs from 13 phyla remained, with the most abundant ASVs belonging to Arthropoda (38%), followed by Chordata (18%) and Mollusca (6%) (Figure 3.3)

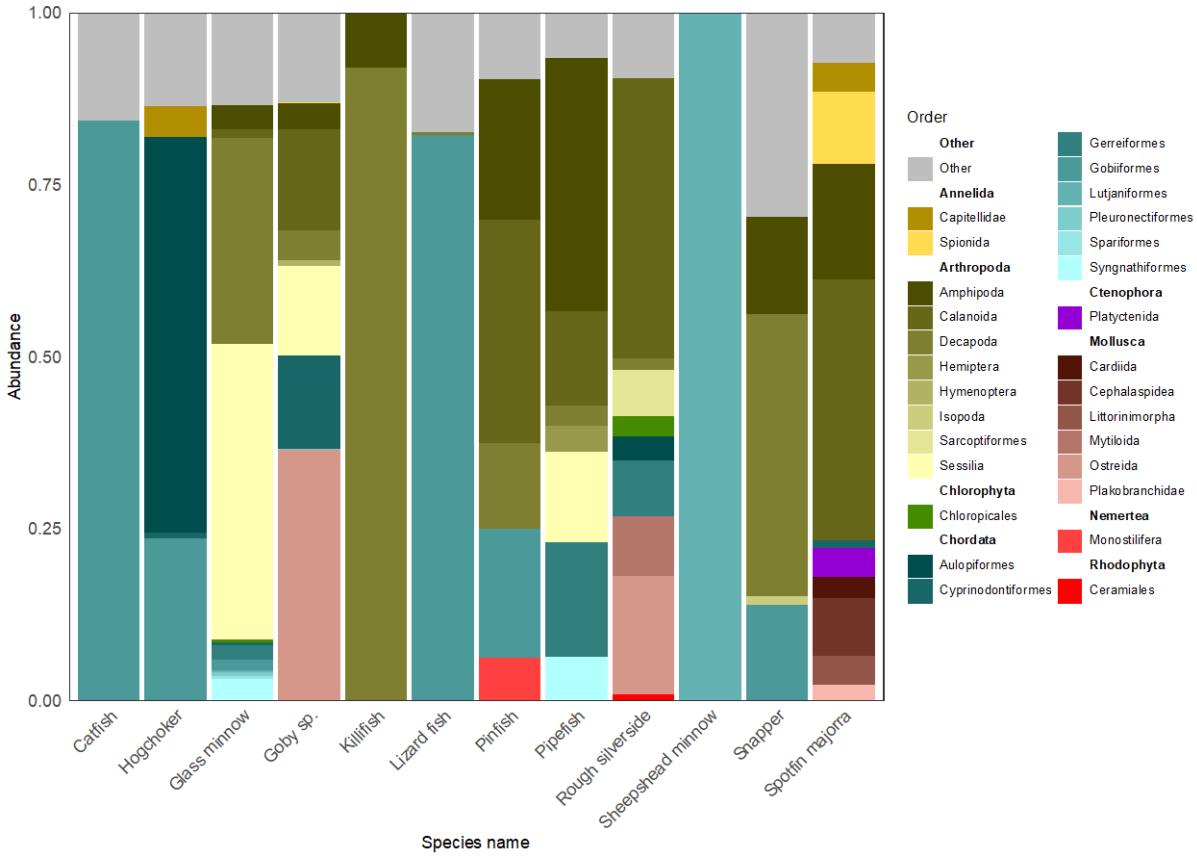


Figure 3.3. Proportional abundance of mitochondrial cytochrome c oxidase subunit I (COI) ASVs from fish stomach contents.

PERMANOVA analysis resulted in significant differences between species (Df=5, F=1.3866, p=0.001), however a post-hoc analysis looking at pairwise differences with Bonferroni correction did not result in significant differences. When the dataset was analysed against trophic levels (trophic groups based on $\delta^{15}N$ ratios from Chapter 2), results from the PERMANOVA analysis showed significant difference (Df=1, F = 1.6406, p-value = 0.001). A pairwise PERMANOVA resulted in significant difference between trophic levels 3 (n=108) and 4 (n=22) only (Df=1, F = 1967, p-value = 0.002997). The analysis was repeated on a subset of trophic level 3 (n=22) individuals, and the PERMANOVA analysis retained significant difference (Df =1, F= 1.603, p-value = 0.001). On closer inspection, stomach contents of fish from trophic level 3 were abundant in Arthropoda (44%) whereas trophic level 4 largely consisted of Chordata ASVs (20%).

A non-metric multidimensional scaling (nMDS) plot indicated presence of grouping for certain species such as pinfish, snapper, glass minnows and lizard fish despite belonging to different sampling locations (Figure 3.4 and 3.5). Whereas glass minnow individuals from Lover's key and Hendry creek group separated from each other and PERMANOVA resulted in significant difference in beta diversity (df = 1, F = 2.6316, p-value = 0.02397) (Figure 3.5). Glass minnows from Lover's key primarily included decapods (52% that includes crabs from families Panopeidae and Pinnotheridae, along with shrimp from Palaemonidae), Sessilia (15% Balanidae and Chthamalidae - barnacle) and Syngnathiformes (13% specifically from family Syngnathidae comprising seahorse and pipefish). Individuals from Hendry creek comprised Ocypodidae (ghost and fiddler crabs 58%), Sesarmidae (2.7%) and panopeidae (mud crabs – 2.3%) followed by Sessilia (balanidae 26%) and Ampithoidae (8%, amphipod crustacean) (Figure 3.5).

Goby sp. from Hendry creek and Vester differed significantly in the diversity of their stomach contents (df = 1, F = 2.5092, p=0.005994). Individuals from Hendry creek comprised Chordata (28%) mainly from families Fundulidae (topminnows and North American killifish) and Achiridae (American soles) along with Ampithoidae (5%) and Palaemonidae (1%). Conversely, individuals from Vester were abundant in Ostreida (oyster) (55%), Arthropoda from families Acartiidae (20%) and Hippolytidae (4%) and Balanidae (18%) (Figure 3.6).

Similarly, rough silversides from Lover's key and Vester formed two distinct clusters (PERMANOVA results: df = 1, F = 2.6316, p-value = 0.005994). Silversides from Vester were dominated by Calanoida (92% from family Acartiidae - copepod) and Gerridae (7.75%, mojarras) species. Whereas individuals from Lover's key seemed to have consumed a mixture of species including Ostreidae (27%), Gerridae (16%), Acartiidae (14%) and Mytilidae (14% - bivalves) (Figure 3.6).

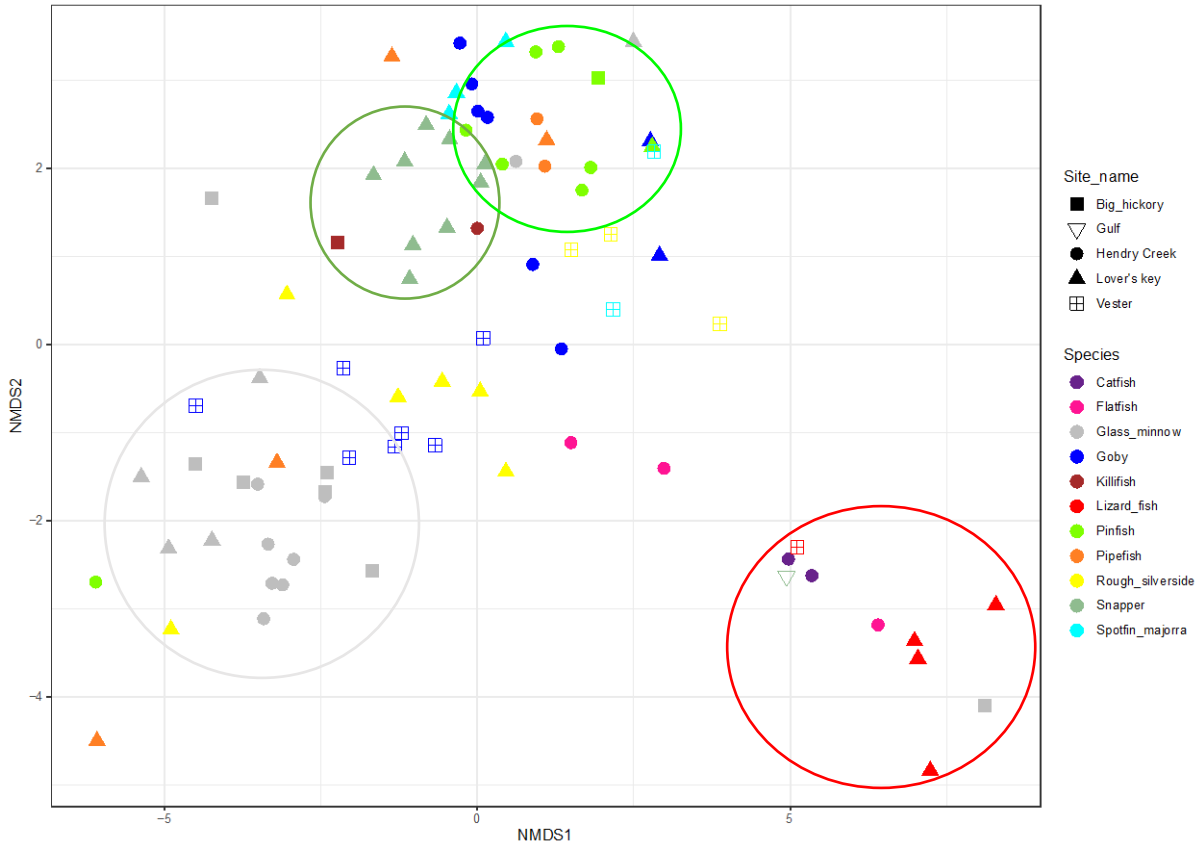


Figure 3.4. Non-metric multidimensional scaling (nMDS) plot of stomach contents from proportional abundance of mitochondrial cytochrome c oxidase subunit I (COI) ASVs. Ellipses represent grouping of species.

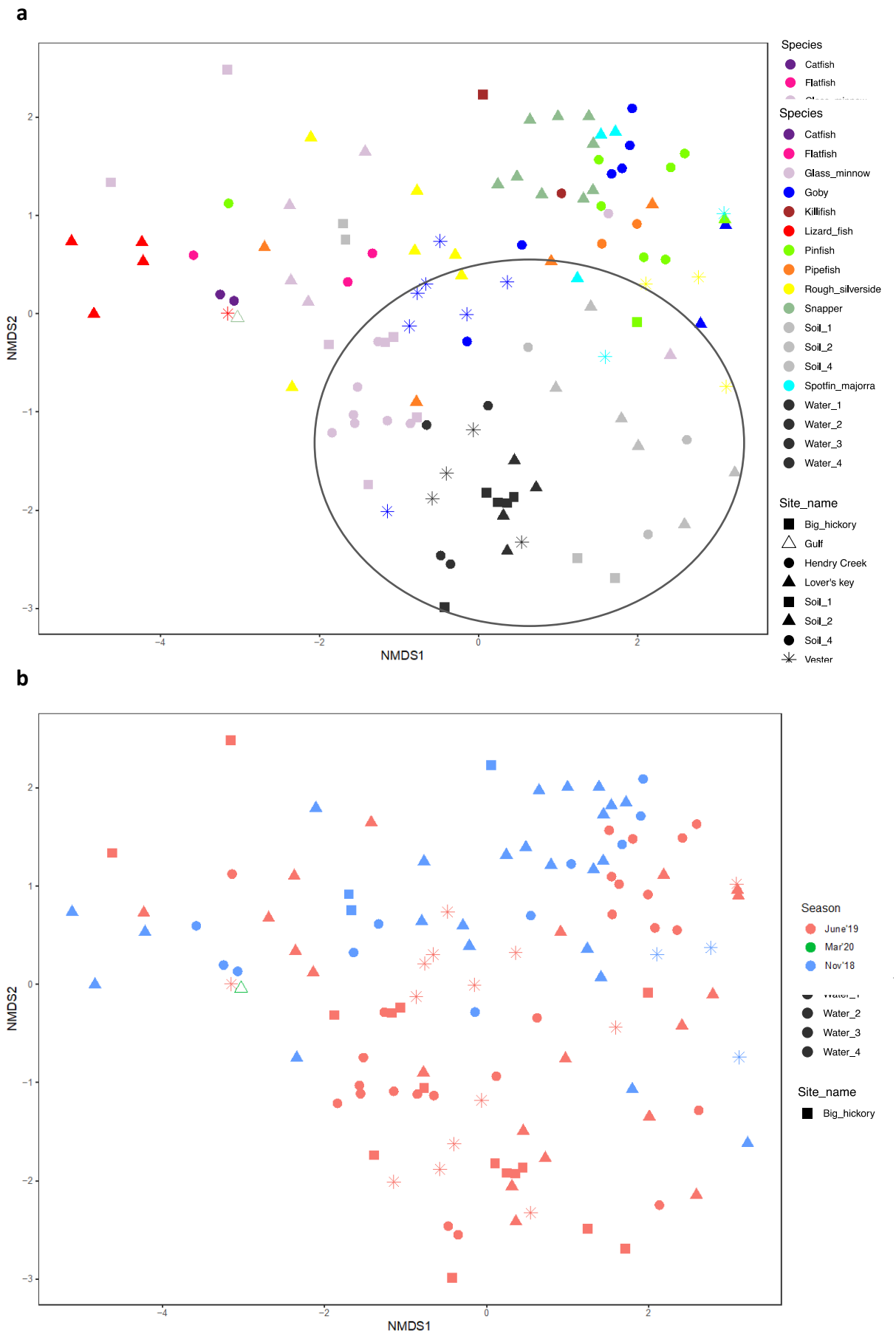


Figure 3.5. Non-metric multidimensional scaling (nMDS) plot of stomach contents from proportional abundance of mitochondrial cytochrome c oxidase subunit I (COI) ASVs. The figures represent how sediment, water, and fish diets change across sampling a) location and b) time. The ellipse shown in a) reflects some separation between environmental samples and gut samples.

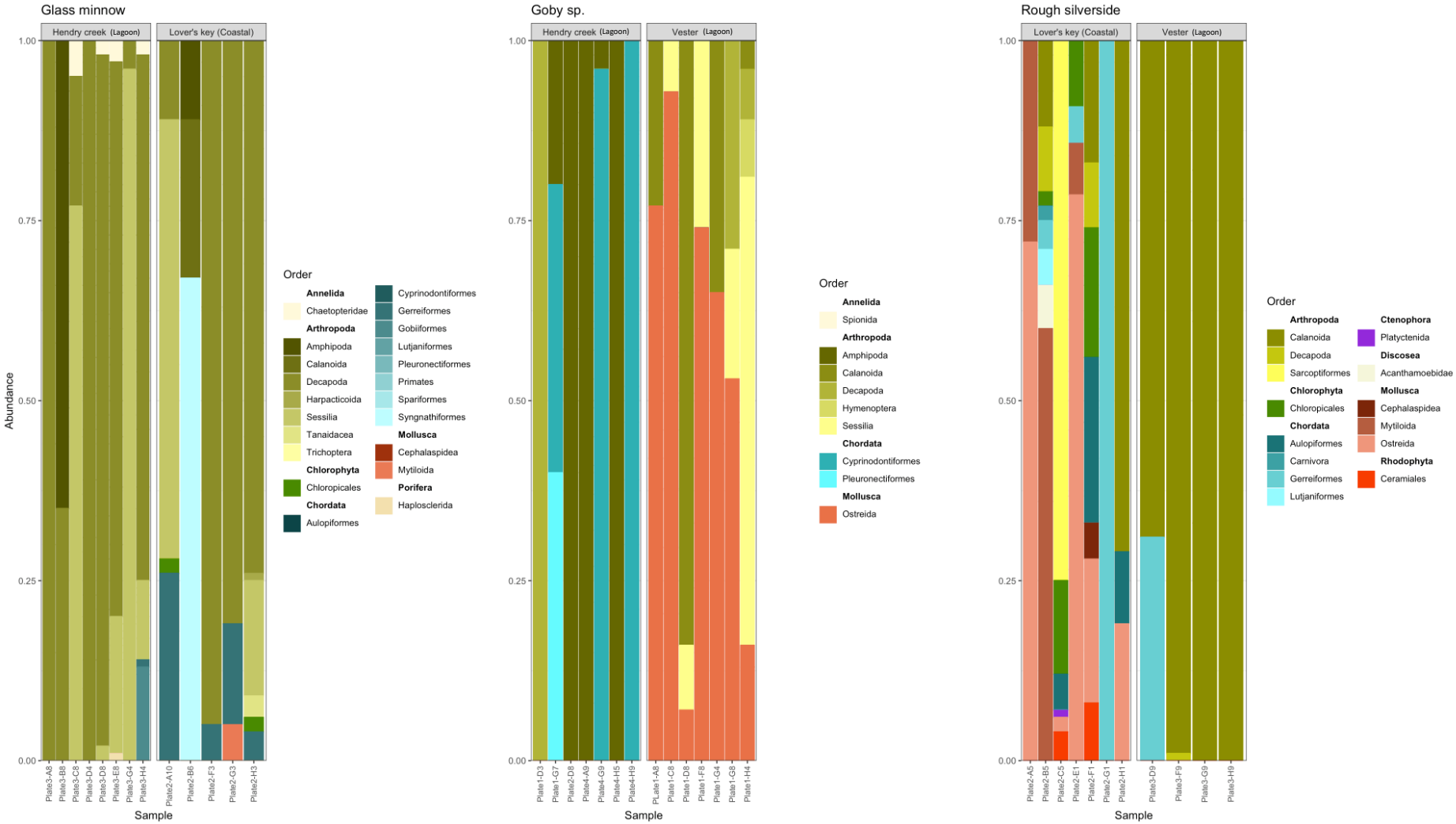


Figure 3.6. Comparisons of proportional abundance of mitochondrial cytochrome c oxidase (COI) ASVs from stomach contents within three species between different sampling locations.

3.4.2.1 COI marker – network analysis

Sampling completeness for all networks was below the 80% threshold level, therefore interpretations from incomplete interaction data may not depict the true connections between species (Evans et al. 2016). However, due to the lack of network studies using molecular data conducted in mangrove ecosystems, results from this study should not be dismissed.

Similar results were obtained for nestedness, extinction slope and robustness for both the observed (Figure 3.7) and null network (Table 3.2), this indicated that the observed and null networks do not differ significantly. When seasonal (wet and dry) and habitat (coastal and lagoon) networks were compared, nestedness and the extinction slope in lower trophic level fish were higher for the wet season network. Similarly, nestedness and extinction slope at lower trophic levels were higher for the coastal web. High nestedness values indicate presence of specialist species and high extinction slope (in lower trophic level fish) values reflect that lower trophic level species (prey) are more resilient to species loss. When coastal and lagoon habitats were separated by habitat composition, similarities between habitat compositions for indices; nestedness, no. of compartments, extinction slope and robustness was observed, potentially indicating that a combination of salinity and habitat composition influences network structure and its constituent interactions.

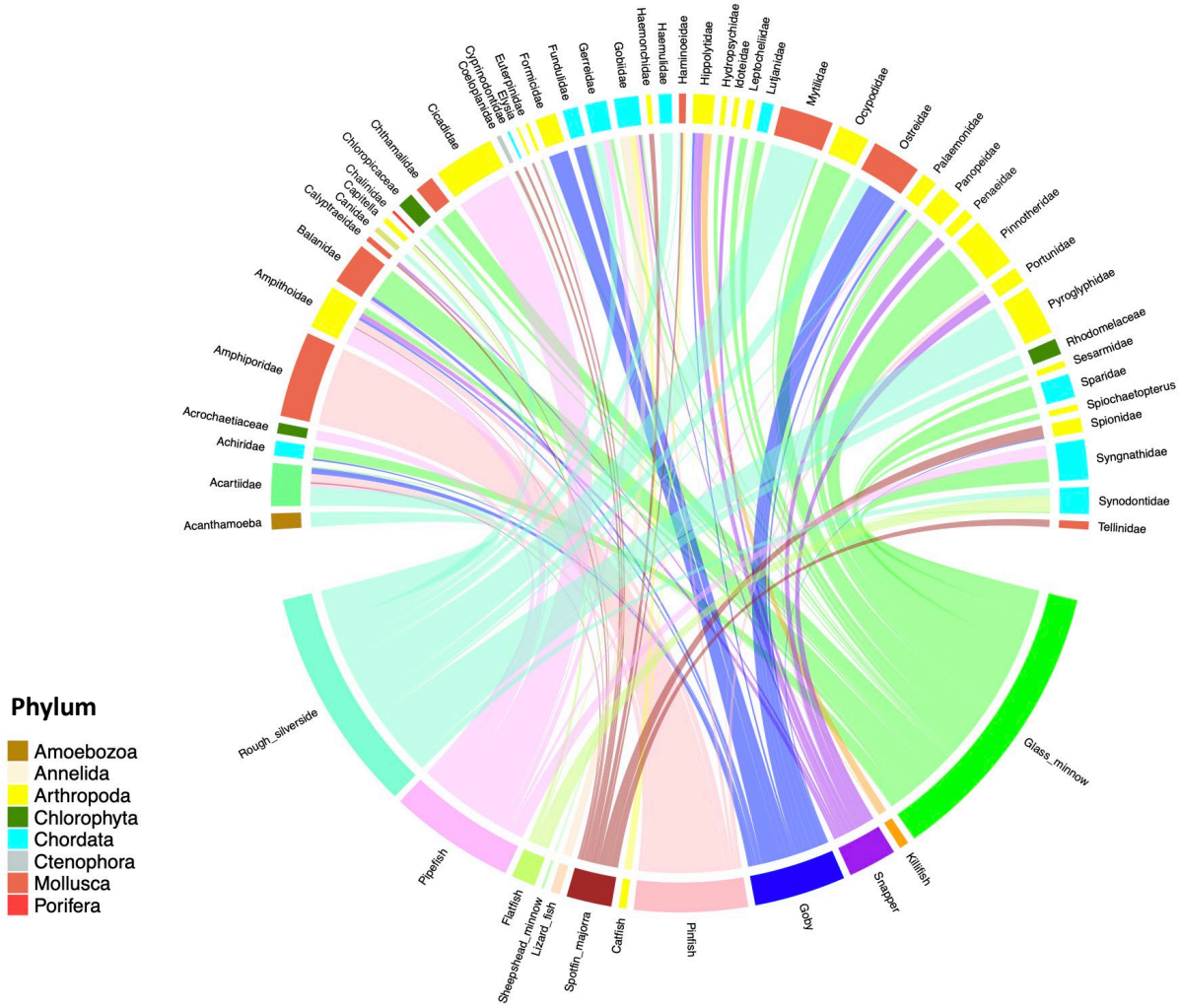


Figure 3.7. Qualitative bipartite plot showing interactions between predatory fish and consumed prey using binary data from dietary metabarcoding analysis of the mitochondrial cytochrome c oxidase subunit I (COI) gene region. Prey items presented are grouped at family level and coloured corresponding to phylum.

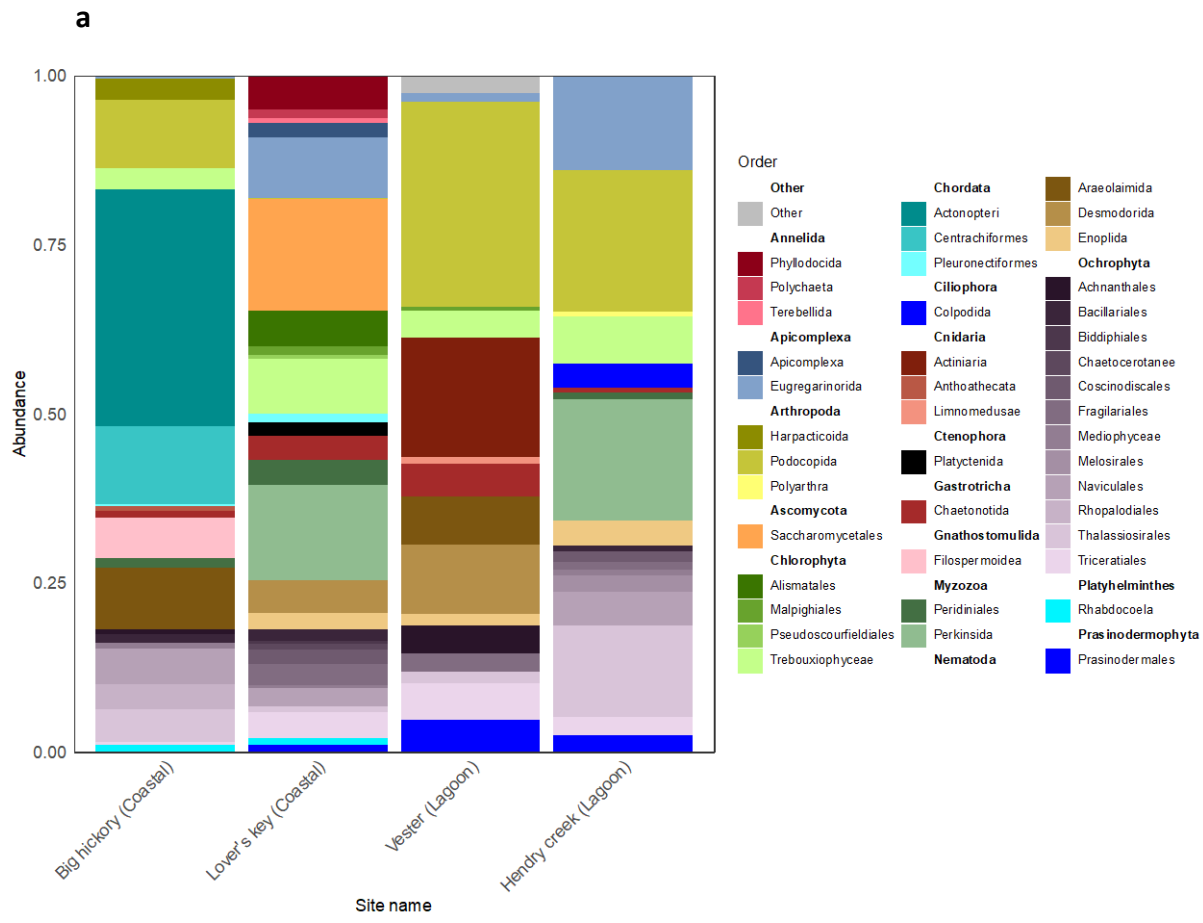
Table 3.2. Network index comparisons between food web structures using binary data from metabarcoding analysis of mitochondrial cytochrome c oxidase subunit I (COI) gene region. No. of nodes – number of nodes, nodes indicate points of interactions, HL- higher trophic level, LL – lower trophic level. Data provided here is for comparisons between networks. **A**: observed vs Null, **B**: seasonal networks – wet vs dry, **C**: habitat networks – coastal vs lagoon, **D**: habitat composition networks, coastal mangrove vs coastal seagrass vs lagoon mangrove, vs lagoon seagrass.

A	**Observed (43.3%)**	**Null**
No. of nodes HL	12	12
No. of nodes LL	47	47
Nestedness	34.618	33.610
Extinction slope HL	1.722	1.742
Extinction slope LL	5.221	5.584
Robustness HL	0.551	0.551
Robustness LL	0.799	0.806
B	**Wet (50.9%)**	**Dry (55.0%)**
No. of nodes HL	6	9
No. of nodes LL	15	28
Nestedness	40.275	26.934
Extinction slope HL	1.610	1.544
Extinction slope LL	6.776	3.555
Robustness HL	0.453	0.498
Robustness LL	0.816	0.734
C	**Coastal (39.0%)**	**Lagoon (70.4%)**
No. of nodes HL	10	11
No. of nodes LL	40	23
Nestedness	33.563	28.409
Extinction slope HL	1.568	1.562
Extinction slope LL	4.442	2.895
Robustness HL	0.512	0.521
Robustness LL	0.771	0.690

D	**BH (48.6%)** ***Coastal Mangrove***	**LK (46.6%)** ***Coastal Seagrass***	**V (79.6%)** ***Lagoon Mangrove***	**HC (71.7%)** ***Lagoon Seagrass***
No. of nodes HL	6	8	6	9
No. of nodes LL	20	31	11	18
Nestedness	18.130	34.0309	19.762	33.122
No. of compartments	3	1	3	1
Extinction slope HL	1.1963	1.698	1.243	1.643
Extinction slope LL	2.627	4.629	1.899	2.761
Robustness HL	0.388	0.505	0.399	0.511
Robustness LL	0.671	0.778	0.563	0.669

3.4.3 18S dataset results (5 353 875 reads)

A large proportion of the ASVs from soil samples were assigned to Ochrophyta (21%, photosynthetic heterokonts/brown algae), Arthropoda (15%) and Myzozoa (10%). PERMANOVA showed an overall difference in the beta diversity between coastal and lagoon sampling locations (df, 1, F = 1.5077, p-value = 0.011989). Ochrophyta (20%), Chordata (18%) and Myzozoa (12%) were most abundant at coastal sites whereas Arthropoda (25%), Ochrophyta (22%) and Nematoda (11%) were dominant at lagoon sites (Figure 3.8).



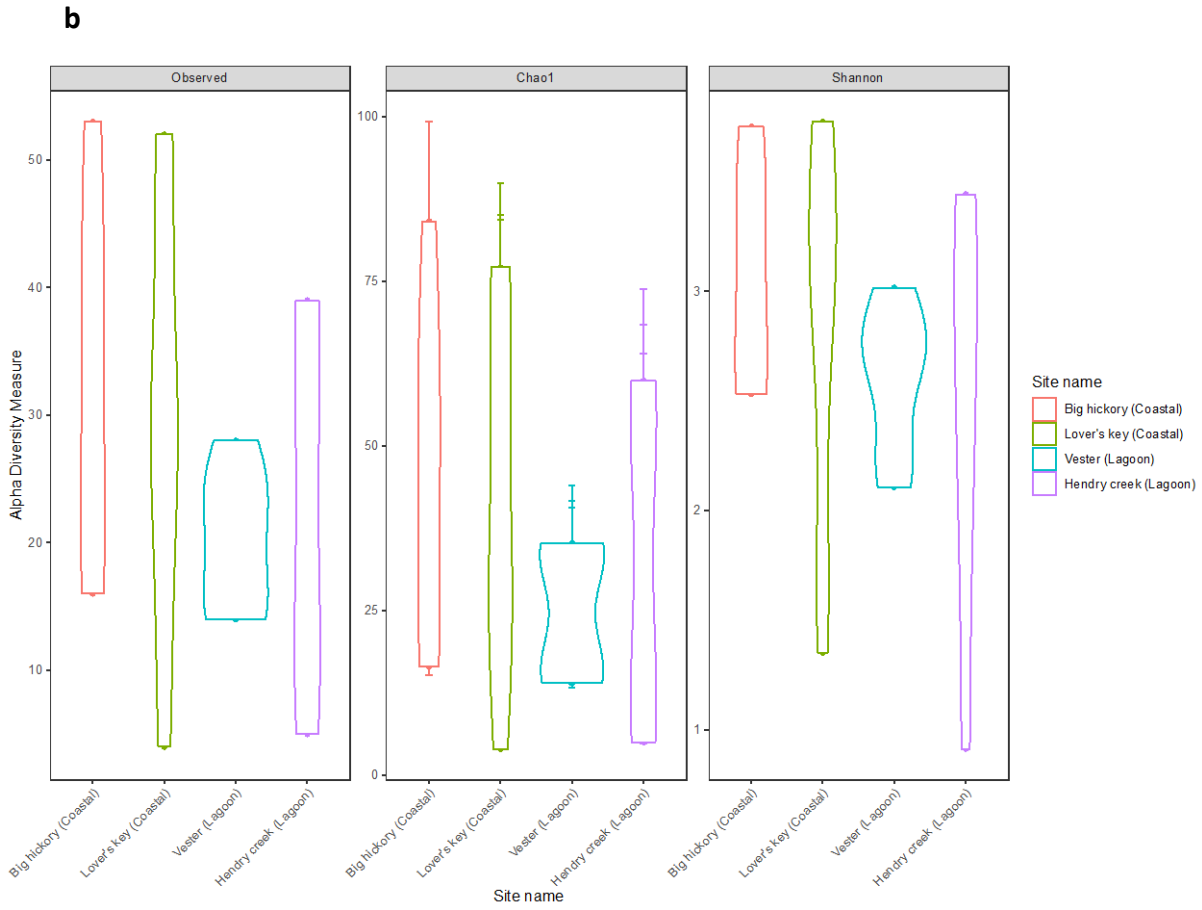


Figure 3.8. 18S V4 region of the nuclear small subunit ribosomal DNA ASVs from major soil biota groups from each sampling location. a) Stacked bar plot reflecting proportional abundance b) violin plots depicting alpha diversity estimates.

Water samples from coastal and lagoon sites differed significantly in beta diversity (df = 1, F = 2.4, p-value = 0.01698). Coastal sites were abundant in Ochrophyta whereas lagoon sites were dominated in Chlorophyta (green algae) (Figure 3.9).

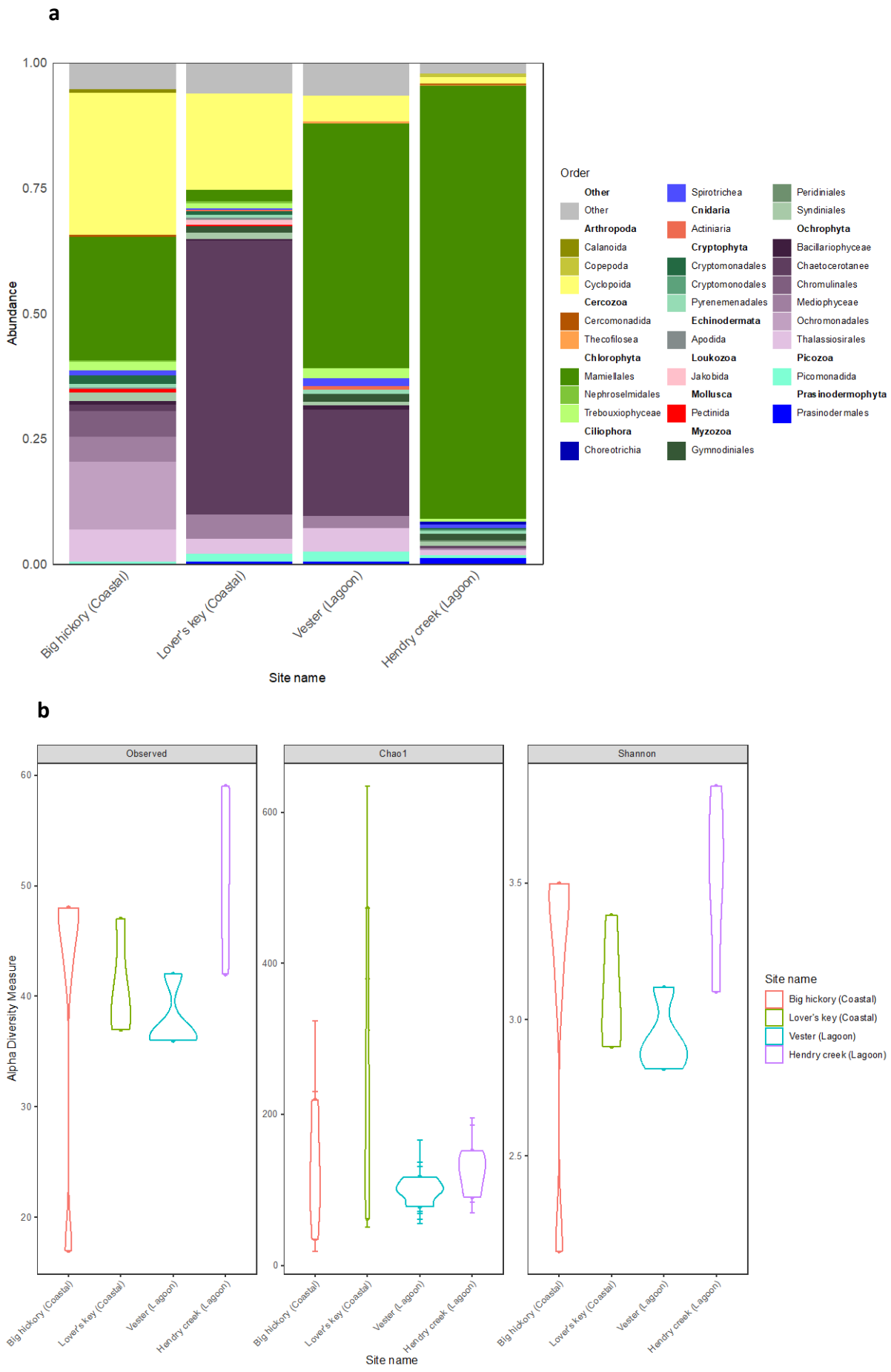


Figure 3.9. 18S V4 region of the nuclear small subunit ribosomal DNA ASVs from major water biota groups from each sampling location. a) Stacked bar plot reflecting proportional abundance b) violin plots depicting alpha diversity estimates.

PERMANOVA analysis on stomach content diversity resulted in significant differences between species (Df=12, F=1.9542 p=0.001) (Figure 3.10), however no difference was observed when a pairwise post hoc analysis with Bonferroni correction was applied. When the dataset was analysed against trophic levels 3 (n=109) (secondary consumers) and 4 (n=20) (tertiary consumers) (trophic groups based on ^{15}N ratios from previous chapter), PERMANOVA resulted in a significant difference (Df=1, F = 1.967, p-value = 0.002997). PERMANOVA was repeated on a subset of trophic level 3 (n=20) individuals, that still resulted in a significant difference between trophic levels 3 and 4 (Df =1, F= 1.5368, p-value = 0.025). The beta diversity differences observed between trophic levels 3 and 4 were concordant with the COI marker results, as stated above.

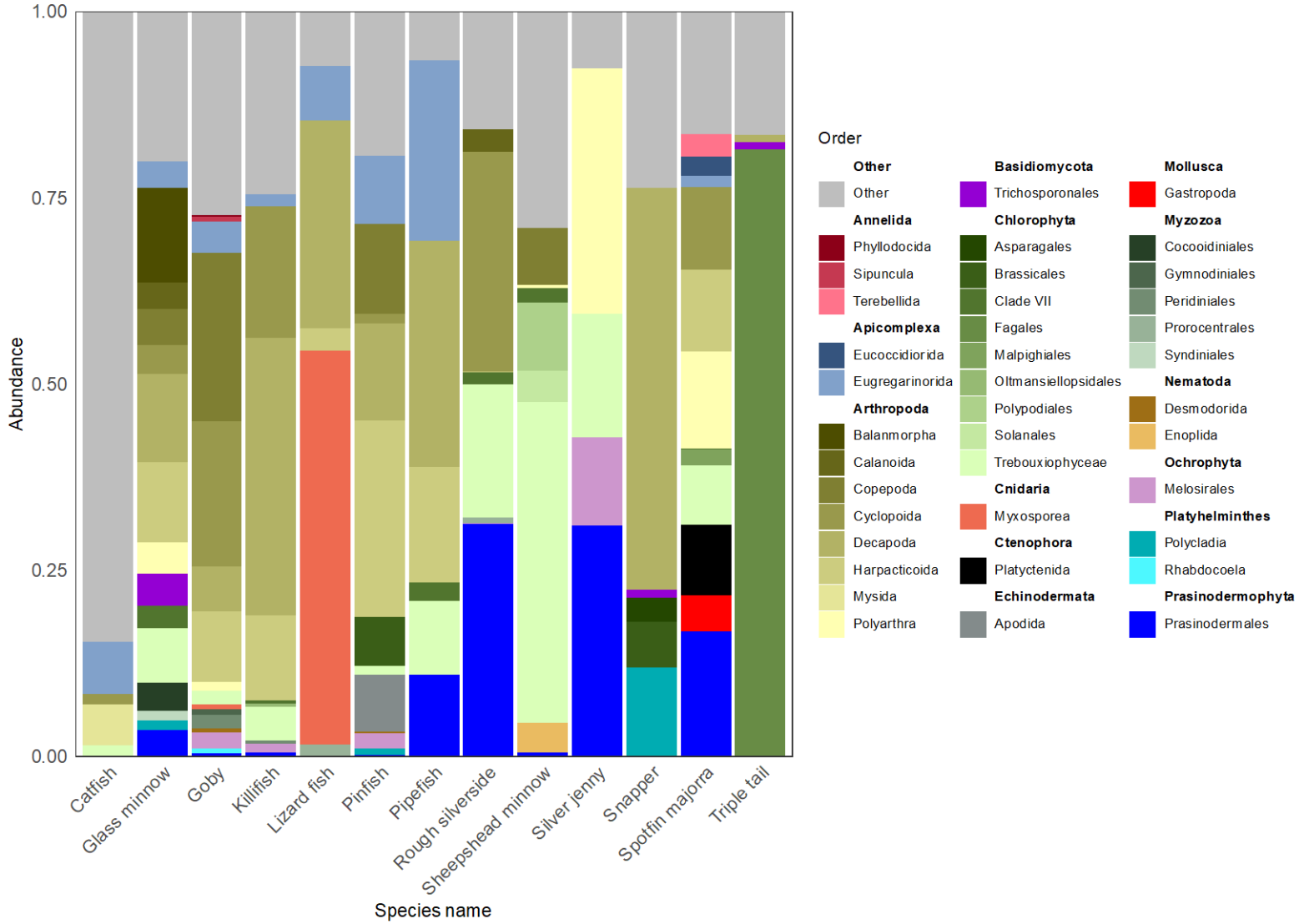


Figure 3.10. Proportional abundance of 18S V4 region of the nuclear small subunit ribosomal DNA ASVs from fish stomach contents.

Non-metric multidimensional scaling (nMDS) was performed to visualise if multivariate grouping of dietary composition within and between species was present. An initial nMDS plot (Figure 3.11A) showed that majority of the points were clustered from -2 to 2 and -2 to 1.5 along NMDS1 and NMDS2 axes respectively. Two conspicuous outlier groups were present skewing the results observed. An additional nMDS was performed with the outliers removed (Figure 3.11B) and grouping within pinfish, rough silversides and spotfin mojarra were observed.

In the previous chapter, the $\delta^{13}C$ values reflected distinct grouping within coastal and lagoon individuals. To investigate if this grouping was present in the metabarcoding data, intraspecific species comparisons were performed on specific species that occurred in both coastal and lagoon sites. Intraspecific comparisons of glass minnows resulted in significant differences in beta diversity of individuals from Big hickory (coastal) and Lover's key (coastal) (df = 1, F= 2.4492, p-value = 0.01798) and Lover's key (coastal) and Hendry creek (lagoon) (df = 1, F= 2.8553, p-value = 0.005994). Diet contents of glass minnows from Lover's key was dominated by Balanmorpha (12%, barnacles), Decapoda (8%) and Copepoda (5%), whereas Big hickory individuals were abundant in Decapoda (14%), Harpicticoida (12%, copepod) and Balanmorpha (11%) similar to Hendry creek that contained Harpicticoida (11%), Balanmorpha (8%) and Trebouxiophyceae (8%, green algae) (Figure 3.13).

PERMANOVA performed on gobies from Hendry creek (lagoon), Lover's key (coastal) and Vester (lagoon) yielded significant differences (df=2, F= 3.2285, p-value=0.001). Post-hoc pairwise analyses also reflected differences between all sites (p-value < 0.01). Diet from all three sites was dominated by arthropods, but differences arose in the proportion, Hendry creek and Vester species only had 24% arthropods whereas Lover's key contained almost twice as much at 43% (Figure 3.13). Differences within the lagoon sites were observed, where individuals from Hendry creek were abundant in Ochrophyta whereas specimens from Vester were dominated by Chlorophyta.

Despite the presence of grouping along the NMDS1 scale, beta diversity of stomach contents from rough silversides and spotfin mojarras between Lover's key (coastal) and Vester (lagoon) were significantly different (df=1, F=6.4444, p-value<0.001, df=1, F=3.896887, p-value= 0.011988). Diet of rough silversides from Lovers' key were dominant in Clycopoida (34%) and Trebouxiophyceae (14%) whereas individuals from Vester were abundant in Prasinodermales

(49%, microalgae). Spotfin mojarras from Lovers' key were observed to be abundant in Platyctenida (29%, comb jelly) and Harpacticoida (21%), conversely to Vester individuals that contained Prasinodermales (30%) and Cyclopoida (20%) (Figure 3.13).

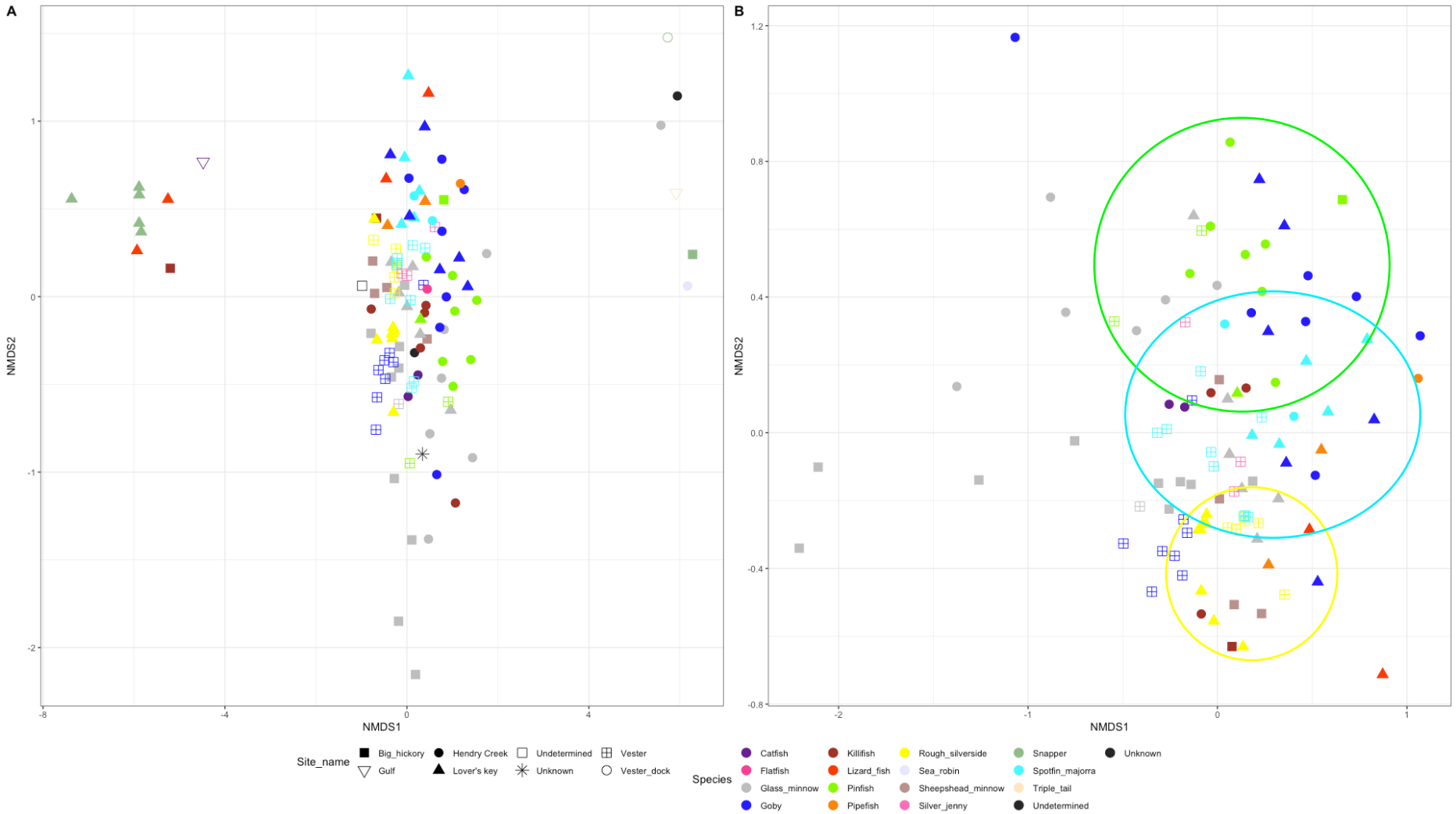


Figure 3.11. Non-metric multidimensional scaling (nMDS) plot of stomach contents from proportional abundance of 18S V4 region of the nuclear small subunit ribosomal DNA ASVs. **A**) contains group of outliers that skew the results for a large proportion of the data. **B**) nMDS was repeated with outliers removed and ellipses represent grouping of species.

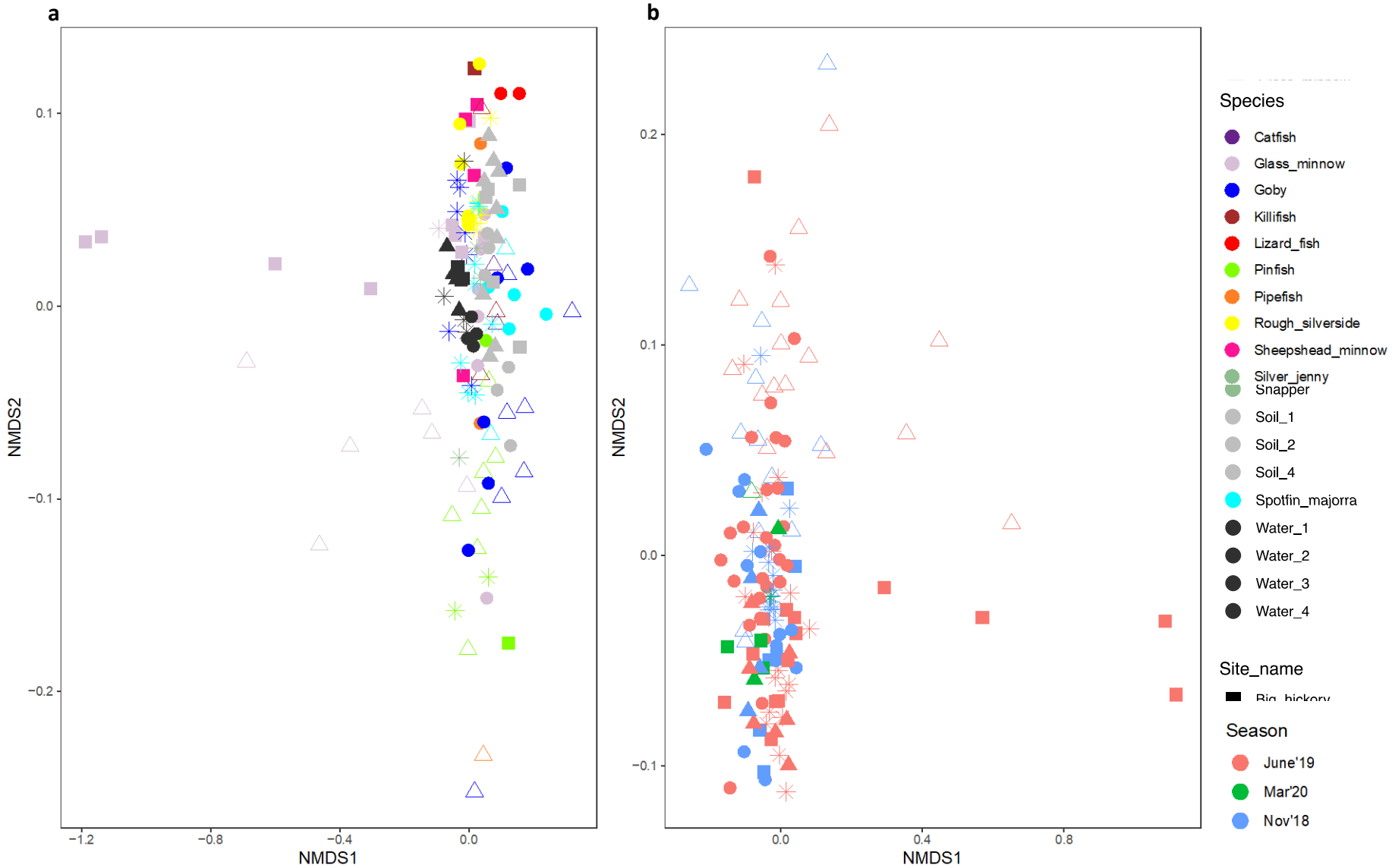


Figure 3.12. Non-metric multidimensional scaling (nMDS) plot of stomach contents from proportional abundance of 18S V4 region of the nuclear small subunit ribosomal DNA ASVs The figures represent how sediment, water, and fish diets change across sampling a) location and b) time. Outliers are present due to combination of environmental samples included in the analysis.

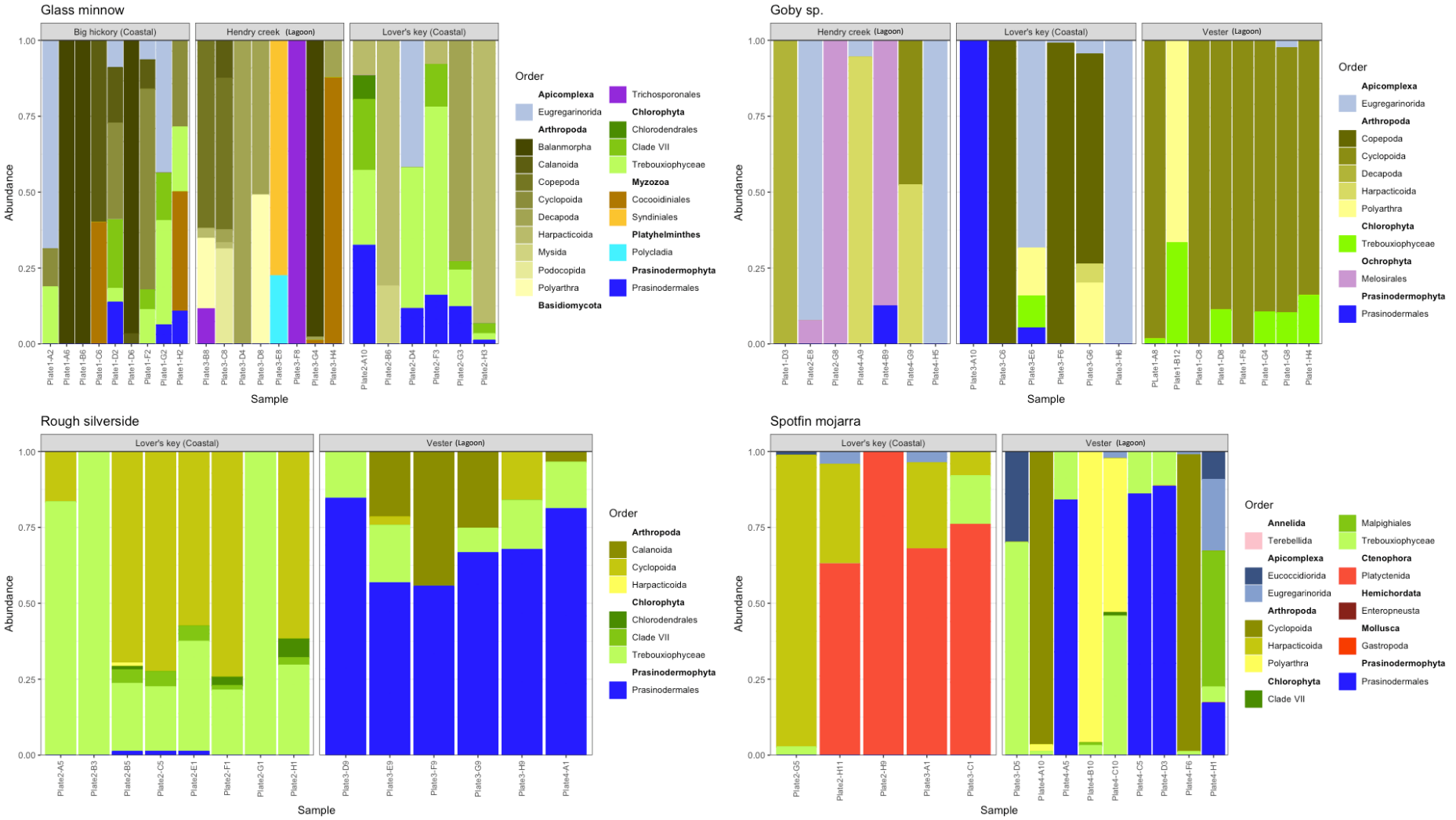


Figure 3.13. Comparisons of proportional abundance of 18S V4 region of the nuclear small subunit ribosomal DNA ASVs from stomach contents between different sampling locations.

3.4.3.1 18S marker - network analysis

Similar to the results obtained from the COI dataset, the networks constructed using 18S metabarcoding data, had sampling completeness values below the 80% threshold. The observed web (Figure 3.14) and null web showed differences in nestedness and extinction slope (Table 3.3) indicating that the observed web may not be reflecting all possible interactions and is incomplete.

When comparing between seasons, the results reflected higher values for nestedness and extinction slope for the wet season network. Similar results were observed between the coastal and lagoon networks, where coastal network had higher values for nestedness and extinction slope. Higher values of nestedness suggests increased presence of specialisation and higher extinction slopes demonstrate that the fish are less vulnerability to extinction events. Results from Big Hickory were much lower in nestedness, extinction slope and robustness compared to the other three sampling locations, suggesting that species from Big Hickory possess generalised diets but are less resilient towards species extinction and environmental perturbation, contrary to theoretical suggestions.

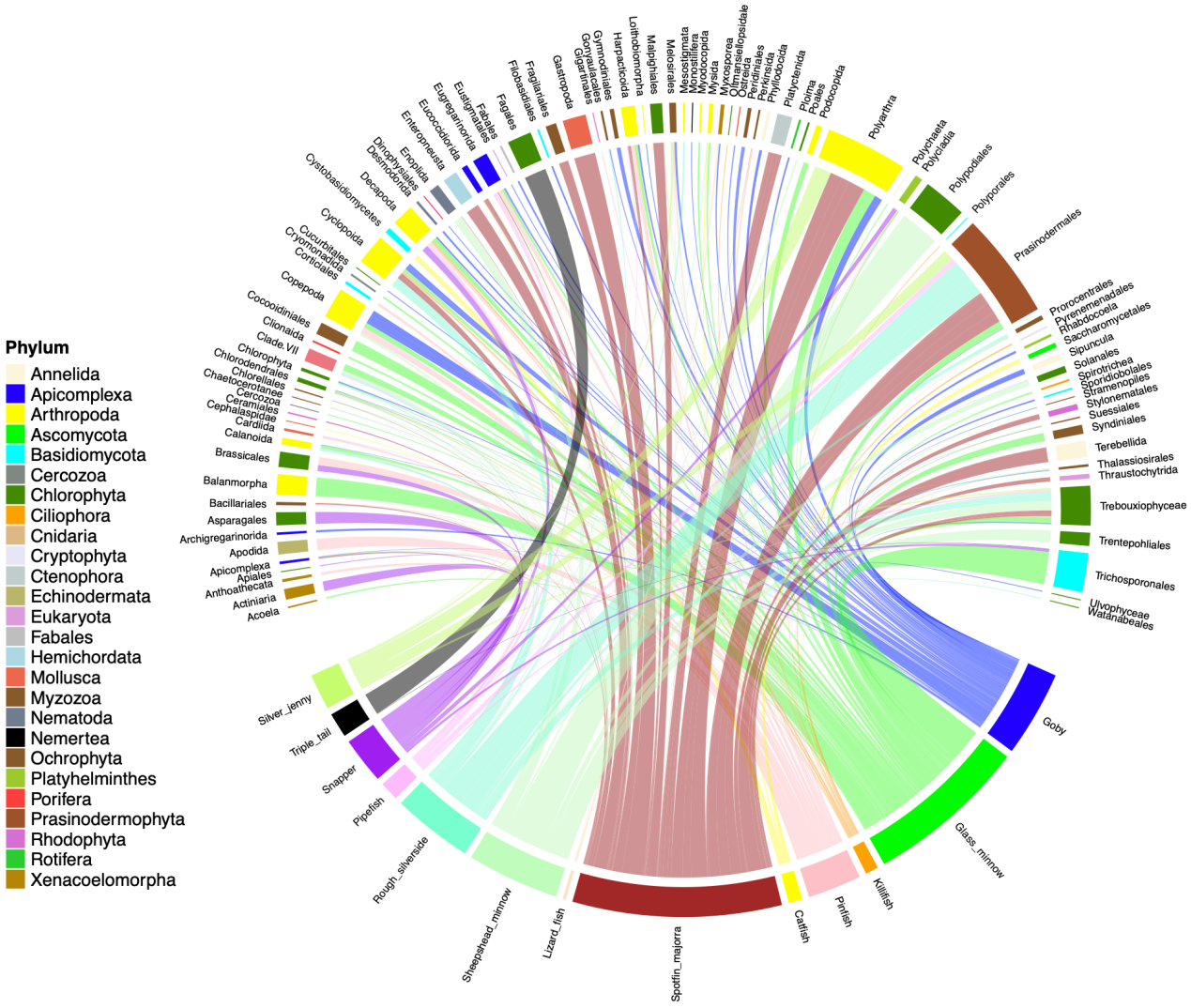


Figure 3.14. Qualitative bipartite plot showing interactions between predatory fish and its consumed prey using binary data from metabarcoding analysis of 18S V4 region of the nuclear small subunit ribosomal DNA. Prey items presented are at order level and are coloured corresponding to its phylum grouping.

Table 3.3. Network index comparisons between food web structures using binary data from metabarcoding analysis of 18S ribosomal DNA gene region. No. of nodes – number of nodes, nodes indicate points of interactions, HL- higher trophic level, LL – lower trophic level. Data provided here is for comparisons between networks. **A**: observed vs Null, **B**: seasonal networks – wet vs dry, **C**: habitat networks – coastal vs lagoon, **D**: habitat composition networks, coastal mangrove vs coastal seagrass vs lagoon mangrove, vs lagoon seagrass.

A	**Observed (48.9%)**	**Null**
No. of nodes HL	13	13
No. of nodes LL	89	89
Nestedness	35.235	19.353
Extinction slope HL	1.807	2.340
Extinction slope LL	12.898	18.189
Robustness HL	0.566	0.620
Robustness LL	0.906	0.936
B	**Wet (61.5%)**	**Dry (40.1%)**
No. of nodes HL	8	9
No. of nodes LL	65	58
Nestedness	44.892	28.341
Extinction slope HL	1.799	1.544
Extinction slope LL	7.100	10.860
Robustness HL	0.519	0.500
Robustness LL	0.841	0.891
C	**Coastal (38.0%)**	**Lagoon (60.0%)**
No. of nodes HL	10	12
No. of nodes LL	63	59
Nestedness	30.233	39.263
Extinction slope HL	1.476	1.875
Extinction slope LL	9.925	6.334
Robustness HL	0.498	0.568
Robustness LL	0.886	0.818

D	**BH (36.8%)** ***Coastal Mangrove***	**LK (39.6%)** ***Coastal Seagrass***	**V (64.6%)** ***Lagoon Mangrove***	**HC (41.8%)** ***Lagoon Seagrass***
No. of nodes HL	6	8	7	8
No. of nodes LL	38	45	35	48
Nestedness	17.630	32.431	36.141	29.347
No. of compartments	2	1	2	1
Extinction slope HL	1.152	1.585	1.628	1.520
Extinction slope LL	4.272	8.634	7.195	7.928
Robustness HL	0.385	0.494	0.481	0.483
Robustness LL	0.766	0.867	0.838	0.837

3.5 Discussion

To understand the feeding interactions underpinning lower trophic network structure in subtropical mangrove fish, we used DNA metabarcoding of stomach contents primarily from the intermediate trophic level of fish to construct ecological networks. DNA metabarcoding offered species level dietary characterisation of prey taxa and how feeding strategies changed based on season (wet or dry), habitat (coastal or lagoon) and habitat composition (mangrove or seagrass). When DNA metabarcoding from this study was coupled with stable isotope analysis performed in the previous chapter we were able to better elucidate prey composition. Indices computed from ecological networks provided an insight into how seasonal changes, salinity and habitat assemblages influence trophic interactions that consequently affects overall robustness and stability of the networks.

3.5.1 Feeding ecology of mangrove fish

Metabarcoding of stomach contents successfully revealed prey composition of the intermediate trophic level fish sampled and provided taxonomic certainty that was unavailable using SIA techniques alone. Arthropoda was found in many of the species sampled, including glass minnow, goby, killifish, pinfish, pipefish, rough silverside, snapper and spotfin mojarra. Gobies (Gobidae spp.) had mostly consumed Arthropoda (barnacles) and supplemented their diet with Mollusca (specifically oysters), Chordata, Annelida and Ochrophyta, highlighting opportunistic feeding behaviours. However, such opportunistic feeding behaviours were not observed in the stable isotope data that could be attributable to low sample numbers. Comparing between habitats, the data suggest that gobies consumed more oysters (Mollusca) (Figure 3.6) and algae (Figure 3.13) at the Vester site, that could be a result of grazing within the algae growing on oysters for Cyclopoida (as seen in Figure 3.13). Previous research has concluded that macroalgae growing on sessile molluscs such as oysters form vital interactions by providing feeding grounds for copepods (who consume algae), and these in turn are consumed by the gobies from Vester where dense aggregation of oysters were present (Doi et al. 2008). This finding reinforces the capability of dietary metabarcoding to uncover feeding interactions over three trophic levels from just a small number of samples.

Gulf killifish (*Fundulus grandis*) supplemented its mainly arthropod diet with amphipods and decapods, similar to findings stated in Rozas and Lasalle (1990). Killifish stomach contents also showed evidence of Chlorophyta which suggest partial grazing on seagrass habitats. Rozas

and Lasalle (1990) suggest that *Eleocharis spp.* (creeping spike rush) found in Gulf killifish diet was a result of foraging on *Eleocharis* where amphipods are abundant. The majority of the killifish that contained Chlorophyta were collected from Hendry Creek (Figure 3.9), which was dominated by seagrass. A diverse range of amphipod species are typically found in seagrass beds (Navarro-Mayoral et al. 2023). Therefore, the metabarcoding data suggest that killifish forage on seagrass beds for Arthropoda, while inadvertently ingesting seagrass. Conversely, the presence of Chlorophyta could also indicate secondary predation where the diet of amphipod prey is reflected in the stomach contents of the killifish.

The native species sheepshead minnow (*Cyprinodon variegatus*) has been observed to preferentially consume detritus with some algae and arthropods (Shepta et al. 2021). The 18S data reflected that sheepshead minnow primarily consumed Chlorophyta with a single individual shown to consume Arthropoda (Figure 3.10). The Chlorophyta taxa was primarily made up of Trebouxiophyceae (microscopic algae) but also contained orders Solanales and Polypodiales. The order Solanales constitutes flowering plants and Polypodiales comprises ferns. The sheepshead minnow included in this study were from the Big Hickory site (Figure 2.2, Chapter 2), that comprised a small channel surrounded by vegetation. Buttonwood (*Conocarpus erectus*, order : Solanales) and giant leather fern (*Acrostichum danaeifolium*, order: Polypodiales) are native to Estero Bay (Florida Department of Environmental Protection (3) 2015). Buttonwood are commonly found in the transitional zones between mangroves and upland forests (Lopez et al. 2004). Giant leather ferns are abundant within mangrove forests and flooded river areas and is known to take over rapidly where red mangrove plants have been removed (Morton 1965; Mehltreter et al. 2003). Due to their proximity to aquatic environments, it is possible that decomposed material from Solanales and Polypodiales could have been washed into the channel where sampling took place. This can explain the presence of Solanales and Polypodiales found in sheepshead minnow stomach contents especially since these fish have shown preferential consumption of detritus (Shepta et al. 2021).

Gulf pipefish (*Syngnathus scovelli*) predominantly consumed Arthropoda (Amphipoda, Sessiilia, Decapoda and Harpiticoida) with some Chlorophyta (Trebouxiophyceae) observed, consistent with previously recorded morphological identification of stomach contents (Motta et al. 1995). Unfortunately, Gulf pipefish populations have been predicted to decline due to reduction in seagrass and algal beds (Rydene and Matheson 2003). Time-series data produced by the Fisheries Independent Monitoring programme (FIM) reflects a slight decline in gulf

pipefish population in Florida since 2000 (Matheson et al. 2008). The FIM dataset includes Charlotte Habour, in which Estero Bay is located. Consequently, continued loss of seagrass habitats and associated prey communities may result in further decreases of *Syngnathus* populations (Adams et al. 2022).

The pelagic rough silversides (*Membras martinica)* have been observed to have a narrow feeding pattern and specifically consume small copepods about 1mm in length but when larger copepods become seasonally available, those are additionally exploited (Allen et al. 1995). Metabarcoding data from this study reflected similar results where COI data comprised of Arthropoda (Calanoida – zooplankton) and Mollusca (Ostreida – oysters and Mytiloida – mussels – possibly eggs/larvae) while the 18S data showed presence of Arthropoda (Cyclopoida and copepoda – zooplankton), Chlorophyta (Trebouxiphyceae – microalgae) and Prasinodermophyta (Algae). As rough silversides are pelagic filter feeders it is likely that they consume microalgae such as Trebouxiphyceae and Prasinodermophyta, while filtering water for resources.

Spotfin mojarra (*Eucinostomus argenteus*) and silver jenny (*Eucinostomus gula)* from the Gerridae family are closely related species and co-occur in estuarine habitats and in this study were found to co-exist in the Vester site. Vasconcellos et al. (2018) used morphological diet analysis to investigate dietary shifts during ontogenetic development of these Gerridae species and identified mechanisms that enables coexistence. The study concluded that a small degree of resource partitioning is present between these species as spotfin mojarras preferred Bivalvia siphons (appendage of bivalve) over polychaetes while the opposite was true for silver jennies. In addition, Vasconcellos et al. (2018) found low levels of algae consumption by the silver jenny species only. The results from this study indicated that spotfin majorras consumed Arthropods and Ctenophore to a large degree, with some Annelids and Chlorophyta. Stomach contents of silver jennies consisted Arthropoda (Polyarthra – micro crustaceans and rotifers), Chlorophyta (Trebouxiophyceae) and Prasinodermophyta. However, unlike the previous study, the data did not reflect presence of distinct resource partitioning. This is primarily attributed to the low sample numbers of silver jenny (3) compared to spotfin mojarras (18) and the lack of silver jenny samples limits our capacity to make clear interpretations of resource partitioning from the metabarcoding data.

The majority of glass minnow diet was made up of arthropods with some reads from Chordata, Chlorophyta and Cnidaria. We expected the abundance of fish to be greater as indicated from the high $\delta^{15}N$ isotope data, where it was suspected that glass minnows were consuming ichthyoplankton. However, based on the dietary metabarcoding data, arthropods comprising Copepoda, Balanmorpha and Decapoda were consumed at higher abundances. Individuals from Lover's key and Hendry creek sites (both dominated by seagrass) supplemented the predominantly arthropod diet with some Chordata and Mollusca (Figure 3.5). The most abundant fish diet species found in glass minnows from these sites were *Syngnathids* (seahorse and pipefish) and *Gerridae* (mojarras) that have known to be abundant in seagrass habitats (Olney and Boehlert 1988; Tolan et al. 1997). As mentioned above metabarcoding only provides snapshot of prey ingested, unlike stable isotope analysis that provides long-term feeding behaviour and assimilation data (Maloy et al. 2013). However, the presence *Syngnathids* and *Eusinostomus* observed in the metabarcoding data confirms the suggestion that glass minnows do consume higher trophic level fish species to some degree.

Pinfish (*Lagodon rhombiodes*) are usually omnivores with smaller individuals consuming plankton and crustaceans while larger individuals consume macrocrustaceans, annelids and macrophytes (Canto-Maza and Vega-Cendejas 2008). Pinfish specimens collected in this study were small (35-90 mm) and their diet consisted of primarily zooplankton such as Amphipoda, Calanoida, Harpiticoida and Cyclopoida (Figures 3.3 and 3.10). Due to the size restriction of the fish collected, its omnivorous nature was not captured in this dietary metabarcoding study. Morphological identification of prey composition conducted by Stoner (1980) stated that pinfish of sizes 36-80 mm (similar to pinfish from this study) consisted of 30% plant material, mainly micro epiphytes. However, the specimens from this study reflected feeding preferences of pinfish belonging to size ranges 16 – 35 mm (Stoner 1980).

Stomach contents of predatory fishes; snapper (*Lutjanus griseus*), lizard fish (*Synodus foetens*) and hardhead catfish (*Ariopsis felis*) were predominately abundant in Chordata and Arthropoda. All three fish consumed benthic dwelling Gobiiformes (Goby spp.). Diets of the economically important snapper reflected a high abundance of Arthropoda, specifically Decapoda (crabs and shrimp), consistent with previous morphological analyses of prey composition (Franks and VanderKooy 2000; Yeager et al. 2014). Yeager et al. (2014) concluded that the diet of snappers shifts significantly across the estuarine gradient, where snappers from upstream habitats were more reliant on intertidal prey whereas downstream snapper diets were

more reflective of marine based food webs, feeding on soft-bottom habitats. However, differences between lagoon and coastal dwelling snappers were not observed in this study, as only juvenile snappers (length ranging from 35 – 78 mm, total length) from a single habitat, Lover's Key, were included in this study.

Unlike the snapper, lizardfish do not have commercial importance and thus, are usually discarded when caught in shrimp trawls (Jeffers et al. 2008). However, they do have important ecological value as they interact with or consume commercially and ecologically important species (Cruz-Escalona et al. 2005). From this study we observed that the juvenile lizard fish (40 - 100 mm, total length) stomach contents were abundant in Gobiiformes followed by shrimp (Hippolytidae). Cruz-Escalona et al. (2005) explain that ontogenetic shifts in lizardfish diet is observed and as they grow larger their prey species and prey area increase in size. This makes them an apex predator and consequently have an influence on the mangrove food web to a greater extent.

The hardhead catfish is a common fish found in the Gulf of Mexico and are considered to be opportunistic feeders that feed on detritus, crustaceans and other fish (Lee 1980). Our dietary metabarcoding data reflects this observation to some degree, however it also reflects that catfish consumed Prasinodermophyta, Chlorophyta, Myzozoa and Apicomplexa. It is likely that these microorganisms were consumed while foraging in the detritus or reflect the parasitic loads of the consumed prey. Pensinger et al. (2021) suggests that there is no ontogenetic shifts in diet within this species as nitrogen isotope signatures did not reflect a linear relationship with total length of fish. Therefore, catfish are able to compete for resources against other estuarine fish, subsequently having an effect on more commercially important fish species.

3.5.2 Overall observation of the feeding ecology

The metabarcoding data performed in this study uncovered feeding interactions over multiple trophic levels. Lower trophic level species such as sheepshead minnow, pipefish, killifish, goby, silver jenny and spotfin mojarra reflected high levels of dietary overlap as they consisted of Arthropods (mainly zooplankton) shrimp, crab and barnacle. However, some levels of trophic partitioning was evident that enables them to co-exist. Trophic partitioning is also seen between gobies, killifish and sheepshead minnow where they supplement their arthropod diets with other organisms. Gobies feed on Annelida and Ochrophyta, killifish on Chlorophyta in

seagrass beds and sheepshead minnow on detritus. Such feeding behaviours are described as omnivory where organisms feed on multiple trophic levels (Kratina et al, 2012). Similar feeding behaviours were also observed in the predatory fish where a combination of arthropods, fish and detritus are consumed. Strong omnivorous links have shown to reduce the magnitude of trophic cascades and increases stability in complex food webs (Bascompte et al. 2005; Bruno and O'Connor 2005; Kratina et al. 2012). Omnivory has previously been recorded in estuarine and marine food webs (Hall and Raffaelli 1991; Thompson et al. 2007; Selleslagh et al. 2012). In addition, Lerner et al. (2022) suggests that in coastal ecosystems, the increased presence of zooplankton found in the omnivorous fish is a result of increased phytoplankton production. Based on this theory, we can infer that Estero Bay is a highly productive coastal environment and the presence of omnivory stabilises interactions between species, that could dampen the effects of trophic cascades.

3.5.3 Comparisons between metabarcoding data and SIA at resolving prey composition

In the previous chapter a large overlap was observed along the $\delta^{13}C$ carbon axis at approximately -20. The species included in this overlap were glass minnow, lizard fish, snapper, spotfin mojarra, killifish and pinfish. The observed overlap was speculated to be due to the consumption of similar basal organisms or a reflection of the mean $\delta^{13}C$ values across multiple food webs (Abrantes and Sheaves 2009). A shortcoming of SIA is its inability to provide detailed prey composition data without prior knowledge of isotopic signatures of prey components (Hoenig et al. 2022). Metabarcoding analysis revealed that these fish had a shared diet comprising juvenile Decapoda (crustaceans), Cyclopoida (zooplankton), Calanoida (zooplankton) and Balanamorpha (barnacle). Juvenile crab and shrimp feed primarily on plankton (Dittel et al. 2006; Abrantes and Sheaves 2009) and these Cyclopida and Calanoida zooplankton prey consume a variety of organisms such as diatoms, algae, rotifers and nauplii (Vega 1999; Dhont and Dierckens 2013). Balanamorpha are omnivorous and consume suspended planktonic material (Newman and Abbott 1980). As the majority of the fish sampled were from lagoon sampling locations and consumed prey within the same food web, we can conclude the overlap observed is due to consumption of organisms that have derived energy from a mixture of lagoon algae and phytoplankton.

Data from the stable isotope analysis revealed overlap in $\delta^{13}C$ carbon signatures within lagoon and coastal locations indicating presence of shared resources within each location. Both COI

(Figure 3.2) and 18S (Figure 3.9) markers of water samples revealed significant differences in beta diversity between lagoon and coastal locations. However, the prey contents of only some species such as rough silverside and spotfin mojarra reflected differences between lagoon and coastal locations (Figure 3.13). Other species such as gobies (Figure 3.6) and glass minnows (Figure 3.13) displayed dietary differences within lagoon and coastal locations. On closer inspection the dietary data did not always correspond to the environmental data. In the absence of comparative studies in the estuarine and marine ecosystem, a dietary study performed in companion with eDNA demonstrated that some plant families amplified from rodent scats were not present in the soil samples (Lopes et al, 2020). They concluded that the combined effects of small sampling effort and stringent sequence filtering protocol led to the risk of not recovering true interactions. Studies should be mindful of the home ranges of different species. Benthic species such as gobies and blennies are known to have small home ranges or territories (<5 m) (Harding et al. 2020) while larger pelagic species like grey snapper are known to migrate and forage among waters across salinity gradients (Serafy et al. 2003; Serrano et al. 2010). Furthermore, presence of diel variability seen in glass minnows that are active during the night and silversides that are active during the day (Castillo-Rivera et al. 2010) can under represent respective diets due to improper sampling. Therefore, greater environmental sampling effort that covers a larger area will provide accurate estimates of the biodiversity present in each location (Lopes et al. 2020). In addition, metabarcoding data provides only a single snapshot of stomach contents and to capture the full dietary breath, they have to be sampled over a wide range of space and time (Casey et al. 2019), though SIA data has been proven to provide this long-term dietary information successfully. The combination of SIA and dietary metabarcoding has characterised fish diet in greater detail compared to if these methods were employed independently.

3.5.4 Network analysis

The high taxonomic resolution provided by DNA metabarcoding enabled construction of ecological networks to investigate how salinity, season and habitat composition affected network structure, thereby modelling the implications of future ecological change. Sampling completeness was used to measure if the interactions in the network had been sampled sufficiently. Network completeness is affected by sampling effort, as the chances of identifying new and rare species increases as effort increases (Henriksen et al. 2019) and poor sampling completeness can lead to the underestimation of network metrics and represent a biased

network structure (Costa et al. 2016). However, achieving high sampling completeness for large networks is unrealistic and is often lower than 60% (Chacoff et al. 2012). Only networks that are species poor or highly specialised reflect high sampling completeness (Costa et al. 2016). In this study, sampling completeness of more than 60%, with a reduced number of nodes, was observed for lagoon habitats (Table 3.2), suggesting that lagoon habitats are species poor or are comprised of highly specialised networks. Though all sampling locations were sampled using the same technique with even sampling effort, coastal networks had lower sampling completeness (38% – 39%), suggesting that marine coastal networks are large. Several published studies have shown that species diversity and richness changes as habitats transform along the freshwater, estuarine, marine continuum (Martino and Able 2003; Whitfield 2015). Species diversity tend to peak in marine habitats followed by estuarine and tidal freshwater zones (Breine et al. 2011) and in this study, the difference in number of species between coastal and lagoon habitats is reflected in the number of lower level nodes (Table 3.2C and 3.2D). Since species richness is the greatest in marine sites, sampling effort for coastal/marine habitats must be increased to capture interactions accurately. The use of molecular approaches have been acknowledged to construct more taxonomically comprehensive networks compared to traditional observed datasets, despite technical biases associated with PCR amplification (Bennett et al. 2019; Cuff et al. 2022). To date, insect-pollination networks using molecular data have been studied extensively (Falcão et al. 2016) while research on molecular mangrove networks are poor. Since mangrove networks have been understudied, the lack of sampling completeness observed in this study should not invalidate the results of this research.

3.5.5 Comparisons between observed and null models

The observed and null model generated by COI data were highly similar indicating that the observed web reflects true predator-prey interactions. Conversely, the observed model deviated substantially from the computed null model for the 18S dataset. The observed model for 18S had extinction slope values lower than the null model and was more nested. Reduced extinction slope value suggests that species are more vulnerable to extinction, possibly due to increased specialisation that reduces stability and overall robustness to extinction events (Dormann et al. 2009). However, as null models reflect random interactions, they tend to overestimate extinction slopes as seen in the null model value here (Dormann et al. 2009). Similar theory can be applied to explain the higher nested value in the observed web, where specialised

interactions have not been accounted for in the null model (computed via random interactions) resulting in lower nestedness values (Nielsen and Bascompte 2007). In addition, because the 18S marker has the ability to capture a larger range of diversity compared to the COI marker, it can cause the null model interactions to be skewed.

3.5.6 Differences between lagoon and coastal networks

The most obvious difference between lagoon and coastal networks is the lower trophic level extinction slope values (Tables 3.2 and 3.3). Extinction slope values show how affected the network is when species in another trophic level is lost (Dormann et al. 2009). A higher extinction slope value observed in the coastal network for both COI and 18S dataset suggest that the fish from coastal systems are more resistant to extinctions or environmental perturbations. Networks that are species rich per functional group are more resistant to secondary species loss (Borrvall et al. 2000). The number of species (nodes at lower level) for the coastal networks is higher than for lagoon networks and therefore they are predicted to be more resistant to secondary extinctions (Tables 3.2 and 3.3). Dunne et al. (2002) concludes that the stability of a food web increases with increasing number of interactions as it dampens the effects of population fluctuations. Furthermore, as discussed above, a large proportion of the fish included in this study display omnivorous feeding behaviours and omnivory is known to increase resilience towards secondary extinctions (Borrvall et al. 2000). Albouy et al. (2019) also suggested that robustness of coastal networks can be attributed to greater interaction redundancy, that enables interactions in coastal networks to be replaced by other species within functional groups. Therefore, due to a combination of species richness and omnivory, coastal networks are expected to be more resilient to secondary extinction than lagoon networks.

3.5.7 Inconsistencies in intraspecific comparisons

Stable isotope analysis performed in the previous chapter demonstrated distinct differences in carbon contribution between coastal and lagoon sampling sites. However, this difference was only observed in specific species such as glass minnows, rough silversides, goby and spotfin mojarra (Figures 3.6 and 3.13). Prey composition of the dietary differences observed within similar species is a reflection of resource availability in specific habitats, this is observed in the presence of Ctenephora species in spotfin mojarra gut contents (Figure 3.13), that also featured in the soil dataset (Figure 3.8). However, the high abundance of Prasinodermophyta in rough silversides from Vester and Trebouxiphyceae from Lovers' key is not reflected in similar abundances in soil or water samples. Therefore the contribution of marine or freshwater algae

is not pronounced in the metabarcoding datasets. By focussing environmental sampling on phytoplankton community, we would be able to identify the coastal and lagoon contributions to fish diet as phytoplankton species are strongly influenced by salinity as changes in phytoplankton community have been observed along the freshwater, estuarine and marine gradient (Muylaert et al. 2009). Typically, the lower estuary is highly saline as waters experience high exchange of marine water that is dominated by marine diatoms while lagoon estuarine wetlands are abundant in a mixture of diatoms and blue-green algae (Roshith et al. 2018; Bharathi et al. 2022).

The aggregate COI dataset from this study showed that the genus *Micromonas* (phylum Mamiellas, green algae) was dominant in Big Hickory and Lover's key coastal habitats while the 18S dataset presented an overlap in algae and diatom; the sites Big Hickory, Vester and Hendry creek were abundant in the genus *Ostrecoccus* (Phylum Mamiellales, unicellular green algae) while Lover's key was dominant in the diatom *Chaetoceros spp.* and this species was the second most dominant at Vester site. Since the primers used in this study were not specifically targeting phytoplankton communities, they were not efficient at characterising phytoplankton diversity. To characterise phytoplankton and primary producer communities to a finer scale, primers optimised to recover phytoplankton DNA (Cavalier-Smith et al. 2009; Bråte et al. 2010; Herlemann et al. 2011) should be utilised in future studies. However, because stable isotope analysis was performed in the previous chapter that reflected long term dietary assimilation data, we are still able to conclude that there was a distinct coastal and lagoon influence on the fish. If targeted metabarcoding was used independently from SIA the differences in coastal and lagoon contributions would have been captured effectively.

3.5.8 Differences between wet and dry season networks

Subtropical mangrove habitats such as Estero Bay experience two distinct seasons wet (June to October) and dry (November to March). Species richness in mangrove ecosystems are generally higher in the wet season compared to the dry season due to recruitment of species during the wet season along with an increase in abundance of food sources and shelter (Idelberger and Greenwood 2005; Sheaves et al. 2010). The wet season network for both COI and 18S datasets produced higher nestedness (used to infer the extent to which organisms are generalists) values in contrast to the dry season network, suggesting increased specialisation during the wet season. In a study conducted on juvenile *Pimelodus maculatus* (catfish) from a Brazilian floodplain, it was observed that the catfish was able to shift its diet from generalist

detritus during droughts to specialised benthic fauna and fruit/seeds during the wet season (Da Cunha et al. 2018). Heng et al. (2018) also concluded that during the wet season, competitive pressure reduces, enabling fish in floodplain habitats to have narrow dietary preferences, but when resources are scarce, fish are forced to diversity their diet and become more generalist to reduce competition. In this study we can conclude that the wet season increases resource availability through the recruitment of ichthyoplankton and larvae enabling fish to consume a narrower range of dietary materials consequently displaying more nested behaviour and expanding their range during dry season when resources are scarce resulting in a lower nested value (Tables 3.2 and 3.3). However, increased specialisation during wet season diet can lead to greater susceptibility to extinction when desired prey items are no longer available (Dormann et al. 2009).

3.5.9 Habitat composition influences foraging mechanisms

Networks constructed from the COI dataset reflected that fish species from mangrove habitats were less nested (reflective of generalist feeding behaviours) and contained more compartments compared to seagrass habitats. Some studies have shown that mangrove habitats are higher in fish abundance and diversity compared to seagrass habitats (Kathiresan and Bingham 2001; Harper et al. 2022). Specifically at high tide, fish species and abundance are higher at mangrove sites than seagrass beds, while at low tide when mangrove forests are emergent, fish would move to seagrass beds near the mangroves to stay underwater (Laegdsgaard and Johnson 1995; Jelbart et al. 2007). Since our sampling only took place during high tide, the results (high nestedness and low compartments) suggest that the diet of seagrass dwelling fish is only a subset of the resources found in estuarine habitats. Nagelkerken and van der Velde (2004) observed that the stable isotope data from of juvenile seagrass fish adjacent to mangrove forests indicated little contribution of mangrove food sources to the diet of seagrass fish. This finding indicates that select fish species occupying seagrass beds have more specialised diets compared to mangrove fish who have a generalist feeding pattern. The generalist behaviour of mangrove fish is predicted to be influenced by a higher species assemblage and abundance at mangrove sites enabling the fish to exploit a greater diversity of resources. It is noteworthy to address that the networks constructed in this study were transformed into binary presence/absence data. Cuff et al. (2022) proposed that using frequency of occurrence data, where each detected interaction is represented as equal proportion, will

produce more complete representation of a quantitative network that can be compared against observed networks.

3.5.10 High abundance of protist and harmful algae (*Karina brevis*)

High abundance of Synurophyceae or Ochromonadales specifically from the genus *Poterioochromonas* is seen in both COI and 18S environmental datasets from Big hickory (coastal) and Hendry creek (lagoon) sites (Figures 3.2 and 3.9). *Poterioochromonas* species are described as mixotrophs because they are capable of photosynthesising and undergoing phagotrophy. This protist consumes important phytoplankton dietary sources to fish such as *Chlorella spp.*, *Scenedesmus spp.*, *Nanchloropsis oceania* and *Synechocystis spp.* (Wei et al. 2020). In addition, high abundances of *Poterioochromonas* coupled with environmental variables can inhibit bacterial growth and be responsible for fish and zooplankton mortality as they contain toxic elements (Boenigk and Stadler 2004). Therefore, continued monitoring of *Poterioochromonas* abundances is key to understand its effects on zooplankton and fish populations.

Another mixotrophic algae from the phylum myzozoa (specifically family Kareniaceae, order: Gymnodiniales) was observed in water samples from all sites and in dietary samples of fish from Big hickory and Hendry creek (Figure 3.7 and 3.9). Diet samples of glass minnows from Big Hickory and Hendry Creek consisted of myzozoa species. Since glass minnows in this study have consumed a large proportion of filter feeders such as Copepoda, Balanmorpha and Decapoda, the presence of Myzozoa observed in glass minnows could be attributed to secondary predation. Species *Karina brevis* (family Kareniaceae) is a photosynthetic dinoflagellate found in the Gulf of Mexico and has been responsible for the harmful algae blooms common in the coastal waters of Florida. When *K. brevis* concentrations increase, their brevetoxins have been known to affect vertebrate nervous systems, killing associated marine life. Shellfish can filter out brevetoxins from the seawater but it results in accumulation of toxins in the shellfish tissue, which is known to harm its human consumers (Brand and Compton 2007). Aerosolised *K. brevis* toxins can also cause respiratory issues in humans and marine mammals (Backer et al. 2003). These harmful algal blooms are aggravated by eutrophication (Medina et al. 2022) and rising water temperatures due to climate change (Errera et al. 2014). The first *K. brevis* bloom, also known as red tide, was detected in Estero Bay in 2011 (McFarland et al. 2016) and since has been occurring on an annual basis (Rolton et al.

2015). To date a several studies have been conducted investigating the effects of *K.brevis* on marine life in Estero bay with specific importance to mollusc species (McFarland et al. 2015; Rolton et al. 2015).

The presence of toxins post bloom has shown to increase mortality of benthic feeding fish (Landsberg et al. 2009) and consequently on dolphins that prey on these fish due to accumulation of toxins (Brand and Compton 2007). Management practices have been put in place to prioritise the mitigation of *K. brevis* blooms but its continued presence in Estero Bay poses not only a threat to aquatic life, but also to lagoon fisheries, associated businesses and tourism, resulting in a detrimental impact on the ecosystem and the economy (Hoagland et al. 2020).

3.5.11 Causes of sample loss

An unexpected result from the metabarcoding data was the loss of samples during chimera removal during bioinformatic analysis. Chimeras occur when DNA does not extend completely, and this incomplete fragment acts as a primer resulting in incorrect hybridization with another fragment of DNA and formation of novel sequences that does not exist in the natural environment (Bradley and Hillis 1997). The number of amplification cycles and duration of extension are crucial determiners in the formation of chimeras during PCR (Wang and Wang 1997; Lahr and Katz 2009). Fonseca et al. (2012) have also suggested that phylogenetically diverse and rich datasets can increase chimera formation. In this study, the presence of chimeras was identified post sequencing, and the only outcome was to remove these sequences, reducing the total samples and read numbers. Though the sample number and read numbers were lower than expected, we were still able to use the remaining data to address the questions proposed in this study.

Some samples had low read numbers (<100, as identified in the rarefaction curves) and this could be a result of low concentration of DNA template or PCR product. During the dissection of fish stomachs in the field, some individuals had very little contents while some had none at all, that could have resulted in the low read numbers observed. Sample degradation could be a possible cause for low read numbers and this is influenced by digestion rates that is affected by the predator species, metabolic rates, prey sizes, food types and feeding frequencies (Hilton et al. 1998). Errors caused by differences in digestion rates can be negated when feeding

behaviours of predators are addressed (McInnes et al. 2017). In this study, to minimise errors caused by differences in digestion rates, we only collected samples during high tide, when feeding activity is at its peak in mangrove ecosystems (Sheaves 2005). Similar feeding behaviour is also observed in summer flounders in salt marshes (Rountree and Able 1992), Gulf killifish in Mississippi brackish marsh habitat (Rozas and Lasalle 1990; Sheaves 2009) and European flounder in the Scottish Ythan estuary (Summers 1980). A driver for this behaviour is the rising abundance of resources observed during high tides in estuarine environments (Sheaves 2005). Large samples numbers (at least 20 individuals per species per site) were collected to mitigate discrepancies induced by digestion rates.

3.5.12 Effects of Bonferroni correction on pairwise analysis

Bonferroni correction is commonly applied to reduce Type I errors where the null hypothesis is rejected when in fact it is true (Nakagawa 2004). In this study we found that the application of Bonferroni correction to multiple comparisons in PERMANOVA yielded in non-significant results repeatedly even though there was an overall significance found. Nakagawa (2004) suggested that Bonferroni correction reduces power and is especially exacerbated in situations where power (sample size, significance level, effect size) is already low. Jennions and Møller (2003) propose that effect sizes are reported along with p-values such as Pearson's r (r^2), as it is the proportion of variance explained or R^2 where linear trend is not tested for. In this study, R^2 (Eta-squared) values and sample size of each group is reported along with p-values and F statistics. Eta-square values are commonly produced during ANOVA analysis and can be used as a measure for effect size and are calculated by dividing the sum of squares of a variable by the total number of squares in the model. By Cohen's rule of thumb, small effect size, medium and large effect sizes are 0.01, 0.06 and 0.14. R^2 values presented in this study (Appendix C and D) after PERMANOVA analysis do not exceed more than 5%, falling between the small and medium effect size range and where majority of the variance is not accounted for in the analysis. Therefore, the p-values returned from pairwise PERMANOVA analysis can be indicative of the variations detected, and the lack of significance observed can be attributed to small sample sizes and low power.

3.6 Conclusion

Overall, the metabarcoding results of stomach contents revealed detailed dietary information of intermediate trophic level fish from Estero Bay. Ecological networks have traditionally been

used to predict and mitigate the effects of environmental and human induced change on ecosystem functioning (Ings et al. 2019). The construction of ecological networks from dietary metabarcoding showed that a combination of salinity, season and habitat composition influenced interactions within bipartite food webs providing some clarity to the functioning of a complex estuarine system.

Our results illustrate that the combination of dietary metabarcoding and stable isotope analysis provides complementary data and subsequently insights into interactions not possible with either technique alone. Stable isotope data provided a longer-term perspective of energy flow within food webs and showed distinct separation in carbon sources that was not evident in the metabarcoding analysis. The complimentary metabarcoding data additionally provided a high-resolution snapshot of prey ingested by omnivorous fish and discriminated the overlap observed along the $\delta^{13}C$ axis. We believe the application of multiple techniques will produce the most comprehensive and unbiased understanding of species' diet and presently this has only been demonstrated by a handful of studies (Compson et al. 2019; Bonin et al. 2020; Hoenig et al. 2022).

Appendix C

Results of permutational ANOVA (PERMANOVA) with pairwise comparisons of beta diversity calculated with Bray-Curtis distance matrix for mitochondrial cytochrome oxidase I (COI) marker.

Species	Comparison species	F.Model	R^2	p-value	Bonferroni corrected p-value
Glass minnow (n=22)	Killifish	1.266	0.0501	0.0849	1.0000
	Snapper	2.308	0.0654	0.0010	0.0659
	Goby	1.951	0.0488	0.0010	0.0659
	Pinfish	1.783	0.0544	0.0020	0.1319
	Catfish	2.083	0.0865	0.0020	0.1319
	Spotfin mojarra	1.608	0.0509	0.0060	0.3956
	Lizard fish	1.666	0.0581	0.0120	0.7912
	Sheepshead minnow	2.860	0.0991	0.0010	0.0659
	Flatfish	1.541	0.0628	0.0190	1.0000
	Pipefish	1.215	0.0446	0.1389	1.0000
	Rough silverside	1.665	0.0510	0.0030	0.1978
Killifish (n=9)	Snapper	1.178	0.0728	0.1958	1.0000
	Goby	1.200	0.0566	0.1029	1.0000
	Pinfish	1.103	0.0782	0.1469	1.0000
	Catfish	1.958	0.3286	0.0667	1.0000
	Spotfin mojarra	1.019	0.0782	0.5495	1.0000
	Lizard fish	1.168	0.1148	0.2228	1.0000
	Sheepshead minnow	2.032	0.2026	0.0430	1.0000
	Flatfish	1.258	0.2010	0.1369	1.0000
	Pipefish	1.082	0.1191	0.2148	1.0000
	Rough silverside	1.070	0.0760	0.1518	1.0000
Snapper (n=14)	Goby	1.762	0.0573	0.0010	0.0659
	Pinfish	1.470	0.0626	0.0200	1.0000
	Catfish	1.923	0.1289	0.0330	1.0000
	Spotfin mojarra	1.338	0.0599	0.0410	1.0000
	Lizard fish	1.674	0.0851	0.0070	0.4615
	Sheepshead minnow	2.871	0.1445	0.0010	0.0659
	Flatfish	1.573	0.1010	0.0160	1.0000
	Pipefish	1.360	0.0741	0.0410	1.0000
	Rough silverside	1.683	0.0710	0.0010	0.0659
Goby (n=18)	Pinfish	1.355	0.0478	0.0579	1.0000
	Catfish	2.014	0.1006	0.0170	1.0000
	Spotfin mojarra	1.042	0.0385	0.3467	1.0000
	Lizard fish	1.555	0.0633	0.0040	0.2637
	Sheepshead minnow	2.684	0.1087	0.0010	0.0659
	Flatfish	1.469	0.0718	0.0330	1.0000
	Pipefish	1.173	0.0506	0.1459	1.0000
	Rough silverside	1.090	0.0388	0.2637	1.0000

Pinfish (n=11)	Catfish	2.006	0.1542	0.0140	0.9231
	Spotfin mojarra	1.048	0.0523	0.3776	1.0000
	Lizard fish	1.418	0.0814	0.0020	0.1319
	Sheepshead minnow	2.526	0.1441	0.0010	0.0659
	Flatfish	1.436	0.1069	0.0160	1.0000
	Pipefish	0.949	0.0595	0.6414	1.0000
	Rough silverside	1.305	0.0613	0.0040	0.2637
Catfish (n=3)	Spotfin mojarra	1.908	0.1603	0.0340	1.0000
	Lizard fish	1.671	0.1927	0.0519	1.0000
	Sheepshead minnow	3.366	0.3594	0.0749	1.0000
	Flatfish	2.212	0.4244	0.2000	1.0000
	Pipefish	2.003	0.2503	0.0390	1.0000
	Rough silverside	1.910	0.1480	0.0120	0.7912
Spotfin mojarra (n=10)	Lizard fish	1.314	0.0805	0.0160	1.0000
	Sheepshead minnow	2.342	0.1433	0.0010	0.0659
	Flatfish	1.338	0.1084	0.0809	1.0000
	Pipefish	1.099	0.0728	0.2767	1.0000
	Rough silverside	1.184	0.0586	0.0360	1.0000
Lizard fish (n=10)	Sheepshead minnow	2.431	0.1810	0.0020	0.1319
	Flatfish	1.092	0.1201	0.4046	1.0000
	Pipefish	1.316	0.1069	0.0210	1.0000
	Rough silverside	1.330	0.0768	0.0040	0.2637
Sheepshead minnow (n=6)	Flatfish	2.377	0.2535	0.0519	1.0000
	Pipefish	2.281	0.1857	0.0120	0.7912
	Rough silverside	2.373	0.1366	0.0010	0.0659
Flatfish (n=3)	Pipefish	1.365	0.1632	0.0759	1.0000
	Rough silverside	1.260	0.0950	0.0400	1.0000
Pipefish (n=6)	Rough silverside (n=11)	1.141	0.0707	0.0599	1.0000

Appendix D

Results of permutational ANOVA (PERMANOVA) with pairwise comparisons of beta diversity calculated with Bray-Curtis distance matrix for 18S V4 region of the nuclear small subunit ribosomal DNA marker.

Species	Species comparison	F.Model	R^2	p-value	Bonferroni corrected p-value
Triple tail (n=2)	Spotfin majorra	0.983	0.0518	0.5415	1.0000
	Snapper	1.201	0.1072	0.3516	1.0000
	Silver jenny	1.000	0.2500	1.0000	1.0000
	Sheepshead minnow	1.244	0.1992	0.1518	1.0000
	Rough silverside	1.129	0.0746	0.1249	1.0000
	Pipefish	1.256	0.2389	0.2667	1.0000
	Pinfish	1.180	0.0832	0.2408	1.0000
	Lizard fish	0.986	0.1647	0.6993	1.0000
	Killifish	1.171	0.1433	0.4326	1.0000
	Goby	1.063	0.0482	0.2138	1.0000
	Glass minnow	1.267	0.0501	0.2627	1.0000
	Catfish	1.000	0.2500	1.0000	1.0000
Spotfin majorra (n=18)	Snapper	1.309	0.0479	0.0969	1.0000
	Silver jenny	1.149	0.0570	0.1518	1.0000
	Sheepshead minnow	1.304	0.0585	0.0649	1.0000
	Rough silverside	1.372	0.0437	0.0490	1.0000
	Pipefish	1.492	0.0694	0.0120	0.9351
	Pinfish	1.559	0.0510	0.0120	0.9351
	Lizard fish	1.044	0.0474	0.3596	1.0000
	Killifish	1.660	0.0673	0.0010	0.0779
	Goby	1.518	0.0394	0.0040	0.3117
	Glass minnow	2.677	0.0627	0.0010	0.0779
	Catfish	1.045	0.0521	0.3457	1.0000
Snapper (n=10)	Silver jenny	1.355	0.1097	0.1329	1.0000
	Sheepshead minnow	0.894	0.0643	0.5245	1.0000
	Rough silverside	1.233	0.0531	0.1978	1.0000
	Pipefish	1.548	0.1142	0.1059	1.0000
	Pinfish	1.506	0.0669	0.0809	1.0000
	Lizard fish	0.883	0.0636	0.5784	1.0000
	Killifish	1.942	0.1146	0.0130	1.0000
	Goby	1.974	0.0637	0.0010	0.0779
	Glass minnow	2.553	0.0739	0.0010	0.0779
	Catfish	1.234	0.1008	0.2587	1.0000
Silver jenny (n=3)	Sheepshead minnow	1.530	0.2032	0.0160	1.0000
	Rough silverside	1.142	0.0707	0.1628	1.0000
	Pipefish	1.168	0.1893	0.3337	1.0000
	Pinfish	1.249	0.0819	0.1698	1.0000
	Lizard fish	1.014	0.1445	0.2288	1.0000

	Killifish	1.095	0.1204	0.3706	1.0000
	Goby	1.012	0.0440	0.3956	1.0000
	Glass minnow	1.478	0.0558	0.0759	1.0000
	Catfish	0.854	0.1760	1.0000	1.0000
Sheepshead minnow (n=5)	Rough silverside	1.226	0.0673	0.1818	1.0000
	Pipefish	1.355	0.1622	0.1688	1.0000
	Pinfish	1.158	0.0675	0.2587	1.0000
	Lizard fish	0.994	0.1105	0.4406	1.0000
	Killifish	1.624	0.1397	0.0400	1.0000
	Goby	1.472	0.0578	0.0210	1.0000
	Glass minnow	1.348	0.0476	0.1419	1.0000
	Catfish	1.433	0.1927	0.0150	1.0000
Rough silverside (n=14)	Pipefish	1.122	0.0655	0.2697	1.0000
	Pinfish	1.242	0.0473	0.1878	1.0000
	Lizard fish	0.736	0.0415	0.8242	1.0000
	Killifish	1.551	0.0755	0.0220	1.0000
	Goby	1.479	0.0429	0.0080	0.6234
	Glass minnow	2.437	0.0634	0.0020	0.1558
	Catfish	1.163	0.0719	0.1249	1.0000
Pipefish (n=4)	Pinfish	1.039	0.0648	0.4316	1.0000
	Lizard fish	1.035	0.1288	0.4356	1.0000
	Killifish	1.316	0.1275	0.1309	1.0000
	Goby	0.932	0.0389	0.5884	1.0000
	Glass minnow	1.635	0.0592	0.0340	1.0000
	Catfish	1.220	0.1962	0.3536	1.0000
Pinfish (n=13)	Lizard fish	0.956	0.0564	0.5335	1.0000
	Killifish	1.631	0.0831	0.0250	1.0000
	Goby	1.427	0.0427	0.0460	1.0000
	Glass minnow	2.168	0.0583	0.0060	0.4675
	Catfish	1.198	0.0788	0.1928	1.0000
Lizard fish (n=5)	Killifish	0.818	0.0756	0.7842	1.0000
	Goby	1.069	0.0427	0.2458	1.0000
	Glass minnow	1.418	0.0499	0.1269	1.0000
	Catfish	0.960	0.1379	0.8282	1.0000
Killifish (n=7)	Goby	1.289	0.0472	0.0639	1.0000
	Glass minnow	1.870	0.0606	0.0230	1.0000
	Catfish	1.188	0.1293	0.2458	1.0000
Goby (n=21)	Glass minnow	1.792	0.0400	0.0180	1.0000
	Catfish	1.023	0.0445	0.3566	1.0000
Glass minnow (n=24)	Catfish (n=3)	1.278	0.0486	0.2108	1.0000

Chapter 4: Investigating diet differentiation among sympatric ecomorphs of the cichlid fish *Astatotilapia calliptera* from Lake Masoko (Kisiba), Tanzania.

4.1 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. Cichlids are one of the most ecologically diverse families of freshwater fishes and they vary greatly in ecology, morphology and behaviour. *Astatotilapia calliptera* from crater Lake Masoko in Tanzania has diverged sympatrically into littoral (shallow-water) and benthic (deep-water) ecomorphs. Previous research has indicated that the ecomorphs differ in head and pharyngeal jaw morphology, and carbon stable isotope analysis has shown presence of trophic specialisation. Here, we explored the role of diet and trophic niche divergence in the context of sympatric speciation in *Astatotilapia calliptera* using metabarcoding on stomach contents. A combination of the 18S V4 region from the eukaryotic nuclear small subunit ribosomal DNA and mitochondrial COI region were used to target eukaryotic taxonomic groups and invertebrate diversity respectively, revealing divergent dietary compositions between the ecomorphs. Diets of benthic *A. calliptera* individuals mainly consisted of Nematodes, Bacillariophyta (diatoms) and copepods while the diet of littoral individuals diet comprised molluscs, annelids and fungi. Homologous apicomplexa, arthropods (mainly crustacean zooplankton) and Bacillariophyta taxa were found in both benthic and littoral stomach contents, suggesting that the cichlids are generalist consumers foraging on common resources such as Arthropoda when in high abundance. However, they likely switch to specialised diets such as grazing diatoms and Mollusca when arthropods are scarce. The findings from this study demonstrated the ability of metabarcoding to identify potential drivers of sympatric speciation through trophic niche divergence. As the present study reflected a single time point, future studies should consider the effects of temporal changes in feeding habitats and resource availability in Lake Masoko. Complimentary studies will therefore contribute to understand the full breadth of dietary specialisation seen in *Astatotilapia calliptera* ecomorphs, thereby fully exploring the role of divergent trophic strategies in the ongoing process of sympatric speciation.

4.2 Introduction

Schluter (2000) defines adaptive radiation to be the "evolution of ecological diversity within a rapidly multiplying lineage". He elaborates this further by explaining that it is the differentiation of a single ancestor into a range of different species with features that are adapted better to exploiting ecological opportunities.

Textbook examples of adaptive radiation in vertebrates include Darwin's finches from the Galapagos islands and Anolis lizards from the Caribbean islands. The diversification of *Geospiza* species resulting in a range of species differing in beak size and shape has been attributed to natural selection and introgressive hybridisation. Natural selection occurs when the environment changes and finches with morphology that is best adapted to the environment are at an advantage as they are able to survive when resources are restricted (Almén et al. 2016). Introgressive hybridisation leads to novel alleles, increasing genetic variation, that has the potential to create new phenotypes and species (Grant and Grant 2008). Lizards of the genus *Anolis* have experienced independent variations producing morphologically and behaviourally specialised adaptations in heterogeneous environments (Butler et al. 2007). There are 143 Caribbean species of *Anolis* lizards and they have been thought to have risen from two colonisations from the malagoon, suggesting that diversification occurred within the Caribbean as opposed to multiple colonisations (Jackman et al. 1999). Individuals from the Greater Antilles islands occupy diverse ecological niches and as many as 11 species have been recorded to live in sympatry (Losos 1994). Each island has a short-legged and long-legged species. The short-legged individuals use twigs, whereas the long-legged individuals are typically found on tree-trunks near the ground, but these species are not related to anoles on other islands despite morphological and ecological similarities (Leal et al. 2002). Contrastingly, anoles from the Lesser Antillean islands are either solitary or live in pairs only, exhibiting biogeographic patterns as each island is inhabited by an endemic anole species with limited sympatry (Thorpe et al. 2008). An example of adaptive radiation in plants includes the Columbine flower from the genus *Aquilegia,* a member of the very earliest diverging branch of the eudicots (Hodges and Kramer 2007) and is thought to have undergone rapid radiation due the development of nectar spurs (Hodges and Arnold 1995). There are approximately 70 species of *Aquilegia* that have evolved nectar spurs that differ in length, curvature, orientation and colour to attract different types of pollinators such as bumblebees, hummingbirds and moths (Whittall and Hodges 2007; Kramer 2009). In addition, the length of nectar spur is correlated to male fitness

as it facilitates pollen removal (Fulton and Hodges 1999). The development and variation of floral spurs enables *Aquilegia* species to specialise towards different environments and pollinators (Bastida et al. 2010).

4.2.1 Cichlid fish from African lakes

Cichlid fish have become one of the most iconic model systems for speciation research and, in particular, African lakes are recognised as natural laboratories for studying evolutionary processes (Kocher 2004; Burress 2015). Cichlids are widespread throughout the southern supercontinent with their natural distribution ranging from Africa, Latin America, Madagascar and a few species native to Asia (Turner 2007). There are approximately 3000 to 4000 cichlid species from 200 genera but there are still many more species that have been identified and yet to be formally described (Turner et al. 2001; Turner 2007; Svardal et al. 2021). The rapid evolutionary rate observed amongst cichlids has been attributed to exploitation of novel habitats (Hulsey et al. 2006), hybridisation (Seehausen 2004; Genner and Turner 2012), multiple colonisation (Loh et al. 2013; Tyers and Turner 2013) and sympatric speciation driven by natural and sexual selection (Turner et al. 2001; Kocher 2004).

4.2.2 Haplochromine cichlids

Lake Malawi has thought to have been invaded by cichlids more than 700 000 years ago and produced the greatest number of endemic species (between 450 – 600 Genner et al. 2004) compared to Lake Victoria and Tanganyika (Danley and Kocher 2001). Most cichlid species found in Lakes Malawi and Victoria are haplochromines, where they exhibit clear sexual dimorphism. Male haplochromines have distinct colour patterns that make them easily distinguishable from closely related species unlike their female counterparts who are cryptically coloured (Koblmüller et al. 2008). Due to the presence of divergence in mating colours, sexual selection is believed to be a driver of speciation in haplochromine cichlids (Seehausen et al. 1998; Danley and Kocher 2001; Lande et al. 2001; Genner et al. 2004; Salzburger 2009).

4.2.3 *Astatotilapia calliptera*

Cichlids from Lake Malawi can be characterised into seven groups differing in ecology and morphology. One of the seven groups includes *Astatotilapia calliptera* a maternal mouth-

brooding species that occupies littoral regions of Lake Malawi and is also found in neighbouring rivers, swamps, ponds and shallow lakes (Parsons et al. 2017; Malinsky et al. 2018). Populations of *A. calliptera* exhibit variation in male colour and morphological traits associated with assertive mating (Tyers and Turner 2013; Nichols et al. 2014; Malinsky et al. 2015). Molecular analysis has shown that the seven groups of Lake Malawi cichlids stemmed from an ancestral *Astatotilapia*-type generalist species making *A. calliptera* an ideal model for investigating drivers of adaptive radiation and sympatric speciation (Malinsky et al. 2018; Clark et al. 2022).

4.2.4 *Astatotilapia calliptera* from Lake Masoko

Astatotilapia calliptera has also colonised a variety of aquatic habitats ranging from small creeks and rivers to small lakes around the East African Rift Valley (Parsons et al. 2017). One such successful colonisation includes Lake Masoko (or Lake Kisiba, Figure 4.1 A and B), a crater lake north of Lake Malawi formed approximately 50 000 years ago (Thevenon et al. 2003). Lake Masoko is a steep sided freshwater lake, 700m in diameter and reaches up to a maximum of 39m in depth (Gibert et al. 2002). Currently, it is a closed lake and isolated from external water bodies (Turner et al. 2019). Lake Masoko is surrounded by Zambezian forest, and the highlands are made of Afromontane formations (Barker et al. 2003).

Besides *A. calliptera* other distantly related cichlid species such as *Coptodon rendalli* (redbreast tilapia) and *Oreochromis squamipinnis* along with *Clarias gariepinus* (African sharptooth 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 sympatric speciation can be studied (Munby et al. 2021).

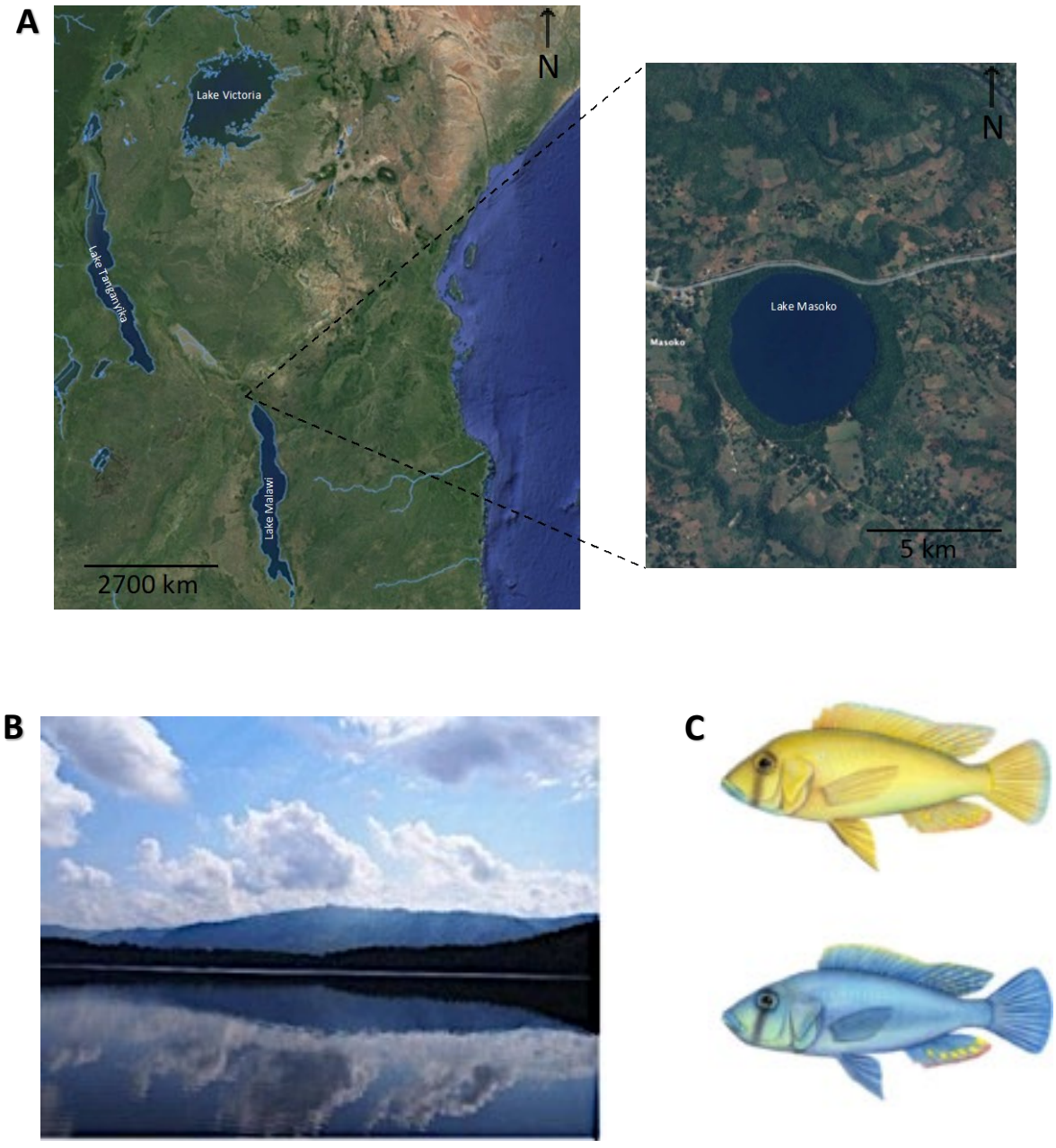


Figure 4.1. Location of Lake Massoko and its associated morphotypes of Astatotilapia calliptera. A) Map of the three great lakes in East Africa – Lake Victoria, Lake Tanganyika and Lake Malawi. Map of lake Masoko on the right in relation to the great lakes (Google Earth 2022). B) Photograph of Lake Massoko taken from the southern shore looking north. C) Yellow (littoral) and Blue (benthic - male) ecomorph of *Astatotilapia calliptera* species from Lake Massoko (images from Malinsky et al. 2015 and Clark et al. 2022).

Malinsky et al. (2015) have shown that approximately 10 000 years ago, *A. calliptera* individuals colonised shallow littoral habitats from nearby riverine systems and successively extended their habitat into deeper benthic waters about 1 000 years ago. The shallow and deep populations can be easily distinguished by male breeding colours where the littoral individuals are yellow and benthic males are blue (Figure 4.1 C). Females from both populations, like other haplochromine cichlids, are dull with silvery brown hues. Landmark based geometric morphometrics indicate phenotypically distinct body shapes and lower pharyngeal jaw morphology between the yellow and blue morphs (Malinsky et al. 2015). In addition to benthic and littoral ecomorphs, an intermediate exists where it could not be readily assigned to either ecomorph, consisting mainly of smaller fish and individuals of intermediate morphology. These intermediates are speculated to be hybrids between benthic and littoral individuals and tend to occur in a zone between benthic and littoral habitats (Turner, pers comm).

Mate choice experiments have revealed that littoral females preferred mating with yellow littoral males (genetically similar to them) whereas no assortative mating was observed in benthic females (benthic females mated with both littoral and benthic males i.e no preference) (Tyers 2013). Whole genome sequencing showed distinct population structure between blue and yellow morphs (Malinsky et al. 2015). Stable isotope analysis of carbon and nitrogen was performed indicating potential diet segregation where benthic individuals had a planktivorous diet compared to littoral yellow individuals who consumed molluscs (Malinsky et al. 2015; Carruthers et al. 2022). Stable isotope ratios have the potential to classify diets into broad functional groups but does not have the ability to elucidate species-level prey composition (Maloy et al. 2013). Hence, there is a significant gap in understanding the level of diet diversification between the ecomorphs and consequently, towards the role of dietary divergence in emergent sympatric species. Morphological analysis of stomach contents was performed to identify the differences in prey composition between the ecomorphs, however, the specimens analysed were dominated by sedimentary material resulting in little identifiable matter (Unpublished data). Thus, a molecular-based technique is necessary obtain species specific information of prey composition.

4.2.5 Metabarcoding of dietary material

Traditionally, diet contents have been analysed through visual morphological analysis that requires minimal equipment and is inexpensive. However, it is time consuming, labour

intensive and depends heavily on taxonomic expertise (Sheppard and Harwood 2005). In addition, it is acknowledged that observational data underrepresents small prey items, soft-tissue prey and easily digested food as they are undetectable whereas hard to digest prey items such as otoliths, mollusc shells and exoskeleton parts are reported at a disproportionally higher abundance (de Sousa et al. 2019; Traugott et al. 2021). With a shortage of taxonomists, it is challenging to apply morphological approach as a reliable technique for species identification (Elbrecht et al. 2017).

DNA metabarcoding of gut contents is able to simultaneously identify various prey taxa present and with uniquely tagged primers and enables the processing of large numbers of samples in a cost-effective manner (De Barba et al. 2014). DNA metabarcoding is also the preferred method for identification of cryptic species and especially beneficial when looking at specimens with plastic feeding behaviours (De Barba et al. 2014).

Metabarcoding of stomach contents have been used extensively in dietary studies to inform trophic positioning, food web dynamics (Carreon-Martinez & Heath, 2010) and prey-predator interactions and subsequently used to inform management and conservation strategies (Roslin and Majaneva 2016). DNA metabarcoding investigating niche partitioning 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) but has never before been used to determine trophic specialisation in cichlid species.

4.2.6 Objectives and hypothesis

The aim of this study is to complement existing morphological and stable isotope information with metabarcoding data, regarding the trophic specialisation of *Astatotilapia calliptera* in the early stages of adaptive divergence as observed in Lake Masoko. Given the challenges associated with visual gut content analyses and limitations of stable isotope analyses, metabarcoding of stomach contents offers one of the best opportunities to test for associations between diet composition and the phenotypic and genomic divergence of *A. calliptera* ecomorphs. Based on the results from Malinsky et al. (2015) and Carruthers et al. (2022), we expect the benthic (blue) ecomorph to primarily consume zooplankton and phytoplankton while littoral (yellow) individuals to specialise in hard bodied macroinvertebrates.

4.3 Methods

4.3.1 Sample collection and processing

All fish specimens were collected in August 2015 using a mixture of diving and gill net methods. The fish were euthanized using an overdose of clove oil, which was then followed by dissection of the entire intestinal tract. The dissections were stored in 100% ethanol and transported back to the UK for molecular analysis. Once in the UK, stomach contents from individual fish were removed by careful longitudinal incision to prevent contamination with host tissue (performed in 2019). Between each dissection, the dissection instruments were bleached (10%) and ethanol flamed for sterilisation. A total of 112 samples were selected for molecular analysis comprised of 52 benthic, 46 littoral and 14 intermediate morphs. DNA was extracted from the isolated stomach contents using the ammonium acetate salt extraction method (Bruford et al. 1998). This ammonium acetate DNA isolation method has been used widely prior to the current popular pre-made extraction kit methods (e.g Qiagen DNeasy Blood and Tissue kit) and has been effective at not only isolating DNA from stomach contents but also successful at extracting high quality DNA from zebrafish fin tissue (Coe et al. 2009), honeybee wings (Châline et al. 2004), *Anolis* lizard tail tissue (Wordley et al. 2011), blood and embryonic tissue of warblers (Richardson et al. 2001), pollen from fungus gnats (Phillips et al. 2014b) and many more organisms.

4.3.2 PCR and sequencing

Illumina MiSeq paired-end indexed amplicon libraries were prepared using a two-step PCR protocol. Two marker genes were amplified using universal primer pairs mlCOIintF (Leray et al. 2013) and jgHCO2198 (Geller et al. 2013) targeting the cytochrome c oxidase subunit I (COI) region found in the mitochondria, and TAReuk454FWD1 and TAReukREV3r (Stoeck et al. 2010) of the 18S V4 region from the eukaryotic nuclear small subunit ribosomal DNA (18S) (Table 4.1). 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 has been successful at targeting invertebrate DNA from aquatic systems (Hajibabaei et al. 2019) and effective at recovering species-specific DNA from stomach contents (Leray et al. 2013). The V4 region of the ribosomal DNA-encoding gene is known to be variable and using the 18S primer set it has the potential to target a wide diversity of eukaryotic taxonomic groups (Stoeck et al. 2010), although it does lack the power to resolve target sequences down to species level

(Creer et al. 2016). It has been recommended that multiple primer sets should be used to capture the full breadth of dietary niche and prevent biases arising from singular universal primer set (Alberdi et al. 2018). Hence, we hope that using a combination of a taxon specific primer (COI) and a primer able to capture a wide variety of eukaryotic diversity (18S), we would be able to obtain information of the full dietary niche of the *A. calliptera* ecomorphs. A 5' universal tail was added to both forward and reverse primers, and a 6 N sequence was added between the forward universal tail and the template specific primer. The 6 N addition is known to improve clustering and cluster detection on Illumina Miseq sequencing platforms (Miya et al. 2015).

The first round PCR was conducted using the Qiagen Multiplex PCR kit in a final volume of 25 µl, which comprised 0.2 µM of forward and reverse primers, 2x Qiagen Multiplex PCR Master Mix (containing HotStarTaq DNA Polymerase, multiplex PCR buffer and dNTP mix) and 1µl of DNA template. The thermal cycling conditions for amplification of COI region were an initial activation step at 95 ºC for 15 mins; 35 cycles at 94 ºC for 30 seconds, annealing at 54 ºC for 90 seconds and extension at 72 ºC for 90 seconds and a final extension at 72 ºC for 10 minutes. The 18S rDNA PCR amplification differed only at the annealing stage where temperature was 60 ºC. Five negative controls, comprising water instead of 1µl of template DNA, were included in each PCR plate. Products from the first PCR were purified using Agencourt AMPure XP beads (Beckman Coulter), at a 1:1 ratio of PCR product : AMPure XP beads.

The second round PCR was carried out at a final volume of 25 µl, 8 µl of purified template from PCR 1, 2x Qiagen Multiplex PCR Master Mix and 2 µM of Fi5 and Ri7 primers (Table 4.1). Thermal cycling condition were 95 ºC for 15 minutes, followed by 10 cycles of 98 ºC for 10 seconds, 65 ºC for 30 seconds and 72 ºC for 30 seconds and a final extension at 72 ºC for 5 minutes. The amplified samples were quantified using the QuantiFluor dsDNA system (Promega) and pooled at equimolar concentrations to a final volume of 50ul. The pooled metabarcoding libraries were cleaned a second time using Agencourt AMPure XP beads.

Table 4.1. Primer name and sequences used in library preparation. Round 1 primer sequences contain forward and reverse template primers, the forward primer sequence contains 6N's to improve clustering and cluster detection on Illumina MiSeq sequencing platforms. Round 1 primers include mitochondrial cytochrome c oxidase subunit I (COI) (Leray et al. 2013; Geller et al. 2013) and V4 region of the 18S ribosomal DNA-encoding gene (Stoeck et al. 2010). Round 1 and round 2 sequences contain complementary universal tails. Round 2 PCR primers (TruGrade, IDT) sequence contained the P5 and P7 Illumina adapters and a 6bp unique index both in forward and reverse primers used for demultiplexing samples.

Round 1 PCR
Forward Universal Tail – NNNNNN – Template specific primer ***mlCOIintF***   [ACACTCTTTCCCTACACGACGCTCTTCCGATCT]-[NNNNNN]-   [GGWACWGGWTGAACWGTWTAYCCYCC]
Reverse Universal Tail – Template specific primer ***jgHCO2198***   [GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT]-[TAIACYTCIGGRTGICCRAARAAYCA]
Forward Universal Tail – NNNNNN – Template specific primer ***TAReuk454FWD1***   [ACACTCTTTCCCTACACGACGCTCTTCCGATCT]-[NNNNNN]-[CCAGCASCYGCGGTAATTCC]
Reverse Universal Tail – Template specific primer ***jgHCO2198***   [GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT]-[ACTTTCGTTCTTGATYRA]
Round 2 PCR
P5 Illumina adapter – **i5 index** – Forward Universal Tail   [AATGATACGGCGACCACCGAGATCTACAC]-[**i5 index**]-   [TCTACACGTTCAGAGTTCTACAGTCCGACGATC]
P7 Illumina adapter – **i7 index** – Reverse Universal Tail   [CAAGCAGAAGACGGCATACGAGAT]-[**i7 index**]-   [GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT]

Each pool was first mixed at a 1:0.5 ratio of DNA library : AMPure XP beads. At this stage, the beads were discarded after being separated on a magnetic stand, this ensures that the larger non-target fragments were removed. The remaining supernatant was mixed in a ratio of 1:0.9 of DNA library : AMPure XP beads to remove smaller fragments of DNA. After standing on a magnetic rack, the beads were reserved, cleaned with ethanol and resuspended in low TE buffer. Final quantification was performed using qPCR with a serial dilution of the pooled libraries created at 100, 1000 and 10 000 fold concentrations. The final volume used in qPCR reactions was 10 µl, made up of 6µl of SYBR mastermix, 2 µl of the diluted libraries and 2 µl of water. The thermal cycling conditions were 95 ^{o}C for 5 minutes, 35 cycles at 95 ^{o}C for 20 seconds and 60 ^{o}C for 45 seconds. Samples from each library were pooled at 4 µM concentrations. Prior to sequencing, the pools were diluted to 9pM concentration and ran with 10% PhiX using a MiSeq V3 reagent kit (600 cycles).

4.3.3 Bioinformatic analysis

FastQC (v 0.11.8) (Andrews 2010) and MultiQC (Ewels et al. 2016) were used to analyse quality of raw sequences. Sequences were filtered using Cutadapt (v 3.7) (Martin 2011) to only include those that contained forward and reverse primer sequences across both read pairs for each fragment and primer regions were removed from each fragment for the sequences retained. The sequences were denoised (sequence quality control; trimming, filtering and removal of chimeras) using 'DADA2' package (v 1.16) (Callahan et al. 2016) in RStudio (v 4.1.3) (RStudio Team 2020). Default parameters of DADA2 were used unless otherwise stated. Only forward reads were used for both COI and 18S markers in the following analysis. Merged COI sequences resulted in large proportion of host reads, masking any true invertebrate diversity and due to an error in the 18S reverse primer, reverse reads from the 18S data were discarded. COI and 18S sequences were trimmed at 240 bp using the filterAndTrim function in the DADA2 package. Sequences were trimmed based on manual examination of read quality profiles from FastQC results. Subsequently, the sequences were denoised followed by chimera identification and removal to produce an amplicon sequence variant (ASV) by sample table. ASVs are biological sequences that differ by a single nucleotide and are able to provide fine scale resolution, as opposed to molecular operational taxonomic units (OTUs) that are clusters of reads that differ less than a fixed dissimilarity threshold (which is commonly set to 3% or another arbitrary threshold) (Callahan et al. 2017). The denoised ASVs were used for taxonomic assignment using BLASTn (Camacho et al. 2009) against the MIDORI (v GB 241)

(Leray et al. 2018) and SILVA databases (v 138.1) (Quast et al. 2013), above 90% identity matches (e-value: <0.00001, mismatch <10) to order level for both the COI and 18S markers were applied. In some analysis only phylum level (at 90% identity match) was used for visual grouping. Only ASVs above 313 and 390 base pairs were retained for the COI and 18S regions respectively. The ASVs not assigned to taxonomy at 90% were reanalysed and assigned to taxonomy at 80%, however this resulted in spurious taxonomic assignment (e.g marine organisms) and therefore discarded.

4.3.4 Statistical analysis

Shapiro-Wilk test for normality was used on ASV count data followed by Kruskal-Wallis sum rank test to determine differences in dietary richness between the ecomorphs. Alpha diversity was calculated in 'Vegan' (v 2.6-2) (Oksanen et al. 2022) using Shannon-Weiner diversity indices, followed by a Shapiro-Wilk test for normality and Kruskal-Wallis sum rank test. A post hoc test following Kruskal Wallis test was performed using Dunn's test (Dunn 1961).

ASV count data was transformed to proportional data and used in downstream analysis. Heatmaps using the proportional data were created using the amp_heatmap function in 'AmpVis2' package (v 2.7.24) (Albertsen et al. 2015) to visualise and rank the most abundant taxa from each ecomorph. As the majority of the detected ASVs for each ecomorph were assigned to five different phyla, the remaining ASVs were removed to reduce 'noise' and spurious ASVs. A subset of the top 5 most abundant phyla, consisting of multiple ASVs, from each ecomorph was used for beta diversity tests and ordination analysis, because preliminary analysis on the entire dataset was overshadowed by excessive numbers of low read taxa resulting in inaccurate representation of key diversity.

Permutational ANOVA (PERMANOVA) was used to assess the differences in community similarity using the adonis2 function in the 'Vegan' package on a matrix of Bray-Curtis dissimilarity indices calculated within the adonis2 function. Pairwise differences were identified with pairwise.adonis function in 'pairwiseAdonis' package (v 0.4) (Martinez 2020) and Bonferroni correction was applied to the resulting p-values to control for false positives. Non-metric multidimensional scaling (nMDS) ordination was calculated in 'Vegan' package using metaMDS function followed by Envfit function to fit significant ($p < 0.05$) dependent

environmental variables onto the ordinations and result figures were plotted using 'ggplot2' (v 3.3.6) (Wickham 2016).

The exacttest function from 'EdgeR' package (v 3.36.0) (Robinson et al. 2009) was used to compute genewise comparisons (differential abundance analysis) of mean between ecomorph data to identify taxa that were significantly ($p < 0.05$) different. Twenty ASVs provided an adequate number of independent variables with good read coverage (>100) on which to perform the differential abundance analysis. Exploratory analysis indicated that increasing the number of ASVs incorporated an excess of rare taxa with low read coverage masking the evident variation between the ecomorphs. Bipartite maps were created using plotweb function in 'Bipartite' (v 2.17) (Dormann 2022) and 'BipartiteD3' (v 0.3.0) (Terry 2018) packages using proportional data.

4.4 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). The number of raw reads per sample ranged from 235 to 363 660 with an average of 99 779 ± 15 365 (standard error). The number of reads per ASV found in the negative controls were deducted from the entire dataset (that made up 3 – 20% of the reads in each sample). Since the negative control samples showed low level of cross-contamination it did not affect the overall distribution of reads.

4.4.1 18S dataset

The diets of 181 individuals from benthic, intermediate and littoral ecomorphs were sequenced and following bioinformatic processing, 166 ASVs from 104 samples remained after host associated sequences were removed. The remaining 166 ASVs were assigned to 26 different phyla. Only forward sequences were used to assign taxonomy as an error was made in the synthesis of the 18S reverse primer.

Before the dataset was removed of low read ASVs, a Shapiro-Wilk test on the sum of ASV reads per specimen reflected that the data was not normally distributed ($p < 0.05$), therefore a non-parametric Kruskal Wallis rank sum test was performed. There was no significant difference ($p = 0.5789$, chi-squared= 1.09, df=2) in the overall ASV richness between the ecomorphs. The Shannon Weiner index was calculated as a measure of diversity followed by

test for normality ($p < 0.05$) and Kruskal Wallis rank sum test. Consequently, there was no significant difference in alpha diversity between the ecomorphs ($p=0.3267$, chi-squared=2.24, df=2).

As the data included a large number of low-read ASVs, that was likely to not reflect true dietary contents, a subset of the top five most abundant phyla for each ecomorph was used for downstream analysis. This subset contained 12 ASVs from 104 samples; 45 benthic, 36 littoral and 16 Intermediate ecomorph individuals. The phylum Apicomplexa (unicellular endoparasites) were assigned to 60% of the reads followed by Arthropoda (invertebrates) (2 ASVs) at 13% and both Cryptomycota (parasitic relatives of fungi: 2 ASVs) and Chlorophyta at 5 % each (Figure 4.2). The remaining 17% of the reads were assigned to phyla Ciliophora (ciliated unicellular eukaryotes), Nematoda (worms), Mollusca, Bacillariophyta (diatoms: 2 ASVs) and Oomycota (water moulds) (Figure 4.2). ASVs were assigned to the phylum level because the 18S marker does not have species level discriminatory power, while acknowledging the lack of DNA based studies characterising the diversity present in remote locations such as African satellite crater lakes.

Non-metric multidimensional scaling (nMDS) of the gut contents showed overlap in data points to a certain degree with outlying points clustered consistent with their ecomorphological dietary origins (Figure 4.3). Furthermore, pairwise permutational ANOVA (PERMANOVA) models showed significant differences ($p<0.05$) in the diets between ecomorphs at phylum level (Table 4.2).

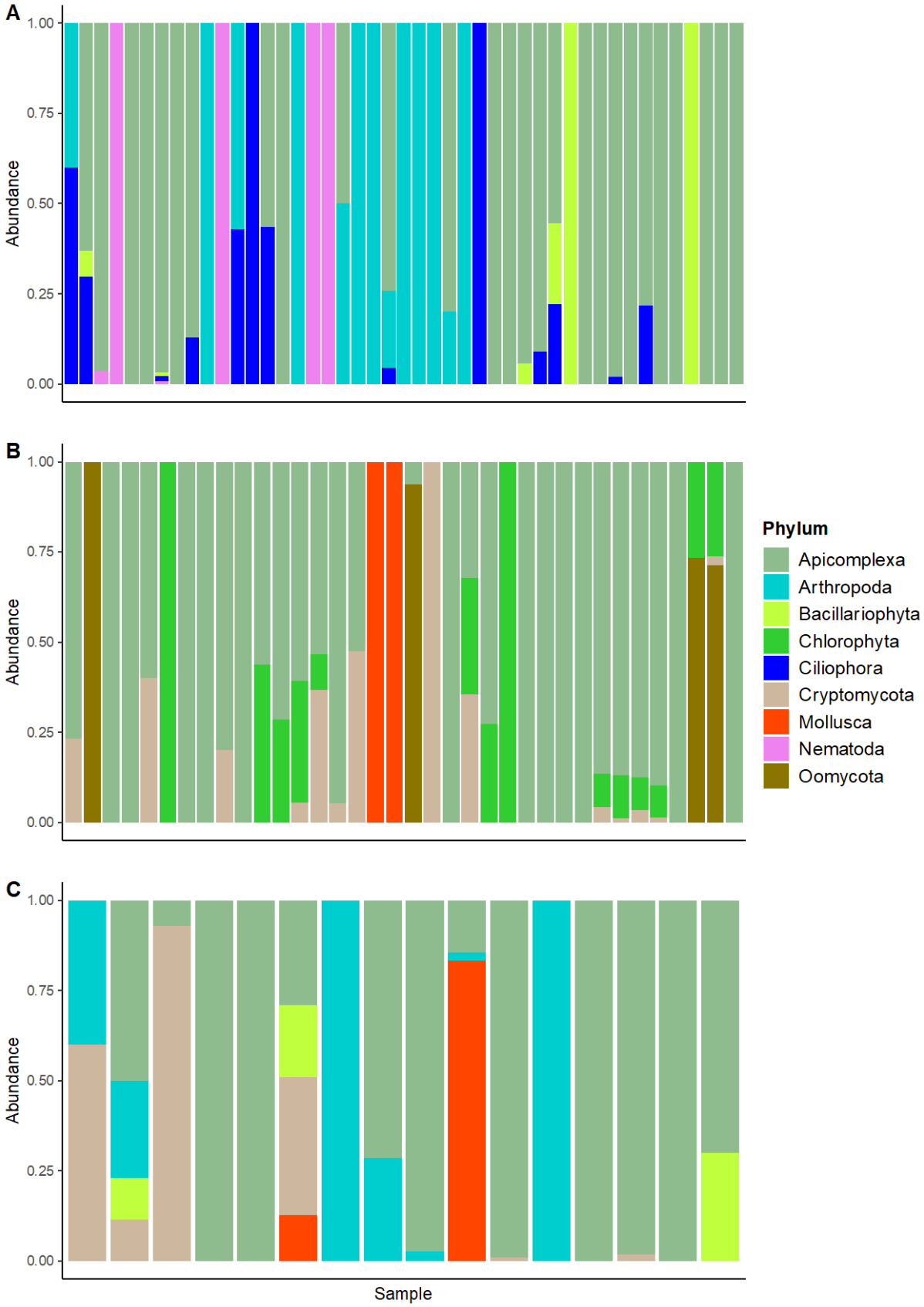


Figure 4.2. Proportional abundance of ASVs across the three ecomorph groups **A**, benthic, **B**, littoral and **C**, Intermediate. Each bar represents an individual fish. ASVs are from metabarcoding of *Astatotilapia calliptera* diet contents from Lake Masoko with 18S – nuclear small subunit ribosomal DNA marker and features the top five phyla from each ecomorph.

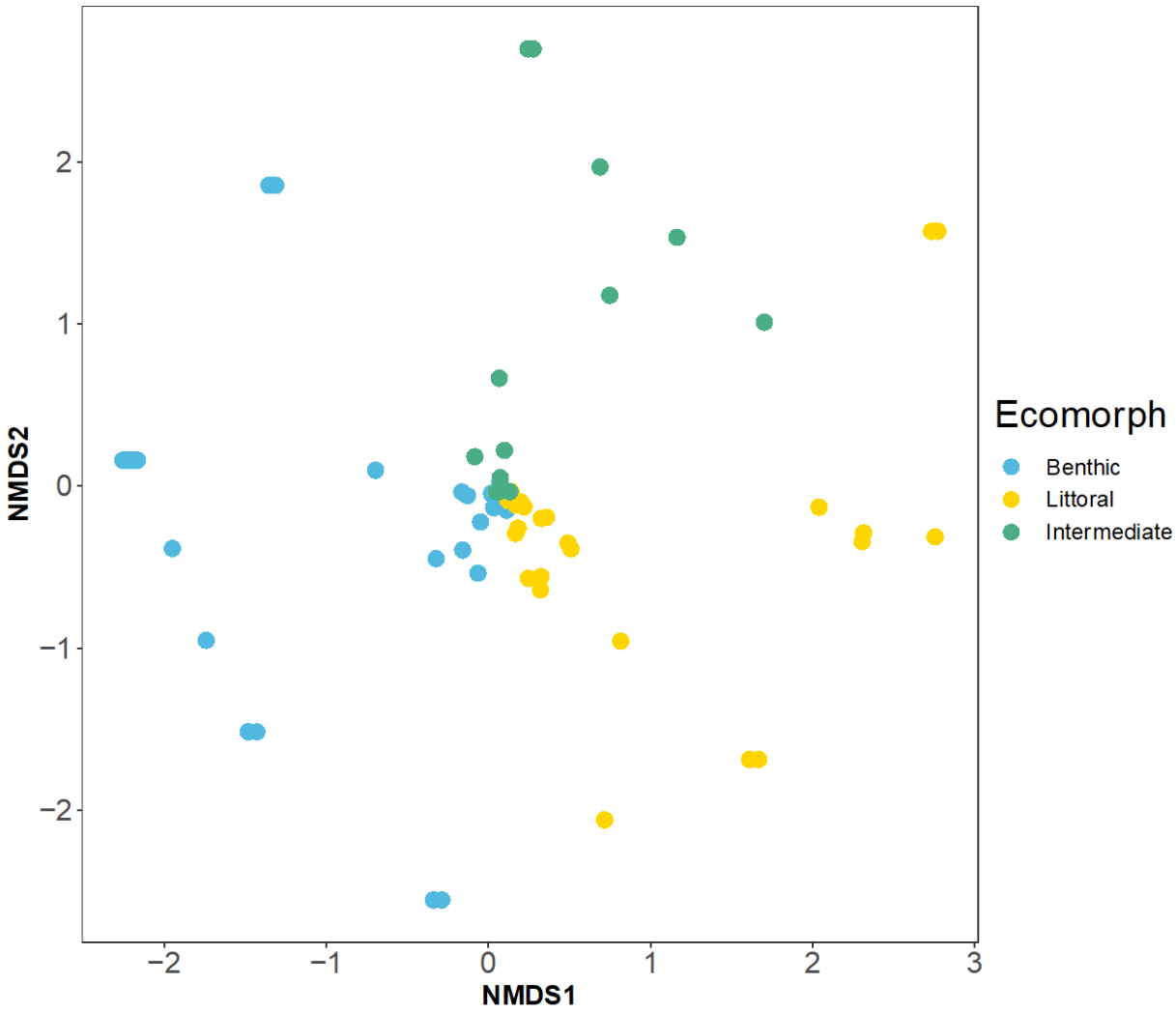


Figure 4.3. Non-metric multidimensional scaling (nMDS) ordination (stress = 0.042) on Bray-Curtis index of amplicon sequence variants (ASV) from metabarcoding of *Astatotilapia calliptera* diets from Lake Masoko with 18S nuclear small subunit ribosomal DNA marker. The three ecomorphs present; benthic, littoral and intermediate are denoted by the colours blue, yellow and green respectively. The figure features the top five phyla from each ecomorph.

Table 4.2. Permutational ANOVA model with pairwise comparison output based on Bray-Curtis index of amplicon sequence variants (ASV) from the top five phyla of each ecomorph benthic, littoral and intermediate *Astatotilapia calliptera* stomach contents from Lake Masoko. Model presented is of 18S nuclear small subunit ribosomal DNA marker.

	Df	SumofSqs	F.Model	R2	p.value	p.adjusted
Benthic vs Littoral	1	1.602	5.870	0.069	0.002	0.006
Benthic vs Intermediate	1	1.130	3.960	0.062	0.010	0.030
Littoral vs Intermediate	1	0.800	3.240	0.061	0.013	0.039

The abundance of Arthropoda, Apicomplexa, Nematoda and Apusozoan (unicellular organisms with flagella) taxa found in benthic ecomorph diets were significantly ($p < 0.05$) higher compared to littoral and intermediate ecomorphs (Figure 4.4). Diet samples from littoral individuals were significantly ($p < 0.05$) more abundant in Chlorophyta and Oomycota in comparison with benthic and intermediate ecomorphs. Intermediate ecomorph diet samples were significantly more abundant in phyla such as Mollusca, Apicomplexa and Cryptomycota when compared to benthic and littoral ecomorphs (Figure 4.4).

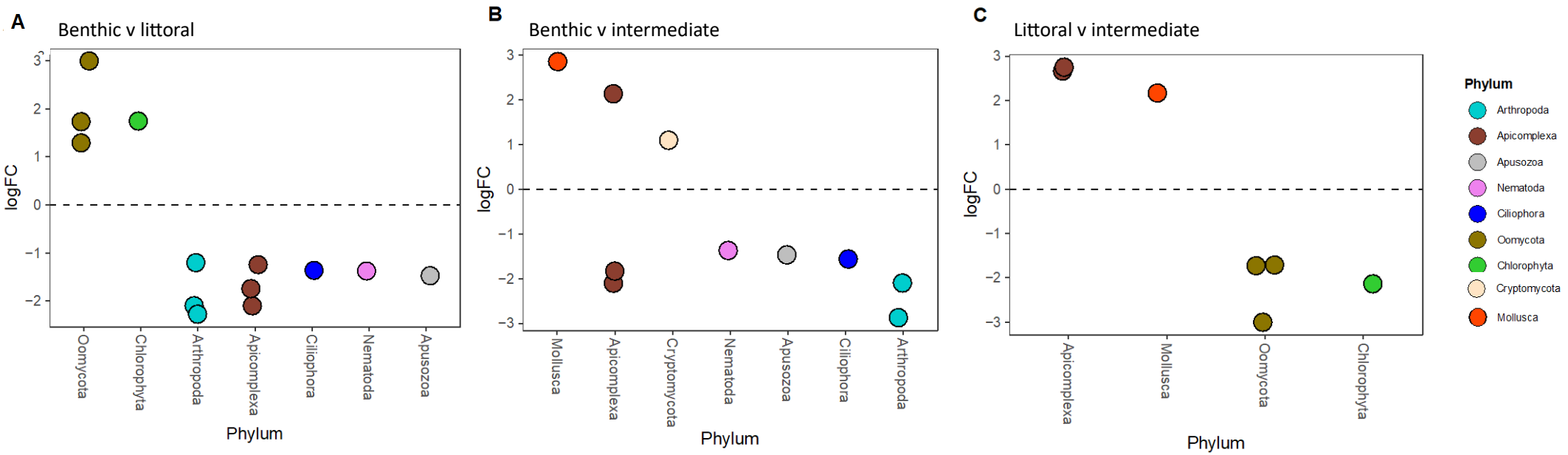


Figure 4.4. Log2fold differential abundance analysis of the different phyla between three ecomorphs benthic, littoral and intermediate *Astatotilapia calliptera* diet contents from Lake Masoko of 18S nuclear small subunit ribosomal DNA marker. Differences were considered significant with p-value (corrected for false positives using Benjamini-Hochberg correction) at 0.05. Log2fold change greater than zero indicated an increase in the relevant taxa, while Log2fold change lesser than zero indicated a decrease. Each point represents a single ASV and the dashed line at value 0 in each plot represents the baseline values of each ecomorph. **A**, Benthic baseline compared against littoral ASVs, **B**, Benthic baseline compared against intermediate ASVs and **C**, littoral baseline compared against intermediate ASVs. A dataset comprised of the top 20 phyla was used in this analysis. The analysis compares at gene level, but only phylum level is reflected to remain conservative.

The diet analysis of all three ecomorphs largely comprised of Apicomplexa. The diet of benthic ecomorphs were further supplemented by Arthropoda (20%), Ciliphora (9%), Nematoda (9%) and Bacillariophyta (5%). In addition to Apicomplexa, diet of littoral ecomorphs comprised Chlorophyta (14%), Mollusca (6%), Oomycota (6%) and Cryptomycota (9%). Intermediate diets consisted of Arthropoda (19%) and Bacillariophyta (4%) like the benthic ecomorph as well as Cryptomycota (13%) and Mollusca (9%) similar to littoral ecomorphs (Figure 4.5).

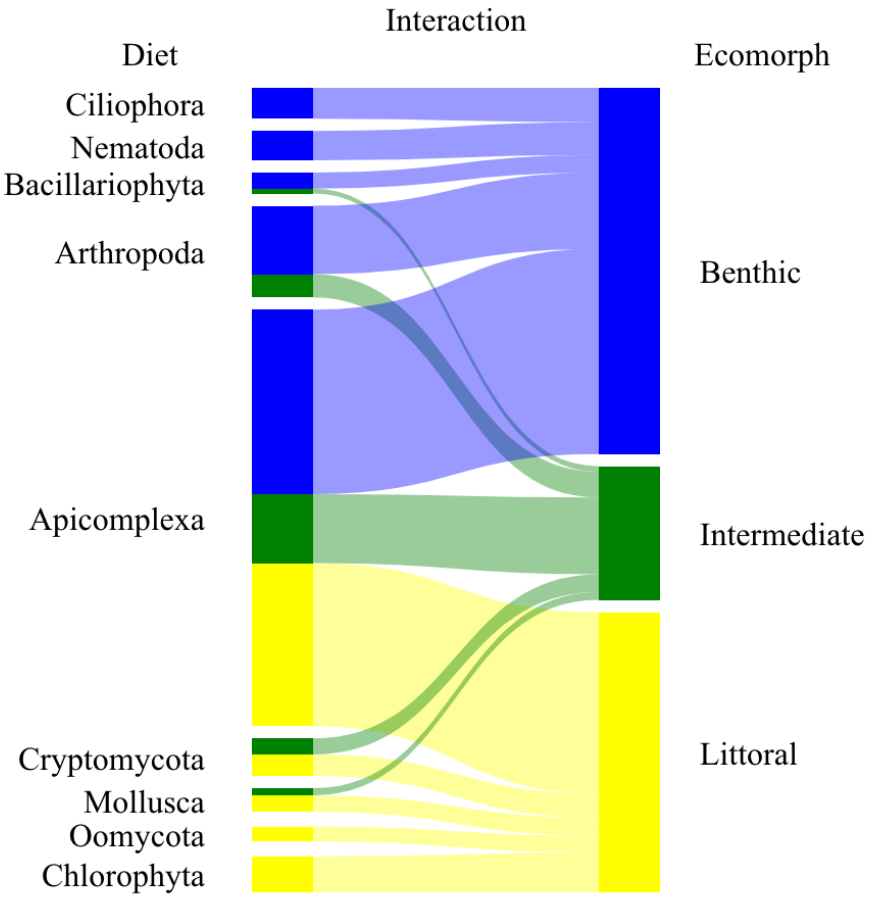


Figure 4.5. Bipartite network of three ecomorphs benthic (n=45), littoral (n=36) and intermediate (n=16) *Astatotilapia calliptera* from Lake Masoko. Prey/diet species are on the left of the network and *A. calliptera* individuals from respective ecomorphs are on the right. The width of the corresponding boxes and the connecting lines are directly proportional to the relative read abundance of the top five phyla from each ecomorph. The figure presented uses data from the 18S nuclear small subunit ribosomal DNA marker.

4.4.2 COI dataset

Amplification of the cytochrome oxidase I (COI) marker resulted in 185 ASVs from 181 samples (68 benthic, 65 littoral and 18 intermediate). After removing host sequences, 171 ASVs from 151 samples remained and the ASVs were assigned to 17 different phyla. Only forward sequences were used to assign taxonomy as large proportion of the sequences were assigned to host when both forward and reverse sequences were used. This error could be due to the poor quality of reverse reads obtained (average quality score ranged from 27 - 11).

The Shapiro-Wilk test on total sum of ASV reads per sample showed that the data was not normally distributed ($p < 0.05$), therefore a non-parametric Kruskal Wallis rank sum test was used followed by a post-hoc Dunn's test used to identify significantly different ecomorph pairs. Both benthic and littoral samples had significantly more reads compared to intermediate ecomorphs ($p < 0.05$). The Shannon Weiner index was calculated as measure of alpha diversity followed by test for normality ($p < 0.05$), Kruskal Wallis rank sum test and post hoc Dunn's test (with Bonferroni correction) to identify differences in alpha diversity. Only intermediate individuals had gut contents that were significantly ($p < 0.05$) less diverse than benthic individuals (Table 4.3). This result could because benthic group consisted of more reads.

Table 4.3. Dunn's test performed on Kruskal-Wallis rank sum test based on Shannon-Weiner diversity index of amplicon sequence variants (ASV) from metabarcoding of ecomorphs benthic, littoral and intermediate *Astatotilapia calliptera* diets from Lake Masoko using COI – Cytochrome Oxidase 1 mitochondrial DNA marker.

	n1	n2	Estimate1	Estimate2	Statistic	p.value	p.adjusted
Benthic vs Littoral	68	65	65.6	79.1	1.79	0.0736	0.221
Benthic vs Intermediate	68	18	65.6	104	3.33	0.000877	0.00263
Littoral vs Intermediate	65	18	79.1	104	2.15	0.0319	0.0956

n1 and n2 refer to sample counts from each group respectively. Estimate 1 and 2 show mean rank value of the 2 groups respectively. Statistic is the Z-value used to compute p-value.

Similar to the 18S dataset, a subset of the COI dataset containing only the top five most abundant phyla per ecomorph was used in downstream analysis after the removal of low read taxa so that true differences between the ecomorphs were not diluted. This subset contained eight ASVs from 135 samples. The phylum Arthropoda contained five ASVs and were assigned 93% of the reads, followed by Eustigmatophyceae (eukaryotic algae) (3%), Bacillariophyta

(diatom) (2.5%) and Rotifera (multicellular organisms making up the zooplankton community) (0.5%) (Figure 4.6). ASVs here were only assigned to phylum level, similar to the 18S dataset, as a threshold of only 90% sequence similarity was applied against the MIDORI reference database.

Permutational ANOVA (PERMANOVA) models showed significant ($p < 0.001$) differences in the diets between the ecomorphs at phylum level. However, non-metric multidimensional scaling (nMDS) of the gut contents showed large overlap marker (Figure 4.3), contrasting to the 18S marker (Figure 4.7). A pairwise PERMAVONA ($p < 0.05$) indicated significant differences between all pairs of the ecomorphs (Table 4.4).

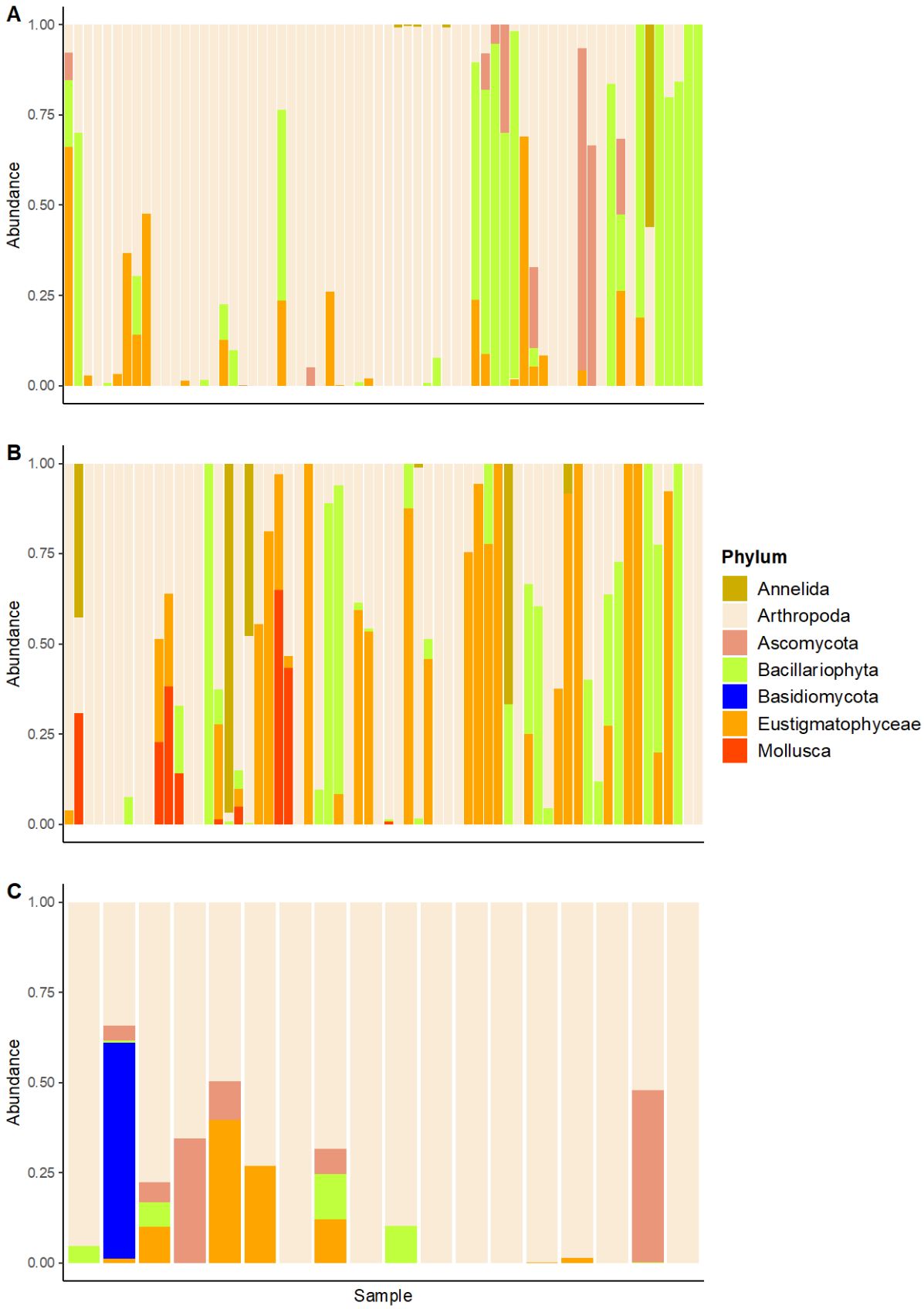


Figure 4.6. Proportional abundance of amplicon sequence variants (ASVs) across the three ecomorph groups **A**, benthic, **B**, littoral and **C**, intermediate. Each bar represents diet of an individual sample. ASVs are from metabarcoding of *Astatotilapia calliptera* diet contents from Lake Masoko with COI – Cytochrome Oxidase 1 mitochondrial DNA marker and features the top five phyla from each ecomorph.

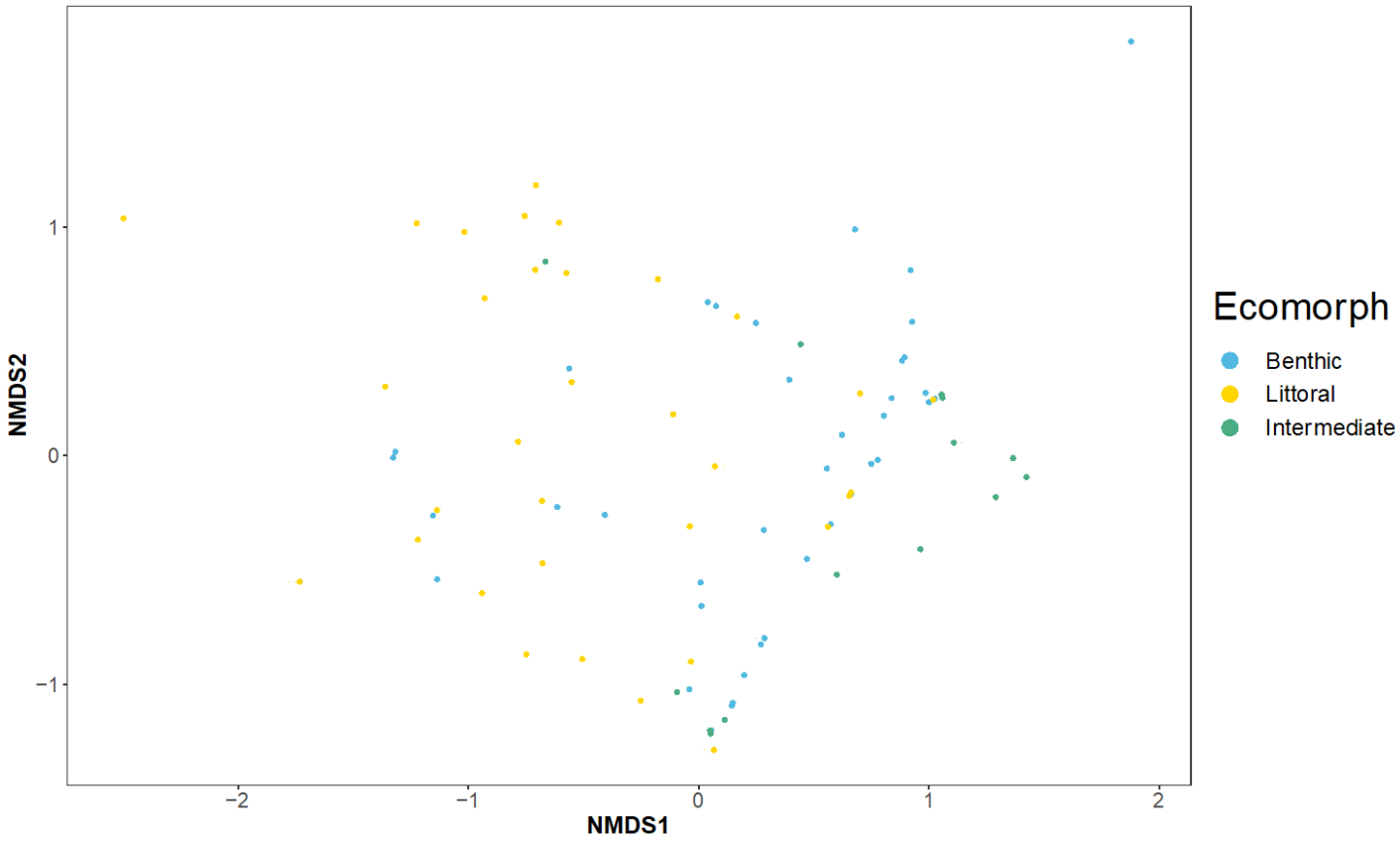


Figure 4.7. Non-metric multidimensional scaling (nMDS) ordination (stress = 0.1229) on Bray-Curtis index of amplicon sequence variants (ASV) from metabarcoding of *Astatotilapia calliptera* diets from Lake Masoko with Cytochrome Oxidase I mitochondrial (COI) DNA marker. The three ecomorphs present; benthic, littoral and intermediate are denoted by the colours blue, yellow and green respectively. The figure features the top five phyla from each ecomorph.

Table 4.4. Permutational ANOVA model with pairwise comparison output based on Bray-Curtis index of amplicon sequence variants (ASV) from the top five phyla of each ecomorph; benthic, littoral and intermediate *Astatotilapia calliptera* diets from Lake Masoko. Model presented is of COI – Cytochrome Oxidase I mitochondrial DNA marker.

	Df	SumofSqs	F.Model	R2	p.value	p.adjusted
Benthic vs Littoral	1	10.671	69.506	0.459	0.001	0.003
Benthic vs Intermediate	1	1.167	5.582	0.042	0.005	0.015
Littoral vs Intermediate	1	9.497	47.122	0.371	0.001	0.003

Bacillariophyta were significantly more abundant in the gut contents of benthic individuals compared to intermediate individuals. Benthic individuals also showed higher abundance in Cyclopoida (Arthropoda: copepods) against littoral individuals. Littoral individuals' diets were

significantly ($p < 0.05$) more abundant in Annelida, Eustigmatophyceae and Mollusca, as well as organisms from order lepidoptera when compared to benthic individuals and more abundant in Bacillariophyta compared to intermediate individuals (Figure 4.8). Intermediate individuals' diet were significantly more abundant in Ascomycota (fungi) and cyclopoida in comparison to the littoral ecomorphs. Additionally, the stomach contents of intermediate individuals featured higher abundance of Mollusca compared to benthic individuals. Results from the COI and 18S dataset revealed similar dietary composition between the ecomorphs.

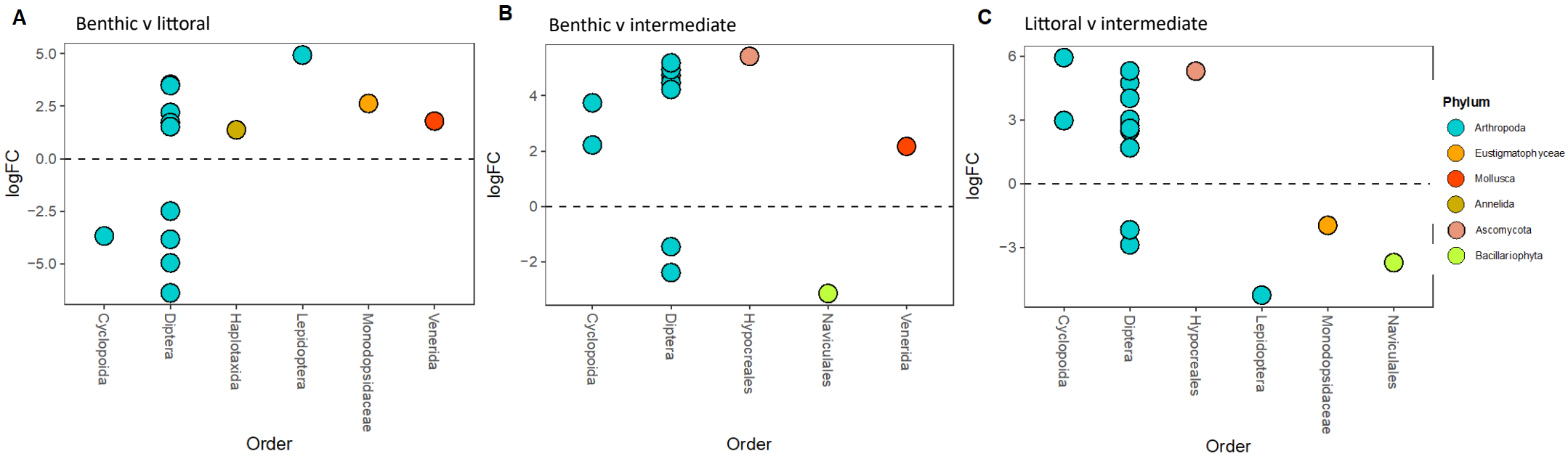


Figure 4.8. Log2fold differential abundance of the different phyla between three ecomorphs benthic, littoral and intermediate *Astatotilapia calliptera* diets from Lake Masoko of Cytochrome Oxidase I mitochondrial DNA marker. Differences were considered significant with p-value (corrected for false positives using Benjamini-Hochberg correction) at 0.05. Log2fold change greater than zero indicated an increase in the relevant taxa, while Log2fold change lesser than zero indicated a decrease. Each point represents a single ASV and the dashed line at value 0 in each plot represents the baseline values of each ecomorph. **A**, Benthic baseline compared against littoral ASVs, **B**, Benthic baseline compared against intermediate ASVs and **C**, littoral baseline compared against intermediate ASVs. The analysis compares at gene level, but only order level is reflected to remain conservative.

The diet of all benthic, littoral and intermediate individuals contained significant amounts of dipterans mainly constituting of flies (Simuliidae sp.), midges (Chironomidae sp.) and mosquitoes (*Culex* sp.), - all species with aquatic life stages and associated with freshwater habitats. Benthic and littoral individuals showed overlap in prey species but at different abundances. The stomach contents of benthic individuals consisted of Diptera, Cyclopoida and Navicuales (diatoms) making up 46%, 28% and 17% of their diet respectively with Monodopsidaceae (Ochrophyta: unicellular algae) only making up 8% of the diet. Whereas, stomach contents of littoral individuals were dominated by Monodopsidaceae (35%) and Cyclopoida (28%) followed by Diptera (21%) and Navicuales (15%). Like benthic and littoral individuals intermediate ecomorph diet comprised Diptera (54%), Cyclopoida (27%) and Monodopsidaceae (8%). Intermediate ecomorphs also consumed Ploima (rotifers: zooplankton, microscopic invertebrates) (10%) which were absent in both benthic and littoral samples.

4.5 Discussion

The major finding of the present study indicates that the two ecological morphs display distinct feeding behaviours. Diets of benthic *Astatotilapia calliptera* individuals mainly consisted of Arthropods (Diptera), Nematodes, Bacillariophyta (diatoms) and copepods while the littoral individuals' diet was made up of molluscs, annelid and fungi (Figure 4.9). Apicomplexa and Bacillariophyta were found in both benthic and littoral stomach contents. The variation observed in the dietary contents between the ecomorphs could indicate presence of resource partitioning driven by availability of prey at each habitat/depth.

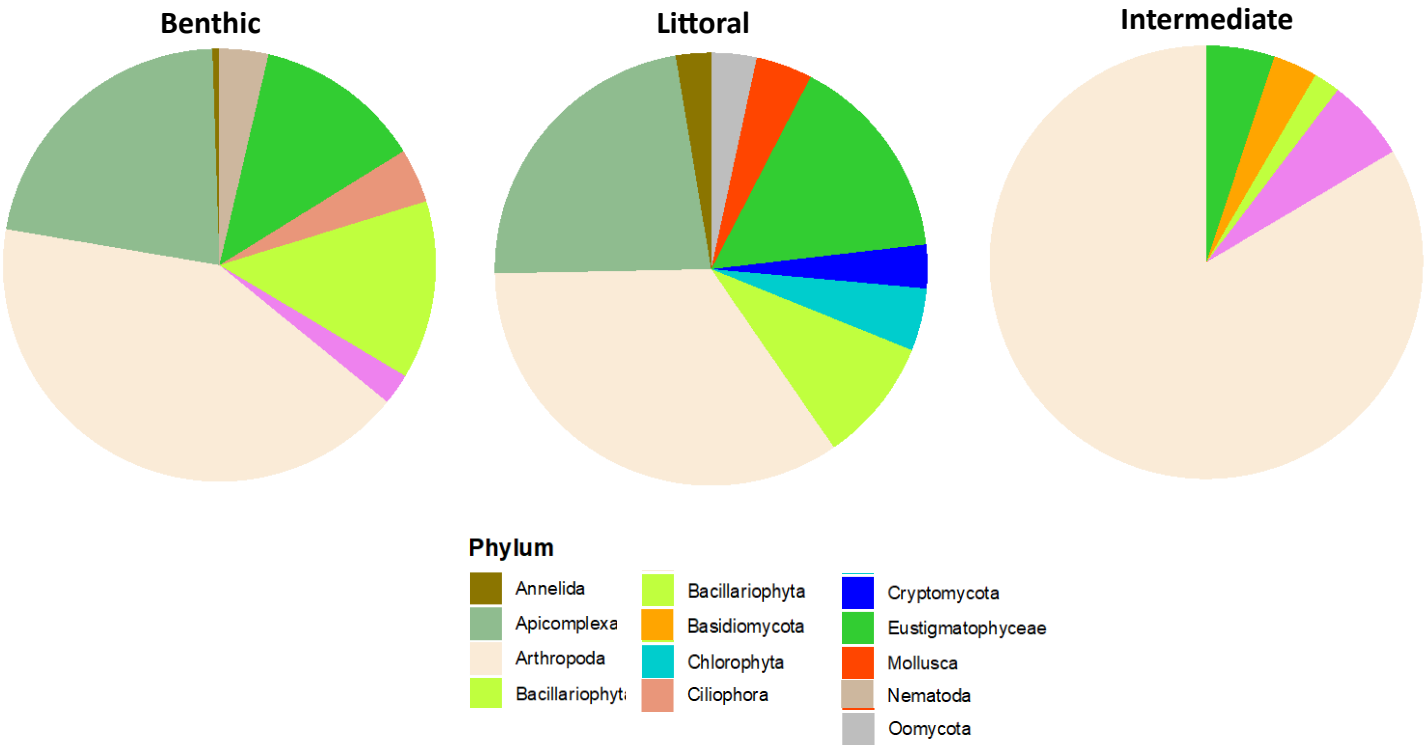


Figure 4.9. Figure shows aggregated proportional abundances of the top five phyla found in the stomach contents of *Astatotilapia calliptera* ecomorphs; benthic, littoral and intermediate, for both the 18S – nuclear small subunit ribosomal DNA marker and cytochrome oxidase I (COI) genetic markers.

4.5.1 Benthic ecomorph diet contents

Benthic individuals are known to have a planktonic diet due to the depleted ^{13}C values observed in stable isotope analysis (Malinsky et al. 2015; Carruthers et al. 2022). Teeth from the lower pharyngeal jaws of benthic individuals are needle-like also known as papilliform teeth and this dentition is usually indicative of a plankton rich diet in fish. The lower pharyngeal jaws of cichlid fish are known to differ to a large extent between species. The formation of dentition has been used to differentiate dietary preferences and associated trophic characteristics of individual species (Muschick et al. 2011). Examples of cichlid species with papilliform teeth include *Cynotilapia afra,* and *Labeotropheus fuelleborni* (blue mbuna) and individuals from these species have been observed to have strong preference for plankton and algae (Streelman and Albertson 2006).

The diet of benthic individuals was also dominated by arthropods and specifically by dipterans (flying insects). It is common to see mayfly, Odonata nymphs, Chironomids and saucer bugs in Lake Masoko (Turner et al. 2019). Even though benthic individuals have not been observed at the surface of the water preying on these insects, it is possible that they travel along the water column whilst foraging for food. Alternatively benthic individuals could be consuming deceased insects that were once the surface of the lake but eventually fall to the bottom enriching the lake benthos in which benthic individuals forage on regularly. Most Chironomid species are aquatic and have been found to be abundant in profundal zones of freshwater lakes (Nyman and Korhola 2005; Adler and Courtney 2019; Gadawski et al. 2022). Female Chironomids lay eggs on the surface of the water that sink to the bottom of the water body, the larvae burrows into the mud or constructs small tubes to live, feed and develop in until it transforms into a pupa where it actively swims to the surface of the water and emerges as an adult (Kranzfelder et al. 2015). The high abundance of Chironomid eggs and larvae is a potential food source for fish foraging on the benthos of water bodies. The diet of *Cyprinus carpio* (Eurasian carp) was found to be largely supplemented by dipteran species and this behaviour was attributed to foraging on zoobenthos (Dadebo et al. 2015). The zoobenthos of lakes consist mostly of dipteran larvae, oligochaetes, nematodes, microcrustaceans, rotifers and bivalves (Strayer 2009). The presence of nematodes, annelids and Arthropoda found in benthic *A.calliptera* individuals suggests extensive foraging on zoobenthos. The diet of *Archocentrus spilurus* (jade-eyed cichlid) from Belize also contained dipteran individuals but this was a result of non-specific consumption when *A. spilurus* individuals consumed algae and

simultaneously ingested dipterans by inadvertently picking them off rocks (Cochran 2008). Similar behaviour could explain the presence of dipterans observed in benthic *Astatotilapia calliptera* stomachs contents as they were abundant in Bacillariophyta (diatoms) as well and the presence of gravel/rocks have been observed under the muddy bottom of the lake floor (Turner et al. 2019).

4.5.2 Littoral ecomorph diet contents

Littoral individuals consisted of molluscs and Chlorophyta (algae) with low abundances of fungi and mould. The lower pharyngeal jaws of littoral individuals were molar like (molariform) that are typically used for crushing shelled organisms. *Herichthys minckleyi* (Minckley's cichlid) occur in two morphological forms, one papilliform morph with needle-like teeth in the lower pharyngeal jaw and the other molariform morph with molar like teeth. Swanson et al. (2003) suggested that adaptations to pharyngeal jaw dentition was developed due to resource partitioning resulting in reduced intraspecific competition. The *Herichthys minckleyi* molariform morph developed molar-like teeth for crushing snails and harder food items while the papilliform morph primarily feed on detritus, algae and soft-bodied invertebrates (Swanson et al. 2008). Hence, the presence of molariform dentition and molluscs observed in the stomach contents suggest that littoral individuals have developed adaptations to consume molluscs or other hard bodied prey that are usually less favoured (Hulsey et al. 2006), enabling them to expand their dietary range and exploit unfavoured resources.

Fungi such as Oomycota and Cryptomycota were also found in the stomach contents of the littoral *A. calliptera* individuals. Littoral zones in lakes experience high abundance of plant litter from aquatic macrophytes that are subsequently colonised by fungi during decomposition (Gulis et al. 2006). Oomycota, especially, thrive in freshwater environments and is vital for the degradation and recycling of nutrients in the shallow zones of lakes (Sigee 2005). Consequently, Oomycota fungi play an important role in the food web dynamics of lake ecosystems (Gleason et al. 2012). Since 40-90% or organic matter found in lakes are terrestrially derived (Tanentzap et al. 2017), there is high probability that Oomycota observed in littoral individuals could have been consumed while foraging through the substrate. In addition, littoral individuals were collected from shallow areas of the lake, where plant debris is in high abundance due to the proximity to terrestrial vegetative material, increasing the likelihood of Oomycota consumption, associated with decomposing organic matter. Parasitic

species from the phylum Cryptomycota infect hosts such as algae, rotifers, crustaceans, nematodes, mosquito larvae and fish (Shearer et al. 2007; Jobard et al. 2010). Since Cryptomycota are parasitic to host taxa that were consumed by littoral individuals, the Cryptomycota reflected in the dataset could be a result of secondary consumption of the host taxa, who were then predated upon by the littoral individuals.

4.5.3 High abundance of Apicomplexa

Over 70% of the 18S sequence reads were made up of Apicomplexa that is a parasitic microbial eukaryote. 18S rDNA markers are able to target apicomplexan diversity (Rueckert et al. 2011; Cleary and Durbin 2016) and they have been highly represented in 18S datasets from marine environments and rainforests (de Vargas et al. 2015; Mahé et al. 2017). In freshwater habitats, Apicomplexa parasitises crustaceans, polychaetes, molluscs, copepods and insects. In aquatic environments, the high prevalence of Apicomplexa has the potential to alter population number and structure by causing disease and death to its host, having a significant impact on food web dynamics as host species (such as polychaetes, molluscs and copepods) form important links between phytoplankton and fish species (Del Campo et al. 2019).The high prevalence of Apicomplexa found in *Astatotilapia calliptera* individuals is almost certainly the result of secondary predation on infected organisms, however the abundance of the Apicomplexa population in Lake Masoko is unknown. Apicomplexa is well studied in human pathogens where contribution to medical studies is prioritised (El Hili et al. 2021), but we are severely lacking information on apicomplexan biodiversity in non-clinical systems such as natural freshwater lakes.

4.5.4 Overlap of benthic and littoral ecomorph diet

Presence of dietary overlap is observed in Figure 4.3 and closer inspection of Figures 4.2 and 4.5 shows that both littoral and benthic ecomorph diets consist of Apicomplexa, Arthropoda and Eustigmatophyceae. The dietary overlap could be indicative of resource partitioning based on space where the ecomorphs feed exclusively at benthic or littoral zones but the availability of similar resources at both habitats allows diet to overlap, similar to elasmobranch feeding patterns observed in Western Australia (O'Shea et al. 2013). However, it is vital to acknowledge that high amounts of Apicomplexa are likely present in the lake, parasitising a large proportion of dietary organisms. Therefore, the overrepresentation of Apicomplexans in the stomach contents blur the discrimination of dietary composition between the ecomorphs.

Alternatively, the large overlap in diet between the ecomorphs suggests high abundance of specific resources and availability of food is likely not a limiting factor. In this study all *A. calliptera* specimen we collected in August (mid dry season, when temperature is at its highest) when some species of Chironomidae are known to increase in abundance (Eggermont and Heiri 2012), thus competition for Arthropod resources between the ecomorphs is low. Prey availability in tropical systems undergo natural variation in abundance (Winemiller 1990; Correa and Winemiller 2014). Seasonal changes of temperature and rainfall influence fluctuations in planktonic diversity in crater lakes, resulting in changes of faunal composition (Umaña-Villalobos 2010). Other abiotic factors such as water level, temperature variability within the lake and internal waves can also alter faunal composition at benthic and littoral sites (Filonov et al. 2022). Niche overlap in sympatric species has been observed when seasons change resulting in opportunistic feeding behaviour on temporally abundant prey, reducing selective pressure on food exploitation (O'Shea et al. 2013; Andriollo et al. 2021). Including specimens that vary across time in metabarcoding analysis could reflect the changes in prey composition and display more intricate specialisation with less overlap between ecomorphs while addressing temporal biases introduced by dietary metabarcoding (Andriollo et al. 2021). Furthermore, metabarcoding only provides a snapshot in time of the diet, unlike stable isotope analysis that provides a longer-term perspective of energy flow patterns (Compson et al. 2019). Bayesian isotope mixing models reflected the largest contributor to benthic diet was zooplankton (depleted ^{13}C) whilst for littoral individuals 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 in this instance reflect the presence of partial/incomplete diet specialisation between *Astatotilapia calliptera* ecological morphs.

Similar diet specialisation has previously been observed in Darwin's finches (genus *Geospiza*). Darwin's finches are known to have undergone adaptive radiation to avoid competition through specialisation of resources. After a five-year study, De León et al. (2014) argue that the finches overlap to a large degree in their diet and consume some organisms exclusively which are best suited to their beak morphology. This observation suggest that the finches are generalists but when resources become limited due to spatial or temporal changes, the finches retreat to consuming resources to which they are best suited (De León et al. 2014) and these feeding patterns possibly promote coexistence. Similar patterns in diet overlap were observed in 11

different species of cichlid species from crater lake Barombi Mbo, Cameroon. The results showed the presence of dietary overlap but the authors concluded that specialised resources are used to supplement their generalist diet, especially when resources are scarce (Galvez et al. 2022).

Feeding studies of *Herichthys minckleyi* morphs concluded that papilliform and molariform individuals only consumed plant material and snails respectively when their preferred food item (in this case Arthropods as they are nutritionally rich and occur in large numbers), was in low abundance (Hulsey et al. 2006). Such feeding patterns enables *H. minckleyi* morphs to be optimal foragers by utilising most-favoured and less-favoured resources while coexisting (Robinson and Wilson 1998; Hulsey et al. 2006). To identify if *Astatotilapia calliptera* ecomorphs from Lake Masoko utilise resources optimally and to understand the drivers of diet specialisation, long-term feeding studies and monitoring of seasonal resource availability is required.

4.5.5 Primer detectability

To detect prey composition of *Astatotilapia calliptera* a combination of molecular taxonomy markers were chosen to provide amplification across a broad range of taxa (18S marker) and deliver enhanced taxonomic annotation power for macroinvertebrates (COI marker). Research has shown that a combination of COI and 18S markers improve the detection of species (Zhang et al. 2018) and are highly complementary with each other for improving diversity estimates (Giebner et al. 2020). The 18S rDNA and COI mitochondrial DNA marker detected a broad range of taxa over nine and seven different phyla respectively. However, in this study we were conservative and did not annotate sequences from the COI marker to species level due to the lower level (90%) of taxonomic coverage across the reference database. Based on the findings from previous research (Malinsky et al. 2015), we predicted littoral ecomorph diets would be dominated by macroinvertebrates. Consequently, the Leray/Geller COI primers were chosen to be used in this study as they have been designed to detect macroinvertebrate diversity in freshwater systems (Leray et al. 2013). Because the diet of both ecomorphs was dominated significantly by microorganisms and photosynthetic material the COI primer chosen was not as effective at characterising the stomach contents compared to the 18S primer. A similar result was reflected by van der Loos and Nijland (2021) where they showed that the COI region was not able to identify microscopic groups such as Apicomplexa that 18S was able to identify. A

study conducted by Alberdi et al. (2018), aimed at characterising the diets of bats from faecal pellets scrutinises the Leray/Geller primer as sequences were assigned to non-invertebrate taxa and more than 60% of the OTUs were assigned to the host species as it designed to cover entire metazoan diversity. To evaluate the performance of primers and address issues of primer biases, scientists have recommended either using mock communities (Piñol et al. 2019), experiments with real DNA extracts from the field (Alberdi et al. 2017) or in silico analysis (Elbrecht and Leese 2017) prior to embarking on a metabarcoding study. Hajibabaei et al. (2019) have recommended the use of multiple markers from COI gene to optimise detection of metazoan taxa. The use of universal primers can also lead to false positives and not detect target DNA if it is present in low concentrations (Cuff et al. 2022). Leese et al. (2021) concluded that the use of universal forward primer paired with a novel reverse primer optimised to capture benthic invertebrate diversity, avoided non-target amplification and improved detection of benthic invertebrates. The use of alternative primers that are optimised to detect the benthic diversity of African freshwater lakes would be more suited to elucidate the dietary composition of *Astatotilapia calliptera* ecomorphs from Lake Masoko to a finer scale.

4.6 Conclusion

The findings from this study demonstrate the presence of divergent dietary preferences in the benthic and littoral ecomorphs of *Astatotilapia calliptera*. With additional evidence of genetic and morphological divergence and sexual selection (Malinsky et al. 2015) in the absence of geographical barrier, *Astatotilapia calliptera* from Lake Masoko is an ideal system to study the preliminary stages of sympatric speciation. We suggest that the results improve our understanding of the behavioural mechanisms and variations in habitat resource availability that possibly facilitates diversification and adaptive radiation of cichlid fishes. Future studies must consider the effects of temporal changes in feeding habitats and resource availability. In addition, careful selection of primers is required to capture greater invertebrate diversity. The fwhf2/EPTDr2n suggested by Leese et al. (2021) has shown promise to specifically detect macroinvertebrates while MiDeca primers (Komai et al. 2019) used to amplify the 16S mitochondrial rRNA region has been proven to be successful at specifically detecting decopods specifically. These, or additional combinations of primers could be used to elucidate the diet of *A. calliptera* to a greater depth. The inclusion of environmental DNA samples (water or sediment) from regular monitoring of the lake would additionally provide information on the available resources and how they are affected by seasonal changes and spatial variation along

the depth continuum observed in Lake Masoko. With temperatures rising and anthropogenic demands for food and water increasing, it is vital to monitor how such changes impact the diversification of these nascent ecomorphs (Kalacska et al. 2017). To our knowledge, this is the first study to use metabarcoding of cichlid stomach contents for dietary analysis, and therefore provides a valuable proof-of-principal methodological resource for the wider community, in understanding trophic niche differentiation in the context of sympatric speciation.

Chapter 5: General Discussion

5.1 Research Highlights

5.1.1 Key findings from stable isotope analysis (SIA)

Stable isotopes analysis has traditionally been successfully applied in ecology to identify sources and trace fluxes of organic matter, delineate key functional traits of trophic webs, model contribution of different carbon sources, determine trophic positions and track movement of animals (Schlacher and Connolly 2014). In chapters 2 and 3, the focal species were intermediate trophic level estuarine forage fish that are numerically dominant and have substantial impact on commercial species as they form important links between basal producers and consumers at the top of the food chain. As forage fish are not directly harvested by fisheries, they are commonly disregarded in trophic ecology studies (Bouillon et al. 2011). Highlighted here are the contributions to the literature from this thesis evaluating the sources of organic matter influencing productivity within an estuarine ecosystem and trophic positioning of associated fauna.

The first key finding of the SIA work is the distinct $\delta^{13}C$ signatures from the coastal and lagoon sampling sites despite similarities in vegetative composition. Many studies debate the role of mangroves in sustaining coastal fisheries (Sheaves and Molony 2000; Kieckbusch et al. 2004; Chong 2007), however, there is no strong evidence to dispute this (Lee et al. 2014). The stable isotope analysis performed in this study and by Abeels et al. (2012) show a strong influence of mangrove carbon supporting lagoon food webs due to the depleted $\delta^{13}C$ signature observed in the fish tissue, that were similar to C_3 plant sources such as mangroves (Duarte et al. 2018). Therefore, we demonstrate the significant influence of mangrove forests on faunal food webs, that includes economically important fisheries species.

The trophic positioning of the forage fish was as predicted for all species except for glass minnows (*Anchoa mitchilli*) who had $\delta^{15}N$ values comparable to snapper (*Lutjanus griseus*) and lizard fish (*Synodus foetuns*), indicating that the glass minnows had predatory feeding habits. The observed $\delta^{15}N$ values for glass minnows are concordant with only one other published research reporting similar range of $\delta^{15}N$ values (Olsen et al. 2014). Since glass minnows are pelagic filter feeders, they consume ichthyoplankton and large zooplankton, contributing to the elevated $\delta^{15}N$ values (Vander Zanden et al. 1998; Olsen et al. 2014; Giménez

et al. 2018). However, as ichthyoplankton and zooplankton tissue samples were not included in stable isotope analysis for this study, additional prey isotopic ratios are required to confirm this association. Thus is the downfall of SIA techniques, where prior information on prey composition is essential (Hoenig et al. 2022). In addition, due to the lack of species-specific trophic interaction information, individuals were placed into discrete functional groups with disproportionate overlap (Compson et al. 2019). Therefore here, dietary metabarcoding was applied to provide species-specific prey composition data that allowed us to elucidate niche overlaps.

5.1.2 Key findings from dietary metabarcoding of intermediate trophic level fish from Estero Bay

Metabarcoding of fish stomach contents was successful at revealing species level prey composition of the intermediate trophic level forage fish. The gut content information was used to discern the dietary overlap observed along the $\delta^{13}C$ scale from the SIA analysis and confirm the presence of omnivory among the forage fish. Omnivory is usually unaccounted for, despite being common in complex aquatic food webs and is additionally known to increase food web stability, decrease strength of trophic cascades and increase productivity (Bascompte et al. 2005; Bruno and O'Connor 2005; Kratina et al. 2012; Wootton 2017; Lerner et al. 2022). The ecological networks constructed from detailed prey composition data demonstrated differences in trophic models between salinities, seasons and habitat compositions, indicating that combinations of factors influence the complexity of interactions and productivity of estuarine systems. Coastal networks are found to be more resistant to perturbations as they are more species rich compared to lagoon networks (Dunne et al. 2002; Breine et al. 2011). Similarly, the wet season network reflected an increase in species number, primarily due to recruitment (Idelberger and Greenwood 2005; Sheaves et al. 2010) but would be predicted to be less robust due to increased specialised feeding preferences (Dormann et al. 2009). The results also indicated that select fish species such as gulf pipefish, gulf killifish and inshore lizard fish, occupying seagrass beds have more specialised diets compared to fish foraging in mangrove habitats, who have a generalist feeding habits. This is the first study to use dietary metabarcoding to elucidate trophic interactions of intermediate trophic level fish from estuarine habitats and the construction of networks provided additional clarity to the functioning of the focal, complex estuarine system.

5.1.3 Key findings from dietary metabarcoding of the sympatric *Astatotilapia calliptera* ecomorphs

Here we found a large overlap in the diet of benthic (deep) and littoral (shallow) *Astatotilapia calliptera* ecomorphs with an identifiable component of specialisation in diet. The diet of benthic specimens consisted of nematodes, diatoms and copepods, while littoral individuals' diet comprised molluscs, annelid and fungi. This finding was consistent with morphological data on the dentition (papilliform and molariform) of each ecomorph and stable isotope analysis of muscle tissues (Malinsky et al. 2015; Carruthers et al. 2022). The lack of distinct niche differences between the ecomorphs can be attributed to the high abundance of Apicomplexa reads detected drowning out the true dietary variations. Additionally, we hypothesise that the dietary overlap observed is due to the high abundance of Arthropoda taxa available at the lake during the time of sampling and additional seasonal sampling is essential to detect variation in dietary patterns alongside environmental DNA samples of water and soil to characterise the resources available.

5.2 Knowledge gaps

5.2.1 The use of complementary data from stable isotope analysis and metabarcoding

In the Floridian ecosystems, the use of stable isotopes provided dietary estimates over long periods of time while DNA analysis of stomach contents provided a comprehensive snapshot prey composition. Only a handful of studies have combined molecular genetics and stable isotope data to provide dietary information of aquatic organisms (Matley et al. 2018; Compson et al. 2019; Kume et al. 2021; Cordone et al. 2022). The first study to do so investigated the source and flow of carbon, energy and nutrients through the food web of small fish from the Murray River in Australia (Hardy et al. 2010). They concluded that the combined data from SIA and molecular analysis was able to reveal food-consumer dynamics in greater detail and provided an insight into ecological connectivity and stresses that impact the whole ecosystem.

Stable isotope analysis requires a priori knowledge of prey isotopic signatures and understanding of isotopic fractionation. DNA based methods suffer from PCR and database biases, are semi-quantitative, not able to elucidate long term dietary trends and do not detect food sources that do not leave DNA signatures (this includes highly degraded detritus and non-organic nutrient sources) (Bennett et al. 2019; Whitaker et al. 2019; Compson 2020; Cuff 2022). Despite the acknowledged drawbacks, stable isotope analysis and DNA metabarcoding

are complementary (Carreon-Martinez and Heath 2010; Maloy et al. 2013; Soininen et al. 2014) and should be adopted as standard methods in future trophic ecology studies.

In this study SIA revealed the distinct effects of coastal and lagoon carbon contribution, however this was not consistently reflected in the metabarcoding dataset. SIA also indicated elevated levels of $\delta^{15}N$ in glass minnows (*Anchoa mitchili*), traditionally considered as a pelagic fish belonging to lower trophic levels. The metabarcoding data provided species level information, confirming the presence of predatory fish DNA in glass minnow guts, suggesting that the SIA data was not spurious. In addition, the metabarcoding data was used to discern the overlap along the $\delta^{13}C$ scale and enabled us to conclude that the overlap observed is primarily due to the consumed prey assimilating similar carbon sources, despite prey composition of predators being diverse. Metabarcoding performed on *Astatotilapia calliptera* stomach contents was supplemented by SIA data from Carruthers et al. (2022) and the combined datasets enabled in-depth characterisation of the dietary differences between the benthic and littoral ecomorphs. The high resolution of metabarcoding provided detailed prey information that was used to construct ecological networks providing a deeper understanding of ecosystem functioning and how variables such as salinity, season, habitat assemblage and depth affects such functions. Hence this study demonstrated the value of using multiple complementary techniques to successfully reveal trophic dynamics in greater detail which would not have been possible if the methods were applied independently.

5.2.2 Using dietary metabarcoding data to construct ecological networks

The comprehensive information resulting from dietary metabarcoding made it possible to construct ecological networks. Ecological networks provide an insight into ecosystem functioning, community dynamics and potential effects of temporal and spatial variation (Roslin et al. 2019). Comparisons between networks can be applied to assess natural or anthropogenic impacts, the evolution of networks and importance of specific nodes/species (Clare et al. 2019). Despite being recommended by previous research (Evans et al. 2016; Roslin and Majaneva 2016), dietary metabarcoding data has not been widely applied into ecological network analysis. The results from this study demonstrate that DNA metabarcoding is a successful alternative to laborious field observations and morphological identification of stomach contents. Only a limited number of recent studies have applied dietary metabarcoding to network ecology (Clare et al. 2019; Cuff et al. 2021; Hemprich-Bennett et al. 2021; Mata et

al. 2021), however, none include interactions of fish in mangrove ecosystems or freshwater lakes. Furthermore, Cuff et al. (2022) highlights that despite the emergence of some studies integrating dietary metabarcoding with ecological methods, metabarcoding has been the primary method used and the integration of additional methods (such as field observation, morphological identification of stomach/faecal content, SIA) to construct networks are yet to be explored.

5.3 Future considerations

The results from this study demonstrate the complementary use of dietary metabarcoding and stable isotope analysis to elucidate trophic dynamics of mangrove and freshwater fish species. One shortcoming of this study is that it does not merge SIA and metabarcoding data to construct ecological networks. SIA data from potential prey taxa is required to identify the contribution of each resource and application of mixing models are vital to make inferences about the composition of consumers' assimilated diet (Phillips et al. 2014a). Mixing models convert isotopic data into estimates of food source contribution from various components of the consumer diet (Phillips 2012). Thus, future research should have comprehensive understanding of the study animals' diet prior to sampling, and this can be achieved through traditional observational techniques or via the literature (Philips et al. 2014).

Located northeast of Estero Bay is the vast freshwater Lake Okeechobee (10.76 km^2) and its associated catchment area. The presence of intensive farming practices around the Okeechobee watershed has resulted in nitrate run-offs due to continued application of ammonium fertilisers (Ma et al. 2020). Fresh water inflow into Estro Bay during the summer months (July to October) is primarily derived from tributaries (such as Matanzas Pass and Mullock Creek) that are connected to the Tenmile Canal which is directly linked to Lake Okeechobee (Thomas and Rumbold 2006). Previous research has shown that contamination from the run-off from fertilisers can contaminate the tissues of bivalves in Estero Bay (Mitra et al. 2011). Assimilation of nitrogen compounds from ammonium inputs affect the $\delta^{15}N$ signature of soil and surface water (Ma et al. 2020). However, the contribution of anthropogenic nitrogen sources was not considered in this study. It is therefore vital to investigate the effects of anthropogenic nitrogen sources on Estero Bay ichthyofauna and subsequently variations in their $\delta^{15}N$ signature. This can be carried out by sampling potential prey items, suspended organic matter and soil from Lake Okeechobee and along the tributaries leading into Estero Bay. Prey items from Lake

Okeechobee will have distinct $\delta^{15}N$ signature that can used as a fingerprint to trace the fate of anthropogenic nitrogen across a spatial scale (Heaton 1986) and its effects on the mangrove food web. The $\delta^{15}N$ signature of organisms from Lake Okeechobee are expected to have elevated $\delta^{15}N$ signatures as human derived/synthesized nitrogen is typically enriched (Medina-Contreras and Arenas 2023). Combined with mixing models mentioned above, the level of contribution from anthropogenic nitrogen assimilated into mangrove fish could therefore be modelled.

Some shortcomings of constructing networks using metabarcoding include the viability of quantification and sampling completeness. PCR based metabarcoding introduces PCR primer bias and random sampling during sequencing making it difficult to quantify (Murray et al. 2011; Leray and Knowlton 2017). Transforming the data to binary presence/absence may be a viable alternative but will represent inaccurate network weightings (Clare 2014). Normalising frequency of interaction data has been recommended as the best type of data to use when integrating dietary metabarcoding to ecological networks (Cuff et al. 2022). Achieving high sampling completeness (>80%) ensures that sampling undertaken is sufficient and provides validity to the network that reflects true interactions (McGregor et al. 2017). In this study sampling completeness was below the recommended threshold but the results can be used as baseline information for future sampling. To increase sampling completeness, sampling effort (inclusion of more specimens and use of additional primers) must be increased as this increases the chances of identifying new interactions and rare species (Henriksen et al. 2019).

5.4 Implications

We demonstrate that SIA coupled with dietary metabarcoding is a useful tool to obtain most comprehensive insights into trophic interactions that is not possible when techniques are applied independently. We show that intermediate trophic level estuarine species exhibit complex feeding interactions and are important conduits of energy transfer to higher trophic levels as they feed on a mixture of autotrophs and heterotrophs. Some studies have suggested that intermediate trophic level species are more susceptible to environmental perturbations and will subsequently affect commercially important species (Cury et al. 2000; Boldt et al. 2022). Even though they are not always commercially exploited (often bycatch species) it is vital to not disregard their contribution to ecosystem function and understand how their complex

interactions have a bottom-up and top-down control on other organisms within the tropical estuarine ecosystem.

Our environmental samples detected the presence of harmful algae (family: Kareniaceae) in Estero Bay and in the fish stomach contents despite not being visible to the human eye during sampling visits. This reflects the ability of DNA based (environmental DNA) methods to be a useful tool for detecting the persistence of toxic organisms that poses harm to both humans and wildlife. Current water quality methods employed in Estero Bay does not include species specific detection of *Karenia brevis* using DNA based monitoring methods (SECOORA 2022). The implementation of such molecular methods will enable scientists to track *K.brevis* even at low levels and at the early stages of blooms.

In conclusion, the results demonstrate how the combination of multiple molecular methods are necessary to construct accurate networks that reflect the true interactions within ecosystems. Describing and quantifying interactions are central to ecology as it provides insights into ecosystem responses to species extinctions, habitat loss, climate change and anthropogenic influences such as habitat modification (Bascompte 2009). Furthermore, integrating molecular based networks with foraging behaviour and metabolic data will provide more predictive power to understanding ecosystem changes (Ings et al. 2009). Using network ecology data to understand structure and functioning of ecosystems has the potential to play a critical role in effective conservation and management, whilst ensuring sustainable growth of associated economies and societal progress.

References

Abeels, H.A., Loh, A.N. and Volety, A.K. 2012. Trophic transfer and habitat use of oyster Crassostrea virginica reefs in southwest Florida, identified by stable isotope analysis. *Marine Ecology Progress Series* 462, pp. 125–142. doi: 10.3354/meps09824.

Abel, K.M. 1984. Inorganic Carbon Source for Photosynthesis in the Seagrass Thalassia hemprichii (Ehrenb.) Aschers.

Abrahamczyk, S. and Kessler, M. 2014. Morphologische und Verhaltensanpassungen um Nektar zu fressen: Wie die Nahrungsökologie den Artenreichtum und die Zusammensetzung von Kolibrigemeinschaften bestimmt. *Journal of Ornithology* 156(2), pp. 333–347. doi: 10.1007/s10336-014-1146-5.

Abrantes, K. and Sheaves, M. 2009. Food web structure in a near-pristine mangrove area of the Australian Wet Tropics. *Estuarine, Coastal and Shelf Science* 82(4), pp. 597–607. doi: 10.1016/j.ecss.2009.02.021.

Abrantes, K.G., Johnston, R., Connolly, R.M. and Sheaves, M. 2015. Importance of Mangrove Carbon for Aquatic Food Webs in Wet-Dry Tropical Estuaries. 38(1), pp. 383–399. Available at: https://www.jstor.org/stable/44851301.

Adams, D.H., Sebastian, A. and Paperno, R. 2022. Population decline of Gulf pipefish (Syngnathus scovelli) in a subtropical estuary: ecosystem changes and habitat loss. *Marine Biodiversity* 52. Available at: https://doi.org/10.1007/s12526-022-01289-x.

Adler, P. and Courtney, G. 2019. Ecological and Societal Services of Aquatic Diptera. *Insects* 10(3), p. 70. doi: 10.3390/insects10030070.

Alberdi, A., Aizpurua, O., Gilbert, M.T.P. and Bohmann, K. 2018. Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution* 9(1), pp. 134–147. doi: 10.1111/2041-210X.12849.

Albertsen, M., Karst, S.M., Ziegler, A.S., Kirkegaard, R.H. and Nielsen, P.H. 2015. Back to basics - The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. *PLoS ONE* 10(7). doi: 10.1371/journal.pone.0132783.

Albouy, C. et al. 2019. The marine fish food web is globally connected. Nature Ecology and *Evolution* 3(8), pp. 1153–1161. doi: 10.1038/s41559-019-0950-y.

Aljanabi, S.M. and Martinez, I. 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Oxford University Press.

Allen, D.M., Johnson, W.S. and Ogburn-Matthews, V. 1995. Trophic relationships and seasonal utilization of salt-marsh creeks by zooplanktivorous fishes. *Environmental Biology of Fishes* 42(1), pp. 37–50. doi: 10.1007/BF00002350.

Almén, M.S., Lamichhaney, S., Berglund, J., Grant, B.R., Grant, P.R., Webster, M.T. and Andersson, L. 2016. Adaptive radiation of Darwin's finches revisited using whole genome sequencing. *BioEssays* 38(1), pp. 14–20. doi: 10.1002/bies.201500079.

American Veterinary Medical Association. 2020. AVMA guidelines for the euthanasia of animals : 2020 edition.

Amundsen, P.A. and Sánchez-Hernández, J. 2019. Feeding studies take guts – critical review and recommendations of methods for stomach contents analysis in fish. Journal of Fish *Biology* 95(6), pp. 1364–1373. doi: 10.1111/jfb.14151.

Andrews, S. 2010. FastQC: A Quality Control Tool for High Throughput Sequence Data. Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [Accessed: 31 August 2022].

Andriollo, T., Michaux, J.R. and Ruedi, M. 2021. Food for everyone: Differential feeding habits of cryptic bat species inferred from DNA metabarcoding. *Molecular Ecology* 30(18), pp. 4584–4600. doi: 10.1111/mec.16073.

Araújo, M.S., Guimarães, P.R., Svanbäck, R., Pinheiro, A., Guuimarães, P., Dos Reis, S.F. and Bolnick, D.I. 2008. Network analysis reveals contrasting effects of intraspecific competition on individual vs. population diets. *Ecology* 89(7), pp. 1981–1993. doi: 10.1890/07-0630.1.

Backer, L.C. et al. 2003. Recreational exposure to aerosolized brevetoxins during Florida red tide events. *Harmful Algae* 2(1), pp. 19–28. doi: 10.1016/S1568-9883(03)00005-2.

Baran, E. and Hambrey, J. 1998. Mangrove Conservation and Coastal Management in Southeast Asia: What Impact on Fishery Resources? *Marine Pollution Bulletin* 37, pp. 431–440. Available at: http://www.odci.gov/cia/publications/nsolo/.

De Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E. and Taberlet, P. 2014. DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. *Molecular Ecology Resources* 14(2), pp. 306–323. doi: 10.1111/1755-0998.12188.

Barker, P., Williamson, D., Gasse, F. and Gibert, E. 2003. Climatic and volcanic forcing revealed in a 50,000-year diatom record from Lake Massoko, Tanzania. Quaternary Research. doi: 10.1016/s0033-5894(03)00114-5.

Bascompte, J. 2007. Networks in ecology. *Basic and Applied Ecology* 8(6), pp. 485–490. doi: 10.1016/j.baae.2007.06.003.

Bascompte, J. 2009. Disentangling the Web of Life. *Science* 325(5939), pp. 416–419. doi: 10.1126/science.1171022.

Bascompte, J., Meliá, C.J. and Sala, E. 2005. Interaction strength combinations and the overfishing of a marine food web. *PNAS* 102(15), pp. 5443–5447. Available at: https://www.pnas.org.

Bastida, J.M., Alcántara, J.M., Rey, P.J., Vargas, P. and Herrera, C.M. 2010. Extended phylogeny of Aquilegia: The biogeographical and ecological patterns of two simultaneous but

contrasting radiations. *Plant Systematics and Evolution* 284(3), pp. 171–185. doi: 10.1007/s00606-009-0243-z.

Beaudoin, C.P., Tonn, W.M., Prepas, E.E. and Wassenaar, L.I. 1999. Individual specialization and trophic adaptability of northern pike (Esox lucius): an isotope and dietary analysis. *Oecologia* 120, pp. 386–396.

Beaugrand, G., Brander, K.M., Lindley, J.A., Souissi, S. and Reid, P.C. 2003. Plankton effect on cod recruitment in the North Sea. *Nature* 426(6967), pp. 661–664. doi: 10.1038/nature02164.

Beaugrand, G. and Reid, P.C. 2012. Relationships between North Atlantic salmon, plankton, and hydroclimatic change in the Northeast Atlantic. *ICES Journal of Marine Science* 69(9), pp. 1549–1562. doi: 10.1093/icesjms/fss153.

Bennett, A.E., Evans, D.M. and Powell, J.R. 2019. Potentials and pitfalls in the analysis of bipartite networks to understand plant–microbe interactions in changing environments. *Functional Ecology* 33(1), pp. 107–117. doi: 10.1111/1365-2435.13223.

Berlow, E.L., Brose, U. and Martinez, N.D. 2008. Success and its limits among structural models of complex food webs. *Journal of Animal Ecology* 77(3), pp. 512–519. doi: 10.1111/j.1365-2656.2008.01362.x.

Bernardino, A.F., Gomes, L.E. de O., Hadlich, H.L., Andrades, R. and Correa, L.B. 2018. Mangrove clearing impacts on macrofaunal assemblages and benthic food webs in a tropical estuary. *Marine Pollution Bulletin* 126, pp. 228–235. doi: 10.1016/j.marpolbul.2017.11.008.

Berry, O., Bulman, C., Bunce, M., Coghlan, M., Murray, D.C. and Ward, R.D. 2015. Comparison of morphological and DNA metabarcoding analyses of diets in exploited marine fishes. *Marine Ecology Progress Series* 540, pp. 167–181. doi: 10.3354/meps11524.

Bharathi, M.D., Venkataramana, V. and Sarma, V.V.S.S. 2022. Phytoplankton community structure is governed by salinity gradient and nutrient composition in the tropical estuarine system. *Continental Shelf Research* 234. doi: 10.1016/j.csr.2021.104643.

Boenigk, J. and Stadler, P. 2004. Potential toxicity of chrysophytes affiliated with Poterioochromonas and related “Spumella-like” flagellates. *Journal of Plankton Research* 26(12), pp. 1507–1514. doi: 10.1093/plankt/fbh139.

Bohmann, K. et al. 2022. Strategies for sample labelling and library preparation in DNA metabarcoding studies. *Molecular Ecology Resources* 22(4), pp. 1231–1246. doi: 10.1111/1755-0998.13512.

Boldt, J.L. et al. 2022. Canada’s forage fish: an important but poorly understood component of marine ecosystems. *Canadian Journal of Fisheries and Aquatic Sciences* 79(11), pp. 1911–1933. doi: 10.1139/cjfas-2022-0060.

Bonin, M., Dussault, C., Taillon, J., Lecomte, N. and Côté, S.D. 2020. Combining stable isotopes, morphological, and molecular analyses to reconstruct the diet of free-ranging consumers. *Ecology and Evolution* 10(13), pp. 6664–6676. doi: 10.1002/ece3.6397.

Borrvall, C., Ebenman, B. and Jonsson, T. 2000. Biodiversity lessens the risk of cascading extinction in model food webs. *Ecology Letters* 3(2), pp. 131–136. doi: 10.1046/j.1461-0248.2000.00130.x.

Bouillon, S., Connolly, R.M. and Gillikin, D.P. 2011. Use of Stable Isotopes to Understand Food Webs and Ecosystem Functioning in Estuaries. Treatise on Estuarine and Coastal *Science* 7, pp. 143–173. doi: 10.1016/B978-0-12-374711-2.00711-7.

Bouillon, S., Connolly, R.M. and Lee, S.Y. 2008. Organic matter exchange and cycling in mangrove ecosystems: Recent insights from stable isotope studies. *Journal of Sea Research* 59(1–2), pp. 44–58. doi: 10.1016/j.seares.2007.05.001.

Boyi, J.O., Heße, E., Rohner, S., Säurich, J., Siebert, U., Gilles, A. and Lehnert, K. 2022. Deciphering Eurasian otter (Lutra lutra L.) and seal (Phoca vitulina L.; Halichoerus grypus F.) diet: Metabarcoding tailored for fresh and saltwater fish species. *Molecular Ecology* 31(19), pp. 5089–5106. doi: 10.1111/mec.16635.

Bradley, R.D. and Hillis, D.M. 1997. Recombinant DNA sequences generated by PCR amplification. *Mol. Biol. Evol*. 14, pp. 592–593.

Braga, R.R., Bornatowski, H. and Vitule, J.R.S. 2012. Feeding ecology of fishes: An overview of worldwide publications. *Reviews in Fish Biology and Fisheries* 22(4), pp. 915–929. doi: 10.1007/s11160-012-9273-7.

Brand, L.E. and Compton, A. 2007. Long-term increase in Karenia brevis abundance along the Southwest Florida Coast. *Harmful Algae* 6(2), pp. 232–252. doi: 10.1016/j.hal.2006.08.005.

Bråte, J., Logares, R., Berney, C., Ree, D.K., Klaveness, D., Jakobsen, K.S. and Shalchian-Tabrizi, K. 2010. Freshwater perkinsea and marine-freshwater colonizations revealed by pyrosequencing and phylogeny of environmental rDNA. *ISME Journal* 4(9), pp. 1144–1153. doi: 10.1038/ismej.2010.39.

Breine, J., Maes, J., Ollevier, F. and Stevens, M. 2011. Fish assemblages across a salinity gradient in the Zeescheldeestuary (Belgium). *Belg. J. Zool* 141(2), pp. 21–44.

Brown, L.E., Edwards, F.K., Milner, A.M., Woodward, G. and Ledger, M.E. 2011. Food web complexity and allometric scaling relationships in stream mesocosms: Implications for experimentation. *Journal of Animal Ecology* 80(4), pp. 884–895. doi: 10.1111/j.1365-2656.2011.01814.x.

Bruford, M.W., Hanotte, O., Brookfield, J.F.Y. and Burke, T. 1998. Multilocus and single-locus DNA fingerprinting. In: Hoelzel, A. R. ed. Molecular Genetic Analysis of Populations: A Practical Approach. Oxford University Press, pp. 287–336.

Bruno, J.F. and O'Connor, M.I. 2005. Cascading effects of predator diversity and omnivory in a marine food web. *Ecology Letters* 8(10), pp. 1048–1056. doi: 10.1111/j.1461-0248.2005.00808.x.

Buelow, C. and Sheaves, M. 2015. A birds-eye view of biological connectivity in mangrove systems. *Estuarine, Coastal and Shelf Science* 152, pp. 33–43. doi: 10.1016/j.ecss.2014.10.014.

Bui, T.H.H. and Lee, S.Y. 2014. Does "you are what you eat" apply to mangrove grapsid crabs? *PLoS ONE* 9(2). doi: 10.1371/journal.pone.0089074.

Burbank, J., DeJong, R.A., Turcotte, F. and Rolland, N. 2022. Understanding factors influencing Atlantic herring (Clupea harengus) recruitment: From egg deposition to juveniles. *Fisheries Oceanography*. doi: 10.1111/fog.12621.

Burress, E.D. 2015. Cichlid fishes as models of ecological diversification: patterns, mechanisms, and consequences. *Hydrobiologia* 748(1), pp. 7–27. doi: 10.1007/s10750-014-1960-z.

Burton, W.H. 1892. Comparative ecology of two sympatric species of atherinids, Comparative ecology of two sympatric species of atherinids, Menidia menidia and Membras martinica. Virginia Institute of Marine Science. Available at: https://dx.doi.org/doi:10.25773/v5-r878-hb37.

Butler, M.A., Sawyer, S.A. and Losos, J.B. 2007. Sexual dimorphism and adaptive radiation in Anolis lizards. *Nature* 447(7141), pp. 202–205. doi: 10.1038/nature05774.

Byrne, M.J. and Gabaldon, J.N. 2007. Hydrodynamic Characteristics and Salinity Patterns in Estero Bay, Lee County, Florida. *Geological Survey Scientific Investigations Report* 2007-5217. Available at: http://www.usgs.gov/pubprod.

Callahan, B.J., McMurdie, P.J. and Holmes, S.P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME Journal* 11(12), pp. 2639–2643. doi: 10.1038/ismej.2017.119.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. and Holmes, S.P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* 13(7), pp. 581–583. doi: 10.1038/nmeth.3869.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. and Madden, T.L. 2009. BLAST+: Architecture and applications. *BMC Bioinformatics* 10. doi: 10.1186/1471-2105-10-421.

Del Campo, J., Heger, T.J., Rodríguez-Martínez, R., Worden, A.Z., Richards, T.A., Massana, R. and Keeling, P.J. 2019. Assessing the diversity and distribution of apicomplexans in host and free-living environments using high-throughput amplicon data and a phylogenetically informed reference framework. *Frontiers in Microbiology* 10(OCT). doi: 10.3389/fmicb.2019.02373.

Canto-Maza, W.G. and Vega-Cendejas, E. 2008. Feeding habits of the fish Lagodon rhomboides (Perciformes: Sparidae) at the coastal lagoon of Chelem, Yucatán, México. Rev. *Biol. Trop. Int. J. Trop. Biol.* ISSN 56(4), pp. 1837–1846.

Careddu, G., Carlini, N., Romano, A., Rossi, L., Calizza, E., Sporta Caputi, S. and Costantini, M.L. 2020. Diet composition of the Italian crested newt (Triturus carnifex) in structurally different artificial ponds based on stomach contents and stable isotope analyses. Aquatic Conservation: *Marine and Freshwater Ecosystems* 30(8), pp. 1505–1520. doi: 10.1002/aqc.3383.

Carreon-Martinez, L. and Heath, D.D. 2010. Revolution in food web analysis and trophic ecology: diet analysis by DNA and stable isotope analysis. Molecular Ecology 19, pp. 25–27.

Carruthers, M. et al. 2022. Ecological speciation promoted by divergent regulation of functional genes within African cichlid fishes. *bioRxiv*. Available at: https://doi.org/10.1101/2022.01.07.475335.

Carter, H.N., Schmidt, S.W. and Hirons, A.C. 2015. An international assessment of mangrove management: Incorporation in integrated coastal zone management. *Diversity* 7(2), pp. 74–104. doi: 10.3390/d7020074.

Casey, J.M. et al. 2021. DNA metabarcoding marker choice skews perception of marine eukaryotic biodiversity. *Environmental DNA* 3(6), pp. 1229–1246. doi: 10.1002/edn3.245.

Casey, J.M., Meyer, C.P., Morat, F., Brandl, S.J., Planes, S. and Parravicini, V. 2019. Reconstructing hyperdiverse food webs: Gut content metabarcoding as a tool to disentangle trophic interactions on coral reefs. *Methods in Ecology and Evolution* 10(8), pp. 1157–1170. doi: 10.1111/2041-210X.13206.

Castillo-Rivera, M., Zárate-Hernández, R., Ortiz-Burgos, S. and Zavala-Hurtado, J. 2010. Diel and seasonal variability in the fish community structure of a mud-bottom estuarine habitat in the Gulf of Mexico. *Marine Ecology* 31(4), pp. 633–642. doi: 10.1111/j.1439-0485.2010.00394.x.

Cavalier-Smith, T., Lewis, R., Chao, E.E., Oates, B. and Bass, D. 2009. Helkesimastix marina n. sp. (Cercozoa: Sainouroidea superfam. n.) a Gliding Zooflagellate of Novel Ultrastructure and Unusual Ciliary Behaviour. *Protist* 160(3), pp. 452–479. doi: 10.1016/j.protis.2009.03.003.

Chacoff, N.P., Vázquez, D.P., Lomáscolo, S.B., Stevani, E.L., Dorado, J. and Padrón, B. 2012. Evaluating sampling completeness in a desert plant-pollinator network. *Journal of Animal Ecology* 81(1), pp. 190–200. doi: 10.1111/j.1365-2656.2011.01883.x.

Châline, N., Ratnieks, F.L.W., Raine, N.E., Badcock, N.S. and Burke, T. 2004. Non-lethal sampling of honey bee, Apis mellifera , DNA using wing tips. *Apidologie* 35(3), pp. 311–318. doi: 10.1051/apido:2004015.

Chevillot, X. et al. 2018. Global Changes Jeopardize the Trophic Carrying Capacity and Functioning of Estuarine Ecosystems. *Ecosystems* 22(3), pp. 473–495. doi: 10.1007/s10021-018-0282-9.

Chong, V.C. 2007. MANGROVES-FISHERIES LINKAGES-THE MALAYSIAN PERSPECTIVE. *BULLETIN OF MARINE SCIENCE* 80(3), pp. 755–772.

Christiansen, P. and Wroe, S. 2007. Bite forces and evolutionary adaptations to feeding ecology in carnivores. *Ecology* 88(2), pp. 347–358. doi: 10.1890/0012-9658(2007)88[347:BFAEAT]2.0.CO;2.

Clare, E.L. 2014. Molecular detection of trophic interactions: Emerging trends, distinct advantages, significant considerations and conservation applications. *Evolutionary Applications* 7(9), pp. 1144–1157. doi: 10.1111/eva.12225.

Clare, E.L. et al. 2019. Approaches to integrating genetic data into ecological networks. *Molecular Ecology* 28(2), pp. 503–519. doi: 10.1111/mec.14941.

Clark, B. et al. 2022. Oca2 targeting using CRISPR/Cas9 in the Malawi cichlid Astatotilapia calliptera. *Royal Society Open Science* 9(4). doi: 10.1098/rsos.220077.

Cleary, A.C. and Durbin, E.G. 2016. Unexpected prevalence of parasite 18S rDNA sequences in winter among Antarctic marine protists. *Journal of Plankton Research* 38(3), pp. 401–417. Available at: https://academic.oup.com/plankt/article/38/3/401/2223599.

Cochran, J.L. 2008. DIET, HABITAT, AND ECOMORPHOLOGY OF CICHLIDS IN THE UPPER BLADDEN RIVER, BELIZE. Saint Mary's University of Minnesota.

Coe, T.S., Hamilton, P.B., Griffiths, A.M., Hodgson, D.J., Wahab, M.A. and Tyler, C.R. 2009. Genetic variation in strains of zebrafish (Danio rerio) and the implications for ecotoxicology studies. *Ecotoxicology* 18(1), pp. 144–150. doi: 10.1007/s10646-008-0267-0.

Cohen, J. 1992. Statistical Power Analysis. Current directions in psychological science 1(3). Cohen, J.E. 1994. Marine and continental food webs: three paradoxes? *Phil Trans R Soc Lond* B 343, pp. 59–69. Available at: https://royalsocietypublishing.org/.

Cohen, J.E., Jonsson, T. and Carpenter, S.R. 2003. Ecological community description using the food web, species abundance, and body size. *PNAS* 100, pp. 1781–1786. Available at: www.pnas.orgcgidoi10.1073pnas.232715699.

Compson, Z.G. et al. 2019. Network-Based Biomonitoring: Exploring Freshwater Food Webs With Stable Isotope Analysis and DNA Metabarcoding. *Frontiers in Ecology and Evolution* 7. doi: 10.3389/fevo.2019.00395.

Compson, Z.G., McClenaghan, B., Singer, G.A.C., Fahner, N.A. and Hajibabaei, M. 2020. Metabarcoding From Microbes to Mammals: Comprehensive Bioassessment on a Global Scale. *Frontiers in Ecology and Evolution* 8. doi: 10.3389/fevo.2020.581835.

Cordone, G. et al. 2022. Metabarcoding, direct stomach observation and stable isotope analysis reveal a highly diverse diet for the invasive green crab in Atlantic Patagonia. *Biological Invasions* 24(2), pp. 505–526. doi: 10.1007/s10530-021-02659-5.

Correa, S.B. and Winemiller, K.O. 2014. Niche partitioning among frugivorous fishes in response to fluctuating resources in the Amazonian floodplain forest. *Ecology* 95(1), pp. 210–224.

Corso, G., De Araujo, A.I.L. and De Almeida, A.M. 2011. Connectivity and nestedness in bipartite networks from community ecology. Journal of Physics: *Conference Series* 285(1). doi: 10.1088/1742-6596/285/1/012009.

Costa, J.M., da Silva, L.P., Ramos, J.A. and Heleno, R.H. 2016. Sampling completeness in seed dispersal networks: When enough is enough. *Basic and Applied Ecology* 17(2), pp. 155–164. doi: 10.1016/j.baae.2015.09.008.

Costanza, R. et al. 2014. Changes in the global value of ecosystem services. Global *Environmental Change* 26(1), pp. 152–158. doi: 10.1016/j.gloenvcha.2014.04.002.

Creer, S. et al. 2016. The ecologist's field guide to sequence-based identification of biodiversity. *Methods in Ecology and Evolution* 7(9), pp. 1008–1018. doi: 10.1111/2041-210X.12574.

Croker, R.A. 1962. Growth and Food of the Gray Snapper, Lutjanus griseus in Everglades National Park. *Transactions of the American Fisheries Society* 91(4), pp. 379–383. doi: 10.1577/1548-8659(1962)91[379:gafotg]2.0.co;2.

Cruz-Escalona, V.H., Peterson, M.S., Campos-Dávila, L. and Zetina-Rejón, M. 2005. Feeding habits and trophic morphology of inshore lizardfish (Synodus foetens) on the central continental shelf off Veracruz, Gulf of Mexico. *Journal of Applied Ichthyology* 21(6), pp. 525–530. doi: 10.1111/j.1439-0426.2005.00651.x.

Cuff, J.P. et al. 2021. Money spider dietary choice in pre- and post-harvest cereal crops using metabarcoding. *Ecological Entomology* 46(2), pp. 249–261. doi: 10.1111/een.12957.

Cuff, J.P., Windsor, F.M., Tercel, M.P.T.G., Kitson, J.J.N. and Evans, D.M. 2022. Overcoming the pitfalls of merging dietary metabarcoding into ecological networks. *Methods in Ecology and Evolution* 13(3), pp. 545–559. doi: 10.1111/2041-210X.13796.

Da Cunha, A.F., Wolff, L.L. and Hahn, N.S. 2018. Seasonal changes at population and individual levels in the diet of juvenile catfish in a Neotropical floodplain. Journal of *Freshwater Ecology* 33(1), pp. 273–284. doi: 10.1080/02705060.2018.1442371.

Currin, C.A., Wainright, S.C., Able, K.W., Weinstein, M.P. and Fuller, C.M. 2003. Determination of Food Web Support and Trophic Position of the Mummichog, Fundulus heteroclitus, in New Jersey Smooth Cordgrass (Spartina alterniflora), Common Reed. *Archives of Environmental Contamination and Toxicology* 59, pp. 495–510.

Curti, K.L. 2005. PATTERNS IN THE DISTRIBUTION, DIET AND TROPHIC DEMAND OF THE HOGCHOKER, TRINECTES MACULATUS, IN THE CHESAPEAKE BAY, USA. University of Maryland.

Curtin, R. and Prellezo, R. 2010. Understanding marine ecosystem based management: A literature review. *Marine Policy* 34(5), pp. 821–830. doi: 10.1016/j.marpol.2010.01.003.

Cury, P., Bakun, A., Crawford, R.J.M., Jarre, A., Quiñones, R.A., Shannon, L.J. and Verheye, H.M. 2000. Small pelagics in upwelling systems: Patterns of interaction and structural

changes in “wasp-waist” ecosystems. In: *ICES Journal of Marine Science*. Academic Press, pp. 603–618. doi: 10.1006/jmsc.2000.0712.

Cury, P., Shannon, L., Shannon, L. and Shin, Y.-J. 2003. The functioning of marine ecosystems: a fisheries perspective 7 The Functioning of Marine Ecosystems: a Fisheries Perspective. Rome, Italy.

Dadebo, E., Eyayu, A., Sorsa, S. and Tilahun, G. 2015. Food and Feeding Habits of the Common Carp (Cyprinus carpio L. 1758) (Pisces: Cyprinidae) in Lake Koka, Ethiopia.

Dame, J.K. and Christian, R.R. 2006. Uncertainty and the Use of Network Analysis for Ecosystem-Based Fishery Management. *Fisheries* 31(7), pp. 331–341. doi: 10.1577/1548-8446(2006)31[331:uatuon]2.0.co;2.

Danley, P.D. and Kocher, T.D. 2001. Speciation in rapidly diverging systems: lessons from Lake Malawi. *Molecular Ecology* 10, pp. 1075–1086.

Darcy, G.H. 1981. FOOD HABITS OF THE CRESTED GOBY, LOPHOGOBIUS CYPRINOIDES, IN TWODADE COUNTY, FLORIDA, WATERWAYS. *BULLETIN OF MARINE SCIENCE* 31(4), pp. 928–932.

Davias, L.A., Kornis, M.S. and Breitburg, D.L. 2014. Environmental factors influencing δ13C and δ15N in three Chesapeake bay fishes. *ICES Journal of Marine Science* 71(3), pp. 689–702. doi: 10.1093/icesjms/fst143.

Deagle, B.E. et al. 2019a. Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? *Molecular Ecology* 28(2), pp. 391–406. doi: 10.1111/mec.14734.

Deagle, B.E. et al. 2019b. Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? *Molecular Ecology* 28(2), pp. 391–406. doi: 10.1111/mec.14734.

Deniro, M. and Epstein, S. 1981. Influence of diet on the distribution of nitrogen isotopes in animals*. *Geochimica et Cosmochimica Acta* 45, pp. 341–351.

DeNiro, M.J. and Epstein, S. 1978. Influence of diet on the distribution of carbon isotopes in animals*. *Geochimica et Cosmochimica Acta* 42, pp. 495–506.

Dhont, J. and Dierckens, K. 2013. Rotifers, Artemia and copepods as live feeds for fish larvae in aquaculture. In: Allan, G. and Burnell, G. eds. Advances in aquaculture hatchery technology. Woodhead Publishing, pp. 157–202.

De Dios Arcos, C., Badillo-Alemán, M., Arceo-Carranza, D. and Chiappa-Carrara, X. 2020. Feeding ecology of the waterbirds in a tropical mangrove in the southeast Gulf of Mexico. *Studies on Neotropical Fauna and Environment* 55(1), pp. 1–9. doi: 10.1080/01650521.2019.1682232.

Dittel, A.I., Epifanio, C.E. and Fogel, M.L. 2006. Trophic relationships of juvenile blue crabs (Callinectes sapidus) in estuarine habitats. *Hydrobiologia* 568(1), pp. 379–390. doi: 10.1007/s10750-006-0204-2.

Doi, H. et al. 2008. Attached microalgae contribute to planktonic food webs in bays with fish and pearl oyster farms. *Marine Ecology Progress Series* 353, pp. 107–113. doi: 10.3354/meps07202.

Donato, D.C., Kauffman, J.B., Murdiyarso, D., Kurnianto, S., Stidham, M. and Kanninen, M. 2011. Mangroves among the most carbon-rich forests in the tropics. *Nature Geoscience* 4(5), pp. 293–297. doi: 10.1038/ngeo1123.

Dorenbosch, M., Grol, M.G.G., Nagelkerken, I. and van der Velde, G. 2006. Seagrass beds and mangroves as potential nurseries for the threatened Indo-Pacific humphead wrasse, Cheilinus undulatus and Caribbean rainbow parrotfish, Scarus guacamaia. *Biological Conservation* 129(2), pp. 277–282. doi: 10.1016/j.biocon.2005.10.032.

Dormann, C.F. 2022. Using bipartite to describe and plot two-mode networks in R. Dormann, C.F., Fründ, J., Blüthgen, N. and Gruber, B. 2009. Indices, Graphs and Null Models: Analyzing Bipartite Ecological Networks. *The Open Ecology Journal* 2, pp. 7–24.

Drake, L.E., Cuff, J.P., Young, R.E., Marchbank, A., Chadwick, E.A. and Symondson, W.O.C. 2022. An assessment of minimum sequence copy thresholds for identifying and reducing the prevalence of artefacts in dietary metabarcoding data. *Methods in Ecology and Evolution* 13(3), pp. 694–710. doi: 10.1111/2041-210X.13780.

Duarte, C.M. et al. 2018. Stable Isotope (δ13C, δ15N, δ18O, δD) composition and nutrient concentration of Red Sea primary producers. *Frontiers in Marine Science* 5(AUG). doi: 10.3389/fmars.2018.00298.

Dunn, O.J. 1961. Multiple Comparisons among Means. Journal of the American Statistical Association 56(293), pp. 52–64. doi: 10.1080/01621459.1961.10482090.

Dunne, J.A., Williams, R.J. and Martinez, N.D. 2002. Network structure and biodiversity loss in food webs: Robustness increases with connectance. *Ecology Letters* 5(4), pp. 558–567. doi: 10.1046/j.1461-0248.2002.00354.x.

Dunne, J.A., Williams, R.J. and Martinez, N.D. 2004. Network structure and robustness of marine food webs. *Marine Ecology Progress Series* 273, pp. 291–302.

Egeter, B., Bishop, P.J. and Robertson, B.C. 2015. Detecting frogs as prey in the diets of introduced mammals: A comparison between morphological and DNA-based diet analyses. *Molecular Ecology Resources* 15(2), pp. 306–316. doi: 10.1111/1755-0998.12309.

Eggermont, H. and Heiri, O. 2012. The chironomid-temperature relationship: Expression in nature and palaeoenvironmental implications. *Biological Reviews* 87(2), pp. 430–456. doi: 10.1111/j.1469-185X.2011.00206.x.

Ehleringer, J.R., Buchmann, N. and Flanagan, L.B. 2000. CARBON ISOTOPE RATIOS IN BELOWGROUND CARBON CYCLE PROCESSES. *Ecological Applications* 10(2), pp. 412–422.

Ehleringer, J.R., Rundel, P.W. and Nagy, K.A. 1986. Stable isotopes in physiological ecology and food web research. *Trends in Ecology and Evolution* 1(2), pp. 42–45. doi: 10.1016/0169-5347(86)90072-8.

Einoder, L.D. 2009. A review of the use of seabirds as indicators in fisheries and ecosystem management. *Fisheries Research* 95(1), pp. 6–13. doi: 10.1016/j.fishres.2008.09.024.

Elbrecht, V. and Leese, F. 2017. PrimerMiner: an r package for development and in silico validation of DNA metabarcoding primers. *Methods in Ecology and Evolution* 8(5), pp. 622–626. doi: 10.1111/2041-210X.12687.

Elbrecht, V., Vamos, E.E., Meissner, K., Aroviita, J. and Leese, F. 2017. Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. *Methods in Ecology and Evolution* 8(10), pp. 1265–1275. doi: 10.1111/2041-210X.12789.

Errera, R.M., Yvon-Lewis, S., Kessler, J.D. and Campbell, L. 2014. Reponses of the dinoflagellate Karenia brevis to climate change: pCO2 and sea surface temperatures. *Harmful Algae* 37, pp. 110–116. doi: 10.1016/j.hal.2014.05.012.

Essington, T.E., Beaudreau, A.H., Wiedenmann, J. and Schindler, D.W. 2006. Fishing through marine food webs. *PNAS* 103(1), pp. 3171–3175. Available at: www.pnas.orgcgidoi10.1073pnas.0510964103.

Estes, J.A. and Palmisano, J.F. 1974. Sea Otters: Their Role in Structuring Nearshore Communities. *Science* 185(4156), pp. 1058–1060. doi: 10.1126/science.185.4156.1058.

Evans, D.M., Kitson, J.J.N., Lunt, D.H., Straw, N.A. and Pocock, M.J.O. 2016. Merging DNA metabarcoding and ecological network analysis to understand and build resilient terrestrial ecosystems. *Functional Ecology* 30(12), pp. 1904–1916. doi: 10.1111/1365-2435.12659.

Ewels, P., Magnusson, M., Lundin, S. and Käller, M. 2016. MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* 32(19), pp. 3047–3048. doi: 10.1093/bioinformatics/btw354.

Falcão, J.C.F., Dáttilo, W. and Rico-Gray, V. 2016. Sampling effort differences can lead to biased conclusions on the architecture of ant-plant interaction networks. *Ecological Complexity* 25, pp. 44–52. doi: 10.1016/j.ecocom.2016.01.001.

FAO. 2020. Global Forest Resources Assessment 2020. Global Forest Resources Assessment 2020. doi: 10.4060/ca9825en.

Feller, I.C., Friess, D.A., Krauss, K.W. and Lewis, R.R. 2017. The state of the world's mangroves in the 21st century under climate change. *Hydrobiologia* 803(1). doi: 10.1007/s10750-017-3331-z.

Filonov, A., Barba-López, M. del R., Ladah, L., Tereshchenko, I., Palacios-Hernández, E., Alcocer, J. and Ávalos-Cueva, D. 2022. The dynamics and temperature regime of the crater lakes in the Nevado de Toluca volcano, Mexico. *Limnologica* 92. doi: 10.1016/j.limno.2021.125938.

Florida Department of Environmental Protection (1). 2022. Florida's Mangroves. Available at: Florida Department of Environmental Protection [Accessed: 29 March 2022].

Florida Department of Environmental Protection (2). 2022. Lee County. Available at: https://floridadep.gov/rcp/coastal-access-guide/content/lee-county [Accessed: 29 March 2022].

Florida Department of Environmental protection (3). 2015. Estero Bay Aquatic Preserve Management Plan.

Florida Fish and Wildlife Conservation Commission. 2021. The Economic Impacts of Saltwater Fishing in Florida. Available at: https://myfwc.com/conservation/value/saltwater-fishing/ [Accessed: 27 September 2022].

Florida Fish and Wildlife Conservation Commission. 2022. Commercial Fisheries Landings in Florida. Available at: https://myfwc.com/research/saltwater/fishstats/commercial-fisheries/landings-in-florida/ [Accessed: 29 March 2022].

Fonseca, V.G., Nichols, B., Lallias, D., Quince, C., Carvalho, G.R., Power, D.M. and Creer, S. 2012. Sample richness and genetic diversity as drivers of chimera formation in nSSU metagenetic analyses. *Nucleic Acids Research* 40(9). doi: 10.1093/nar/gks002.

France, R., Chandler, M. and Peters, R. 1998. Mapping trophic continua of benthic foodwebs: body size - 15N relationships. *Marine Ecology Progress Series* 174, pp. 310–306.

France, R.L. 1995. Carbon-13 enrichment in benthic compared to planktonic algae: foodweb implications. *MARINE ECOLOGY PROGRESS SERIES* 124, pp. 307–312.

Franks, J.S. and VanderKooy, K.E. 2000. Feeding Habits of Juvenile Lane Snapper Lutjanus synagris from Mississippi Coastal Waters, with Comments on the Diet of Gray Snapper Lutjanus griseus. *Gulf and Caribbean Research* 12. doi: 10.18785/gcr.1201.02.

Frederiksen, M., Edwards, M., Richardson, A.J., Halliday, N.C. and Wanless, S. 2006. From plankton to top predators: Bottom-up control of a marine food web across four trophic levels. *Journal of Animal Ecology* 75(6), pp. 1259–1268. doi: 10.1111/j.1365-2656.2006.01148.x.

Fredriksen, S. 2003. Food web studies in a Norwegian kelp forest basedon stable isotope (δ13C and δ15N) analysis. *MARINE ECOLOGY PROGRESS SERIES* 260, pp. 71–81.

Friess, D.A. et al. 2019. The State of the World'sMangrove Forests: Past,Present, and Future. *Annual Reviews Environmental Resource* 44, pp. 89–115.

Fry, B. 2002. Conservative Mixing of Stable Isotopes Across Estuarine Salinity Gradients: A Conceptual Framework for Monitoring Watershed Influences on Downstream Fisheries Production. *Estuarine Research Federation Estuaries* 25(2), pp. 264–271.

Fry, B. and Ewel, K.C. 2003. Using stable isotopes in mangrove fisheries research - A review and outlook. *Isotopes in Environmental and Health Studies* 39(3), pp. 191–196. doi: 10.1080/10256010310001601067.

Fry, B., Mumford, P.L., Tam, F., Fox, D.D., Warren, G.L., Havens, K.E. and Steinman, A.D. 1999. Trophic position and individual feeding histories of fish from Lake Okeechobee, Florida.

Fry, B. and Sherr, E.B. 1989. δ13C Measurements as Indicators of Carbon Flow in Marine and Freshwater Ecosystems. pp. 196–229. doi: 10.1007/978-1-4612-3498-2_12.

Fulton, M. and Hodges, S.A. 1999. Floral isolation between Aquilegia formosa and Aquilegia pubescens. *Proc. R. Soc. Lond. B* 266, pp. 2247–2252.

Gadawski, P., Rossaro, B., Giłka, W., Montagna, M., Zawal, A. and Grabowski, M. 2022. First insights into the diversity and ecology of non-biting midges (Diptera: Chironomidae) of the unique ancient Skadar Lake basin (Montenegro/Albania). *Journal of Great Lakes Research* 48(2), pp. 538–550. doi: 10.1016/j.jglr.2021.02.003.

Galvez, J.R., St. John, M.E., McLean, K., Dening Touokong, C., Gonwouo, L.N. and Martin, C.H. 2022. Trophic Specialization on unique resources despite limited niche divergence in a celebrated example of sympatric speciation. *Ecology of Freshwater Fish*. doi: 10.1111/eff.12661.

Geller, J., Meyer, C., Parker, M. and Hawk, H. 2013. Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources* 13(5), pp. 851–861. doi: 10.1111/1755-0998.12138.

Genner, M.J., Seehausen, O., Cleary, D.F.R., Knight, M.E., Michel, E. and Turner, G.F. 2004. How does the taxonomic status of allopatric populations influence species richness within African cichlid fish assemblages? *Journal of Biogeography* 31, pp. 93–102. Available at: www.blackwellpublishing.com/jbi.

Genner, M.J. and Turner, G.F. 2012. Ancient hybridization and phenotypic novelty within lake Malawi's cichlid fish radiation. *Molecular Biology and Evolution* 29(1), pp. 195–206. doi: 10.1093/molbev/msr183.

Gibert, E., Bergonzini, L., Massault, M. and Williamson, D. 2002. AMS-14 C chronology of 40.0 cal ka BP continuous deposits from a crater lake (Lake Massoko, Tanzania) Modern water balance and environmental implications. Palaeogeography, Palaeoclimatology, *Palaeoecology* 187, pp. 307–322. Available at: www.elsevier.com/locate/palaeo.

Giebner, H. et al. 2020. Comparing diversity levels in environmental samples: DNA sequence capture and metabarcoding approaches using 18S and COI genes. *Molecular Ecology Resources* 20(5), pp. 1333–1345. doi: 10.1111/1755-0998.13201.

Gilmore, R.G. and Greenfield, D.W. 2002. The living marine resources of the Western Central Atlantic. The living marine resources of the Western Central Atlantic. Volume 3: Bony fishes part 2 (Opistognathidae to Molidae), sea turtles and marine mammals. Carpenter, K. E. ed. Rome: FAO Species Identification Guide for Fishery Purposes and American Society of Ichthyol-ogists and Herpetologists Special Publication No. 5.

Giménez, E.M., Winkler, G. and Hoffmeyer, M. 2018. Composition, Spatial Distribution, and Trophic Structure of the ZOOPLANKTON COMMUNITY in San Jorge Gulf, Southwestern Atlantic Ocean. Ferreyra Source: *Oceanography* 31(4), pp. 154–163. Available at: https://www.jstor.org/stable/10.2307/26544302?seq=1&cid=pdf-.

Glaum, P., Cocco, V. and Valdovinos, F.S. 2020. Integrating economic dynamics into ecological networks: The case of fishery sustainability. *Sci. Adv* 6. Available at: https://www.science.org.

Gleason, F.H., Carney, L.T., Lilje, O. and Glockling, S.L. 2012. Ecological potentials of species of Rozella (Cryptomycota). *Fungal Ecology* 5(6), pp. 651–656. doi: 10.1016/j.funeco.2012.05.003.

Gonzalez, O. and Loiselle, B.A. 2016. Species interactions in an andean bird-flowering plant network: Phenology is more important than abundance or morphology. *PeerJ* 2016(12), pp. 1–22. doi: 10.7717/peerj.2789.

Grant, P.R. and Grant, B.R. 2008. How and Why Species Multiply: The Radiation of Darwin's Finches. Princeton, NJ, USA: Princeton University Press.

Greenstone, M.H., Payton, M.E., Weber, D.C. and Simmons, A.M. 2014. The detectability half-life in arthropod predator-prey research: What it is, why we need it, how to measure it, and how to use it. In: *Molecular Ecology*. Blackwell Publishing Ltd, pp. 3799–3813. doi: 10.1111/mec.12552.

Gu, B., Schelske, C.L. and Hoyer, M.V. 1996. Stable isotopes of carbon and nitrogen as indicators of diet and trophic structure of the fish community in a shallow hypereutrophic lake. *Journal of Fish Biology* 49, pp. 1233–1243.

Gu, Z., Gu, L., Eils, R., Schlesner, M. and Brors, B. 2014. circlize implements and enhances circular visualization in R. Bioinformatics 30(19), pp. 2811–2812. doi: 10.1093/bioinformatics/btu393.

Guimarães, P.R. 2020. The Structure of Ecological Networks Across Levels of Organization. Available at: https://doi.org/10.1146/annurev-ecolsys-012220-.

Gulis, V., Kuehn, K. and Suberkropp, K. 2006. The role of fungi in carbon and nitrogen cycles in freshwater ecosystems. In: Gadd, G. M. ed. Funji in Biogeochemical Cycles. Cambridge University Press.

Hajibabaei, M., Porter, T.M., Wright, M. and Rudar, J. 2019. COI metabarcoding primer choice affects richness and recovery of indicator taxa in freshwater systems. *PLoS ONE* 14(9). doi: 10.1371/journal.pone.0220953.

Hall, J. 1999. The Effects of Fishing on Marine Ecosystems and Communities. Oxford: Blackwell Science.

Hall, S.J. and Raffaelli, D. 1991. Food-Web Patterns: Lessons from a Species-Rich Web. Source: *Journal of Animal Ecology* 60(3), pp. 823–841. Available at: https://www.jstor.org/stable/5416.

Hamilton, S.E. and Casey, D. 2016. Creation of a high spatio-temporal resolution global database of continuous mangrove forest cover for the 21st century (CGMFC-21). *Global Ecology and Biogeography* 25(6), pp. 729–738. doi: 10.1111/geb.12449.

Harding, J.M., Allen, D.M., Haffey, E.R. and Hoffman, K.M. 2020. Site Fidelity of Oyster Reef Blennies and Gobies in Saltmarsh Tidal Creeks. *Estuaries and Coasts* 43(2), pp. 409–423. doi: 10.1007/s12237-019-00678-z.

Hardy, C.M., Krull, E.S., Hartley, D.M. and Oliver, R.L. 2010. Carbon source accounting for fish using combined DNA and stable isotope analyses in a regulated lowland river weir pool. *Molecular Ecology* 19(1), pp. 197–212. doi: 10.1111/j.1365-294X.2009.04411.x.

Harper, L.M., Lefcheck, J.S., Whippo, R., Jones, M.S., Foltz, Z. and Duffy, J.E. 2022. Blinded by the bright: How species-poor habitats contribute to regional biodiversity across a tropical seascape. *Diversity and Distributions* 28(11), pp. 2272–2285. doi: 10.1111/ddi.13632.

Havens, K.E. et al. 2001. Nutrient dynamics and the eutrophication of shallow lakes Kasumigaura (Japan), Donghu (PR China), and Okeechobee (USA). *Environmental Pollution* 111, pp. 263–272. Available at: www.elsevier.com/locate/envpol.

Heald, E.J.; and Odum, W.E. 1970. The contribution of mangrove swamps to Florida fisheries Item Type conference_item. *Proc. Gulf Carib. Fish. Inst.* 22, pp. 730–735. Available at: http://hdl.handle.net/1834/28165.

Heaton, T.H.E. 1986. Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: A review. Chemical Geology: Isotope Geoscience section 59, pp. 87–102. doi: 10.1016/0168-9622(86)90059-X.

Hemminga, M.A. and Mateo, M.A. 1996. Stable carbon isotopes in seagrasses: variability in ratios and use in ecological studies. *MARINE ECOLOGY PROGRESS SERIES* 140, pp. 285–298.

Hemprich-Bennett, D.R., Kemp, V.A., Blackman, J., Struebig, M.J., Lewis, O.T., Rossiter, S.J. and Clare, E.L. 2021. Altered structure of bat–prey interaction networks in logged tropical forests revealed by metabarcoding. *Molecular Ecology* 30(22), pp. 5844–5857. doi: 10.1111/mec.16153.

Heng, K., Chevalier, M., Lek, S. and Laffaille, P. 2018. Seasonal variations in diet composition, diet breadth and dietary overlap between three commercially important fish species within a flood-pulse system: The Tonle Sap Lake (Cambodia). *PLoS ONE* 13(6). doi: 10.1371/journal.pone.0198848.

Henriksen, M. V., Chapple, D.G., Chown, S.L. and McGeoch, M.A. 2019. The effect of network size and sampling completeness in depauperate networks. *Journal of Animal Ecology* 88(2), pp. 211–222. doi: 10.1111/1365-2656.12912.

Hernandez Macias, N.E., Wangensteen, O., Toft, S. and Arnedo, M.A. 2016. The role of morphological evolution and prey specialization in adaptive radiations: The spider genus Dysdera in the Canary Islands. *Island Biology*. Available at: http://hdl.handle.net/10138/232472.

Herrera, M., Tubío, A., Pita, P., Vázquez, E., Olabarria, C., Duarte, C.M. and Villasante, S. 2022. Trade-Offs and Synergies Between Seagrass Ecosystems and Fishing Activities: A Global Literature Review. *Frontiers in Marine Science* 9. doi: 10.3389/fmars.2022.781713.

El Hili, R.A., Achouri, M.S. and Verneau, O. 2021. Cytochrome c oxydase I phylogenetic analysis of Haemogregarina parasites (Apicomplexa, Coccidia, Eucoccidiorida, Haemogregarinidae) confirms the presence of three distinct species within the freshwater turtles of Tunisia. *Parasitology International* 82. doi: 10.1016/j.parint.2021.102306.

Hilton, G.M., Houston, D.C. and Furness, R.W. 1998. Which components of diet quality affect retention time of digesta in seabirds? *Functional Ecology* 12(6), pp. 929–939. doi: 10.1046/j.1365-2435.1998.00267.x.

Hoagland, P. et al. 2020. Lessening the Hazards of Florida Red Tides: A Common Sense Approach. *Frontiers in Marine Science* 7. doi: 10.3389/fmars.2020.00538.

Hodges, S.. A. and Arnold, M.L. 1995. Spurring plant diversification: are floral nectar spurs a key innovation? *Proc. R. Soc. Lond. B* 262, pp. 343–348.

Hodges, S.A. and Kramer, E.M. 2007. Columbines. *Current Biology* 17(23). doi: 10.1016/j.cub.2007.09.034.

Hoenig, B.D., Trevelline, B.K., Kautz, A., Latta, S.C. and Porter, B.A. 2022. Two is better than one: Coupling DNA metabarcoding and stable isotope analysis improves dietary characterizations for a riparian-obligate, migratory songbird. *Molecular Ecology* 31(21), pp. 5635–5648. doi: 10.1111/mec.16688.

Hotaling-Hagan, A., Swett, R., Ellis, L.R. and Frazer, T.K. 2017. A spatial model to improve site selection for seagrass restoration in shallow boating environments. *Journal of Environmental Management* 186, pp. 42–54. doi: 10.1016/j.jenvman.2016.10.005.

Hulsey, C.D., Marks, J., Hendrickson, D.A., Williamson, C.A., Cohen, A.E. and Stephens, D.M.J. 2006. Feeding specialization in Herichthys minckleyi: a trophically polymorphic fish. *Journal of Fish Biology* 68, pp. 1399–1410. Available at: https://onlinelibrary.wiley.com/doi/10.1111/j.0022-1112.2006.01021.x.

Hunt, J.M. 1966. The significance of carbon isotope variations in marine sediment. In: Advances in Organic Geochemistry. Pergamon Press, Oxford., pp. 27–35.

Hussey, N.E. et al. 2014. Rescaling the trophic structure of marine food webs. *Ecology Letters* 17(2), pp. 239–250. doi: 10.1111/ele.12226.

Hutchison, J., Spalding, M. and Zu Ermgassen, P. 2014. THE ROLE OF MANGROVES IN FISHERIES ENHANCEMENT. Available at: www.nature.org.

Huxham, M., Dencer-Brown, A., Diele, K., Kathiresan, K., Nagelkerken, I. and Wanjiru, C. 2017. Mangroves and people: Local ecosystem services in a changing climate. In: Mangrove Ecosystems: A Global Biogeographic Perspective: Structure, Function, and Services. Springer International Publishing, pp. 245–274. doi: 10.1007/978-3-319-62206-4_8.

Idelberger, C.F. and Greenwood, M.F.D. 2005. Seasonal variation in fish assemblages within the estuarine portions of the Myakka and Peace rivers, southwest Florida. Gulf of Mexico Science 23(2), pp. 224–240. doi: 10.18785/goms.2302.09.

Igulu, M.M., Nagelkerken, I., Van Der Velde, G., Mgaya, Y.D. and Mgaya5, Y.D. 2013. Mangrove Fish Production is Largely Fuelled by External Food Sources: A Stable Isotope Analysis of Fishes at the Individual, Species, and Community Levels from Across the Globe Fuelled by External Food Sources A Stable Isotope Analysis at the Individual, Species and Community Level the Globel scale. 16(7), pp. 1336–1352.

Ings, T.C. et al. 2009. Ecological networks - Beyond food webs. *Journal of Animal Ecology* 78(1), pp. 253–269. doi: 10.1111/j.1365-2656.2008.01460.x.

Jackman, T.R., Larson, A., De Queiroz, K. and Losos, J.B. 1999. Phylogenetic Relationships and Tempo of Early Diversication in Anolis Lizards. *Syst. Biol* 48(2), pp. 254–285. Available at: https://academic.oup.com/sysbio/article/48/2/254/1670336.

Jackson, M.C., Pawar, S. and Woodward, G. 2021. The Temporal Dynamics of Multiple Stressor Effects: From Individuals to Ecosystems. *Trends in Ecology & Evolution* 36(5), pp. 402–410. doi: 10.1016/j.tree.2021.01.005.

Jarman, W., Hobson, K., Sydeman, W., Bacon, C. and McLaren, E. 1996. Influence of Trophic Position and Feeding Location on Contaminant Levels in the Gulf of the Farallones Food Web Revealed by Stable Isotope Analysis. *Eviron Sci Technol* 30, pp. 654–660. Available at: https://pubs.acs.org/sharingguidelines.

Jeffers, S.A.;, Patterson, W.F.; and Cowan, J.H. 2008. Habitat and bycatch effects on population parameters of inshore lizardfish (Synodus foetens) in the north central Gulf of Mexico Item Type article. *Fish. Bull.* 106, pp. 417–426. Available at: http://hdl.handle.net/1834/25472.

Jelbart, J.E., Ross, P.M. and Connolly, R.M. 2007. Fish assemblages in seagrass beds are influenced by the proximity of mangrove forests. *Marine Biology* 150(5), pp. 993–1002. doi: 10.1007/s00227-006-0419-9.

Jennings, S., Barnes, C., Sweeting, C.J. and Polunin, N.V.C. 2008. Application of nitrogen stable isotope analysis in size-based marine food web and macroecological research. In: *Rapid Communications in Mass Spectrometry*. pp. 1673–1680. doi: 10.1002/rcm.3497.

Jennions, M.D. and Møller, A.P. 2003. A survey of the statistical power of research in behavioral ecology and animal behavior. *Behavioral Ecology* 14(3), pp. 438–445. Available at: https://academic.oup.com/beheco/article/14/3/438/257415.

Jobard, M., Rasconi, S. and Sime-Ngando, T. 2010. Diversity and functions of microscopic fungi: A missing component in pelagic food webs. *Aquatic Sciences* 72(3), pp. 255–268. doi: 10.1007/s00027-010-0133-z.

Jung, A.S., van der Veer, H.W., van der Meer, M.T.J. and Philippart, C.J.M. 2019. Seasonal variation in the diet of estuarine bivalves. *PLoS ONE* 14(6). doi: 10.1371/journal.pone.0217003.

Kalacska, M., Arroyo-Mora, J.P., Lucanus, O. and Kishe-Machumu, M.A. 2017. Land cover, land use, and climate change impacts on endemic cichlid habitats in Northern Tanzania. *Remote Sensing* 9(6). doi: 10.3390/rs9060623.

Kartzinel, T.R. et al. 2015. DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. 112(26), pp. 8019–8024. doi: 10.5061/dryad.2fg10.

Kassambara, A. 2020. ggpubr: "ggplot2" Based Publication Ready Plots.

Kathiresan, K. and Bingham, B.L. 2001. Biology of Mangroves and Mangrove Ecosystems. *ADVANCES IN MARINE BIOLOGY* 40, pp. 81–251.

Kaunisto, K.M., Roslin, T., Sääksjärvi, I.E. and Vesterinen, E.J. 2017. Pellets of proof: First glimpse of the dietary composition of adult odonates as revealed by metabarcoding of feces. *Ecology and Evolution* 7(20), pp. 8588–8598. doi: 10.1002/ece3.3404.

Keough, J.R., Hagley, C.A., Ruzycki, E. and and Sierszen, M.,. 1998. 13C composition of primary producers and role of detritus in a freshwater coastal systems. *Limnol. Oceanogr* 43(4), pp. 734–740.

Kieckbusch, D.K., Koch, M.S., Serafy, J.E. and Anderson, W.T. 2004. TROPHIC LINKAGES AMONG PRIMARY PRODUCERS AND CONSUMERS IN FRINGING MANGROVES OF SUBTROPICAL LAGOONS. *BULLETIN OF MARINE SCIENCE* 74(2), pp. 271–285.

Koblmüller, S., Albertson, R.C., Genner, M.J., Sefc, K.M. and Takahashi, T. 2015. Preface: Advances in cichlid research: behavior, ecology, and evolutionary biology. *Hydrobiologia* 748(1). doi: 10.1007/s10750-015-2196-2.

Koblmüller, S., Schliewen, U.K., Duftner, N., Sefc, K.M., Katongo, C. and Sturmbauer, C. 2008. Age and spread of the haplochromine cichlid fishes in Africa. Molecular Phylogenetics and *Evolution* 49(1), pp. 153–169. doi: 10.1016/j.ympev.2008.05.045.

Kocher, T.D. 2004. Adaptive evolution and explosive speciation: the cichlid fish model. *Nature Reviews Genetics* 5(4), pp. 288–298. doi: 10.1038/nrg1316.

Koenig, C.C., Coleman, F.C., Eklund, A.-M., Schull, J. and Ueland, J. 2007. MANGROVES AS ESSENTIAL NURSERY HABITAT FOR GOLIATH GROUPER (EPINEPHELUS ITAJARA). Available at: www.iucnredlist.org/search/search-basic].

Komai, T., Gotoh, R.O., Sado, T. and Miya, M. 2019. Development of a new set of PCR primers for eDNA metabarcoding decapod crustaceans. *Metabarcoding and Metagenomics* 3, pp. 1–19. doi: 10.3897/mbmg.3.33835.

Kramer, E.M. 2009. Aquilegia: A new model for plant development, ecology, and evolution. *Annual Review of Plant Biology* 60, pp. 261–277. doi: 10.1146/annurev.arplant.043008.092051.

Kranzfelder, P., Anderson, A.M., Egan, A.T., Mazack, J.E., Bouchard, Jr., R.W., Rufer, M.M. and Ferrington, Jr., L.C. 2015. Use of Chironomidae (Diptera) Surface-Floating Pupal Exuviae as a Rapid Bioassessment Protocol for Water Bodies. *Journal of Visualized Experiments* (101). doi: 10.3791/52558.

Kratina, P., LeCraw, R.M., Ingram, T. and Anholt, B.R. 2012. Stability and persistence of food webs with omnivory: Is there a general pattern? *Ecosphere* 3(6), p. art50. doi: 10.1890/es12-00121.1.

Kulatska, N., Neuenfeldt, S., Beier, U., Elvarsson, B.Þ., Wennhage, H., Stefansson, G. and Bartolino, V. 2019. Understanding ontogenetic and temporal variability of Eastern Baltic cod diet using a multispecies model and stomach data. *Fisheries Research* 211, pp. 338–349. doi: 10.1016/j.fishres.2018.11.023.

Kume, G. et al. 2021. Diet niche segregation of co-occurring larval stages of mesopelagic and commercially important fishes in the Osumi Strait assessed through morphological, DNA metabarcoding, and stable isotope analyses. *Marine Biology* 168(1). doi: 10.1007/s00227-020-03810-x.

de la Morinière, E.C., Pollux, B.J.A., Nagelkerken, I., Hemminga, M.A., Huikes, A.H.L. and Velde, G.V.D. 2003. Ontogenetic dietary changes of coral reef fishes in the mangrove-seagrass-reef continuum: stableisotopes and gut-content analysis. *MARINE ECOLOGY PROGRESS SERIES* 246, pp. 279–289.

Ladd, M.C. and Shantz, A.A. 2020. Trophic interactions in coral reef restoration: A review. *Food Webs* 24. doi: 10.1016/j.fooweb.2020.e00149.

Laegdsgaard, P. and Johnson, C.R. 1995. Mangrove habitats as nurseries: unique assemblages of juvenile fish in subtropical mangroves in eastern Australia. *MARINE ECOLOGY PROGRESS SERIES* 126, pp. 67–81.

Lahr, D.J.G. and Katz, L.A. 2009. Reducing the impact of PCR-mediated recombination in molecular evolution and environmental studies using a new-generation high-fidelity DNA polymerase. *BioTechniques* 47(4), pp. 857–866. doi: 10.2144/000113219.

Lai, S., Loke, L.H.L., Hilton, M.J., Bouma, T.J. and Todd, P.A. 2015. The effects of urbanisation on coastal habitats and the potential for ecological engineering: A Singapore

case study. *Ocean and Coastal Management* 103, pp. 78–85. doi: 10.1016/j.ocecoaman.2014.11.006.

Lande, R., Seehausen, O. and Van Alphen, J.J.M. 2001. Mechanisms of rapid sympatric speciation by sex reversal and sexual selection in cichlid fish. *Genetica* 112, pp. 435–443.

Landsberg, J.H., Flewelling, L.J. and Naar, J. 2009. Karenia brevis red tides, brevetoxins in the food web, and impacts on natural resources: Decadal advancements. *Harmful Algae* 8(4), pp. 598–607. doi: 10.1016/j.hal.2008.11.010.

Larsson, A. 2014. AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics* 30(22), pp. 3276–3278. doi: 10.1093/bioinformatics/btu531.

Layman, C.A. et al. 2012. Applying stable isotopes to examine food-web structure: An overview of analytical tools. *Biological Reviews* 87(3), pp. 545–562. doi: 10.1111/j.1469-185X.2011.00208.x.

Leal, M., Knox, A.K. and Losos, J.B. 2002. LACK OF CONVERGENCE IN AQUATIC ANOLIS LIZARDS. *Evolution* 56(4), pp. 785–791.

Ledger, M.E., Edwards, F.K., Brown, L.E., Milner, A.M. and Woodward, G. 2011. Impact of simulated drought on ecosystem biomass production: An experimental test in stream mesocosms. *Global Change Biology* 17(7), pp. 2288–2297. doi: 10.1111/j.1365-2486.2011.02420.x.

Lee, D.S. 1980. Atlas of North American freshwater fishes, 1980- et seq. [Raleigh, N.C.]: North Carolina State Museum of Natural History. doi: 10.5962/bhl.title.141711.

Lee, S. 2005. Exchange of organic matter and nutrients between mangroves and estuaries: myths, methodological issues and missing links. International Journal of Ecology and Environmental Sciences 31(3).

Lee, S.Y. et al. 2014. Ecological role and services of tropical mangrove ecosystems: A reassessment. *Global Ecology and Biogeography* 23(7), pp. 726–743. doi: 10.1111/geb.12155.

Lees, M.J.F. and Stanley S, G.H. 1956. A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES*. The Journal of Biological Chemistry. Available at: www.jbc.org.

Leese, F., Sander, M., Buchner, D., Elbrecht, V., Haase, P. and Zizka, V.M.A. 2021. Improved freshwater macroinvertebrate detection from environmental DNA through minimized nontarget amplification. *Environmental DNA* 3(1), pp. 261–276. doi: 10.1002/edn3.177.

De León, L.F., Podos, J., Gardezi, T., Herrel, A. and Hendry, A.P. 2014. Darwin's finches and their diet niches: The sympatric coexistence of imperfect generalists. *Journal of Evolutionary Biology* 27(6), pp. 1093–1104. doi: 10.1111/jeb.12383.

Leray, M. et al. 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: Application for

characterizing coral reef fish gut contents. *Frontiers in Zoology* 10(1). doi: 10.1186/1742-9994-10-34.

Leray, M., Ho, S.L., Lin, I.J. and Machida, R.J. 2018. MIDORI server: a webserver for taxonomic assignment of unknown metazoan mitochondrial-encoded sequences using a curated database. *Bioinformatics* 34(21), pp. 3753–3754. doi: 10.1093/bioinformatics/bty454.

Leray, M. and Knowlton, N. 2017. Random sampling causes the low reproducibility of rare eukaryotic OTUs in Illumina COI metabarcoding. *PeerJ* 2017(3). doi: 10.7717/peerj.3006.

Lerner, J.E., Marchese, C. and Hunt, B.P.V. 2022. Stable isotopes reveal that bottom-up omnivory drives food chain length and trophic position in eutrophic coastal ecosystems. *ICES Journal of Marine Science* 79(8), pp. 2311–2323. doi: 10.1093/icesjms/fsac171.

Lewis, R.R., Milbrandt, E.C., Brown, B., Krauss, K.W., Rovai, A.S., Beever, J.W. and Flynn, L.L. 2016. Stress in mangrove forests: Early detection and preemptive rehabilitation are essential for future successful worldwide mangrove forest management. *Marine Pollution Bulletin* 109(2), pp. 764–771. doi: 10.1016/j.marpolbul.2016.03.006.

Lindeman, R.L. 1942. The Trophic-Dynamic Aspect of Ecology. *Ecology* 34(4), pp.399-417.

Link, J. 2002. Does food web theory work for marine ecosystems. *MARINE ECOLOGY PROGRESS SERIES* 230, pp. 1–9.

Loh, Y.H.E. et al. 2013. Origins of shared genetic variation in African cichlids. *Molecular Biology and Evolution* 30(4), pp. 906–917. doi: 10.1093/molbev/mss326.

Longmire, J.L., Maltbie, M. and Baker, R.J. 1997. Use of “lysis buffer” in DNA isolation and its implication for museum collections. Occasional Papers the Museum Texas Tech University 163, pp. 1–3.

van der Loos, L.M. and Nijland, R. 2021. Biases in bulk: DNA metabarcoding of marine communities and the methodology involved. In: Molecular Ecology. John Wiley and Sons Inc, pp. 3270–3288. doi: 10.1111/mec.15592.

Lopes, C.M. et al. 2020. Ecological specialization and niche overlap of subterranean rodents inferred from DNA metabarcoding diet analysis. *Molecular Ecology* 29(16), pp. 3144–3154. doi: 10.1111/mec.15549.

LOPEZ, R.R., SILVY, N.J., WILKINS, R.N., FRANK, P.A., PETERSON, M.J. and PETERSON, M.N. 2004. HABITAT-USE PATTERNS OF FLORIDA KEY DEER: IMPLICATIONS OF URBAN DEVELOPMENT. Journal of Wildlife Management 68(4), pp. 900–908. doi: 10.2193/0022-541x(2004)068[0900:hpofkd]2.0.co;2.

Losos, J.B. 1994. Integrative Approaches to Evolutionary Ecology: Anolis Lizards as Model Systems. *Annual Review of Ecology and Systematics* 25, pp. 467–493. Available at: https://about.jstor.org/terms.

Lugendo, B.R., Nagelkerken, I., Van Der Velde, G. and Mgaya, Y.D. 2006. The importance of mangroves, mud and sand flats, and seagrass beds as feeding areas for juvenile fishes in

Chwaka Bay, Zanzibar: Gut content and stable isotope analyses. *Journal of Fish Biology* 69(6), pp. 1639–1661. doi: 10.1111/j.1095-8649.2006.01231.x.

Lynam, C.P., Llope, M., Möllmann, C., Helaouët, P., Bayliss-Brown, G.A. and Stenseth, N.C. 2017. Interaction between top-down and bottom-up control in marine food webs. *Proceedings of the National Academy of Sciences of the United States of America* 114(8), pp. 1952–1957. doi: 10.1073/pnas.1621037114.

M Thompson, R.S., Dunne, J.A., Woodwa Rd, G.U. and Thompson, R.M. 2012a. Freshwater food webs: towards a more fundamental understanding of biodiversity and community dynamics. *Freshwater Biology* 57. Available at: http://onlinelibrary.wiley.com/journal/10.1111/.

M Thompson, R.S., Dunne, J.A., Woodwa Rd, G.U. and Thompson, R.M. 2012b. Freshwater food webs: towards a more fundamental understanding of biodiversity and community dynamics. *Freshwater Biology* 57, pp. 1329–1341. Available at: http://onlinelibrary.wiley.com/journal/10.1111/.

Ma, P., Zhang, L. and Mitsch, W.J. 2020. Investigating sources and transformations of nitrogen using dual stable isotopes for Lake Okeechobee restoration in Florida. *Ecological Engineering* 155. doi: 10.1016/j.ecoleng.2020.105947.

Macgregor, C.J., Evans, D.M. and Pocock, M.J.O. 2017. Estimating sampling completeness of interactions in quantitative bipartite ecological networks: incorporating variation in species' 2 specialisation 3. *BioRxiv*. Available at: https://doi.org/10.1101/195917.

Machemer, E.G.P., Walter, J.F., Serafy, J.E. and Kerstetter, D.W. 2012. Importance of mangrove shorelines for rainbow parrotfish Scarus guacamaia: Habitat suitability modeling in a subtropical bay. *Aquatic Biology* 15(1), pp. 87–98. doi: 10.3354/ab00412.

Macintosh, D.J. and Ashton, E.C. 2002. A Review of Mangrove Biodiversity Conservation and Management A Review of Mangrove Biodiversity Conservation and Management. Available at: www.worldbank.org;

MacKenzie, R.A., Cormier, N. and Demopoulos, A.W. 2020. Estimating the value of mangrove leaf litter in sesarmid crab diets: The importance of fractionation factors. Bulletin of Marine *Science* 96(3), pp. 501–519. doi: 10.5343/bms.2019.0026.

MacNae, W. 1974. MANQROVE FORESTS AND FISHERilllS. Rome.
Mahé, F. et al. 2017. Parasites dominate hyperdiverse soil protist communities in Neotropical rainforests. *Nature Ecology and Evolution* 1(4). doi: 10.1038/s41559-017-0091.

Malinsky, M. et al. 2015. Genomic islands of speciation separate cichlid ecomorphs in an East African crater lake. *Science* 350 (6267), pp.1493-1498.

Malinsky, M., Svardal, H., Tyers, A.M., Miska, E.A., Genner, M.J., Turner, G.F. and Durbin, R. 2018. Whole-genome sequences of Malawi cichlids reveal multiple radiations interconnected by gene flow. *Nature Ecology and Evolution* 2(12), pp. 1940–1955. doi: 10.1038/s41559-018-0717-x.

Maloy, A.P., Nelle, P., Culloty, S.C., Slater, J.W. and Harrod, C. 2013. Identifying trophic variation in a marine suspension feeder: DNA- and stable isotope-based dietary analysis in Mytilus spp. *Marine Biology* 160(2), pp. 479–490. doi: 10.1007/s00227-012-2105-4.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 17(1), p. 10. doi: 10.14806/ej.17.1.200.

Martinelli, M.M. and Volpi, e T.A. 2010. Diet of racoon Procyon cancrivorus (Carnivora, Procyonidae) in a mangrove and restinga area in Espirito Santo state, Brazil. *Natureza on line* 8(3), pp. 150–151.

Martinez Arbizu, P. 2020. pairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.4.

Martino, E.J. and Able, K.W. 2003. Fish assemblages across the marine to low salinity transition zone of a temperate estuary. *Estuarine, Coastal and Shelf Science* 56(5–6), pp. 969–987. doi: 10.1016/S0272-7714(02)00305-0.

Martusubroto, P. and Naamin, N. 1977. RELATIONSHIP BETWEEN TIDAL FORESTS (MANGROVES) AND COMMERCIAL SHRIMP PRODUCTION IN INDONESIA. *Marine Research in Indonesia* 18, pp. 81–86. Available at: www.oseanografi.lipi.go.id.

Mata, V.A. et al. 2021. Combining DNA metabarcoding and ecological networks to inform conservation biocontrol by small vertebrate predators. *Ecological Applications* 31(8). doi: 10.1002/eap.2457.

Matheson, R.E., Adams, D.H., Keenan, S.F. and Switzer, T.S. 2008. Status, trends, and habitat use by estuarine and marine fishes of greatest conservation need in Florida.

Matley, J.K. et al. 2018. Integrating complementary methods to improve diet analysis in fishery-targeted species. *Ecology and Evolution* 8(18), pp. 9503–9515. doi: 10.1002/ece3.4456.

Mazerolle, M.J. 2020. AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c). R package version 2.3-1. https://cran.r-project.org/package=AICcmodavg.

McFarland, K., Jean, F., Soudant, P. and Volety, A.K. 2015. Uptake and elimination of brevetoxin in the invasive green mussel, Perna viridis, during natural Karenia brevis blooms in southwest Florida. *Toxicon* 97, pp. 46–52. doi: 10.1016/j.toxicon.2015.02.005.

McFarland, K., Jean, F., Thébault, J. and Volety, A.K. 2016. Potential impacts of blooms of the toxic dinoflagellate Karenia brevis on the growth, survival and juvenile recruitment of the non-native green mussel Perna viridis in southeastern United States. *Toxicon* 109, pp. 94–102. doi: 10.1016/j.toxicon.2015.11.017.

McGregor, S. and Strydom, N.A. 2020. Feeding ecology and microplastic ingestion in Chelon richardsonii (Mugilidae) associated with surf diatom Anaulus australis accumulations in a warm temperate South African surf zone. *Marine pollution bulletin* 158, p. 111430. doi: 10.1016/j.marpolbul.2020.111430.

McInnes, J.C., Alderman, R., Deagle, B.E., Lea, M.A., Raymond, B. and Jarman, S.N. 2017. Optimised scat collection protocols for dietary DNA metabarcoding in vertebrates. *Methods in Ecology and Evolution* 8(2), pp. 192–202. doi: 10.1111/2041-210X.12677.

McMurdie, P.J. and Holmes, S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* 8(4), p. e61217. doi: 10.1371/journal.pone.0061217.

Medina, M., Kaplan, D., Milbrandt, E.C., Tomasko, D., Huffaker, R. and Angelini, C. 2022. Nitrogen-enriched discharges from a highly managed watershed intensify red tide (Karenia brevis) blooms in southwest Florida. *Science of the Total Environment* 827. doi: 10.1016/j.scitotenv.2022.154149.

Medina-Contreras, D. and Arenas, F. 2023. Are stable isotopes an efficient tool for tracking the effect of anthropogenic activities on mangrove food web structure and function? *Hydrobiologia* 850(6), pp. 1237–1249. doi: 10.1007/s10750-022-05086-2.

Mehltreter, Klaus, Rojas, P. and Palacios-Rios. 2003. Moth Larvae-damaged Giant Leather-fern Acrostichum danaeifolium as Host for Secondary Colonization by Ants. *American Fern Journal*, pp. 49–55. Available at: https://doi.org/10.1640/0002-.

Memmott, J., Waser, N.M. and Price, M. V. 2004. Tolerance of pollination networks to species extinctions. *Proceedings of the Royal Society B: Biological Sciences* 271(1557), pp. 2605–2611. doi: 10.1098/rspb.2004.2909.

Mestre, F., Gravel, D., García-Callejas, D., Pinto-Cruz, C., Matias, M.G. and Araújo, M.B. 2022. Disentangling food-web environment relationships: A review with guidelines. *Basic and Applied Ecology* 61, pp. 102–115. doi: 10.1016/j.baae.2022.03.011.

Meynecke, J.-O., Lee, S.Y., Duke, N.C. and Warnken, J. 2007. Large oil spill impacts on mangrove habitat and their recovery View project Large-scale dieback of mangroves in Australia's Gulf of Carpentaria: a severe ecosystem response, coincidental with an unusually extreme weather event View project. Available at: https://www.researchgate.net/publication/29465179.

Mitra, S., Bartel, J. and Volety, A.K. 2011. Trace Organic Contaminants (PAHS, PCBs, and Pesticides) in Oysters Crassostrea virginica, from the Caloosahatchee Estuary and Estero Bay, SW Florida. In: Stoytcheva, M. ed. INTECH.

Miya, M. et al. 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. *Royal Society Open Science* 2(7). doi: 10.1098/rsos.150088.

Morrill, A., Kaunisto, K.M., Mlynarek, J.J., Sippola, E., Vesterinen, E.J. and Forbes, M.R. 2021. Metabarcoding prey DNA from fecal samples of adult dragonflies shows no predicted sex differences, and substantial inter-individual variation, in diets. *PeerJ* 9, p. e12634. doi: 10.7717/peerj.12634.

Morton, J.F. 1965. Can the Red Mangrove Provide Food, Feed and Fertilizer? *Economic Botany*, pp. 113–123.

Motta, P., Clifton, K. and Patricia Hernandez, L. 1995. Feeding Relationships among Nine Species of Seagrass Fishes of Tampa Bay, Florida Functional morphology of carp View project Feeding mechanics of fishes View project. *Bulletin of Marine Science*, pp. 185–200. Available at: https://www.researchgate.net/publication/233528441.

Mumby, P.J. et al. 2004. Mangroves enhance the biomass of coral reef fish communities in the Caribbean. *Nature* 427(6974), pp. 533–536. doi: 10.1038/nature02286.

Munby, H. et al. 2021. Differential use of multiple genetic sex determination systems in divergent ecomorphs of 2 an African crater lake cichlid. *bioRxiv*. Available at: https://doi.org/10.1101/2021.08.05.455235.

Murdy, E.O. and Musick, J.A. 2013. Field Guide to Fishes of Chesapeake Bay. Johns Hopkins University Press.

Murray, D.C. et al. 2011. DNA-based faecal dietary analysis: A comparison of qPCR and high throughput sequencing approaches. *PLoS ONE* 6(10). doi: 10.1371/journal.pone.0025776.

Muschick, M., Barluenga, M., Salzburger, W. and Meyer, A. 2011. Adaptive phenotypic plasticity in the Midas Cichlid fish pharyngeal jaw and its relevance in adaptive radiation. *BMC Evolutionary Biology* 11(1). doi: 10.1186/1471-2148-11-116.

Muylaert, K., Sabbe, K. and Vyverman, W. 2009. Changes in phytoplankton diversity and community composition along the salinity gradient of the Schelde estuary (Belgium/The Netherlands). *Estuarine, Coastal and Shelf Science* 82(2), pp. 335–340. doi: 10.1016/j.ecss.2009.01.024.

Nagelkerken, I. et al. 2008. The habitat function of mangroves for terrestrial and marine fauna: A review. *Aquatic Botany* 89(2), pp. 155–185. doi: 10.1016/j.aquabot.2007.12.007.

Nagelkerken, I., Roberts, C. m, van der Velde, G., Dorenbosch, M., van Riel, M.C., de la Morinière, E.C. and Nienhuis, P.H. 2002. How important are mangroves and seagrass beds for coral-reeffish? The nursery hypothesis tested on an island scale. *Marine Ecology Progress Series* 244, pp. 244–305.

Nagelkerken, I. and van der Velde, G. 2004. Are Caribbean mangroves important feeding grounds for juvenile reef fish from adjacent seagrass beds? *Marine Ecology Progress Series* 274, pp. 143–151.

Nakagawa, S. 2004. A farewell to Bonferroni: The problems of low statistical power and publication bias. *Behavioral Ecology* 15(6), pp. 1044–1045. doi: 10.1093/beheco/arh107.

Nalley, E.M., Donahue, M.J., Heenan, A. and Toonen, R.J. 2022. Quantifying the diet diversity of herbivorous coral reef fishes using systematic review and DNA metabarcoding. *Environmental DNA* 4(1), pp. 191–205. doi: 10.1002/edn3.247.

Narwani, A. and Mazumder, A. 2012. Bottom-up effects of species diversity on the functioning and stability of food webs.

Nashima, F.P., Strydom, N.A., Connan, M. and Lamberth, S.J. 2020. Stable isotopes reveal trophic linkages among fish species utilizing the Orange River Estuary Continuum. *Food Webs* 24, p. e00145. doi: 10.1016/j.fooweb.2020.e00145.

Navarro-Mayoral, S. et al. 2023. Drivers of variation in seagrass-associated amphipods across biogeographical areas. *Marine Environmental Research* 186, p. 105918. Available at: https://linkinghub.elsevier.com/retrieve/pii/S0141113623000466.

Newman, W.A. and Abbott, D.P. 1980. Cirripedia: the barnacles. Intertidal invertebrates of California.

Newsome, S.D., Martinez del Rio, C., Bearhop, S. and Phillips, D.L. 2007. A niche for isotopic ecology. *Frontiers in Ecology and the Environment* 5(8), pp. 429–436. doi: 10.1890/060150.1.

Nichols, P. et al. 2014. Secondary contact seeds phenotypic novelty in cichlid fishes. *Proceedings of the Royal Society B: Biological Sciences* 282(1798). doi: 10.1098/rspb.2014.2272.

Nielsen, A. and Bascompte, J. 2007. Ecological networks, nestedness and sampling effort. *Journal of Ecology* 95(5), pp. 1134–1141. doi: 10.1111/j.1365-2745.2007.01271.x.

Nielsen, J.M., Clare, E.L., Hayden, B., Brett, M.T. and Kratina, P. 2018. Diet tracing in ecology: Method comparison and selection. *Methods in Ecology and Evolution* 9(2), pp. 278–291. doi: 10.1111/2041-210X.12869.

Nor Zalipah, M., Mohd Sah, S.A. and Jones, G. 2021. Flowering Biology of Mangroves (Genus Sonneratia) and the Role of Bats in Their Pollination in Peninsular Malaysia. In: Handbook of Halophytes. *Springer International Publishing*, pp. 1707–1722. doi: 10.1007/978-3-030-57635-6_60.

Norton, S.L., Wiley, T.R., Carlson, J.K., Frick, A.L., Poulakis, G.R. and Simpfendorfer, C.A. 2012. Designating Critical Habitat for Juvenile Endangered Smalltooth Sawfish in the United States. *Marine and Coastal Fisheries* 4(1), pp. 473–480. doi: 10.1080/19425120.2012.676606.

Nyman, M.T. and Korhola, A.A. 2005. Chironomid-based classification of lakes in western Finnish Lapland. 10, pp. 239–254.

O'Connell, D.P. et al. 2022. Assessing mangrove restoration practices using species-interaction networks. *Restoration Ecology* 30(4). doi: 10.1111/rec.13546.

Oksanen, J. and et al. 2022. vegan: Community Ecology Package. R package.

Olney, J.E. and Boehlert, G.W. 1988. Nearshore ichthyoplankton ichthyoplankton associated with seagrass beds in the lower. *Marine Ecology Progress Series* 45(1), pp. 33–43.

Olsen, Z., Fulford, R., Dillon, K. and Graham, W. 2014. Trophic role of gulf menhaden Brevoortia patronus examined with carbon and nitrogen stable isotope analysis. *Marine Ecology Progress Series* 497, pp. 215–227. doi: 10.3354/meps10519.

O'Shea, O.R., Thums, M., van Keulen, M., Kempster, R.M. and Meekan, M.G. 2013. Dietary partitioning by five sympatric species of stingray (Dasyatidae) on coral reefs. *Journal of Fish Biology* 82(6), pp. 1805–1820. doi: 10.1111/jfb.12104.

Overman, N.C. and Parrish, D.L. 2001. Stable isotope composition of walleye: 15N accumulation with age and area-specific differences in δ13C. *Canadian Journal of Fisheries and Aquatic Science*s 58(6), pp. 1253–1260. doi: 10.1139/cjfas-58-6-1253.

Pagès, H., Aboyoun, P., Gentleman, R. and DenRoy, S. 2022. Efficient manipulation of biological strings.

Papiol, V., Fanelli, E., Cartes, J.E., Rumolo, P. and López-Pérez, C. 2017. A multi-tissue approach to assess the effects of lipid extraction on the isotopic composition of deep-sea fauna. *Journal of Experimental Marine Biology and Ecology* 497, pp. 230–242. doi: 10.1016/j.jembe.2017.10.001.

Parimuchová, A., Dušátková, L.P., Kováč, Ľ., Macháčková, T., Slabý, O. and Pekár, S. 2021. The food web in a subterranean ecosystem is driven by intraguild predation. *Scientific Reports* 11(1). doi: 10.1038/s41598-021-84521-1.

Parsons, P.J., Bridle, J.R., Rüber, L. and Genner, M.J. 2017. Evolutionary divergence in life history traits among populations of the Lake Malawi cichlid fish Astatotilapia calliptera. *Ecology and Evolution* 7(20), pp. 8488–8506. doi: 10.1002/ece3.3311.

Pauly, D., Christensen, V., Dalsgaard, J., Froese, R. and Torres Jr, F. 1998. Fishing Down Marine Food Webs. *Science* 279(5352), pp. 860–863.

Paw, J.N. and Chua, T.E. 1989. An assessment of the ecological and economic impact of mangrove conversion in Southeast Asia. *Marine Pollution Bulletin* 20(7), pp. 335–343.

Pensinger, L.G., Polito, M.J. and Midway, S.R. 2021. Ontogenetic stability in the trophic niche of a common Gulf of Mexico fish, Ariopsis felis. *Environmental Biology of Fishes* 104(5), pp. 569–579. doi: 10.1007/s10641-021-01095-7.

Pereira, A.A., Van Hattum, B., De Boer, J., Van Bodegom, P.M., Rezende, C.E. and Salomons, W. 2010. Trace elements and carbon and nitrogen stable isotopes in organisms from a tropical coastal lagoon. *Archives of Environmental Contamination and Toxicology* 59(3), pp. 464–477. doi: 10.1007/s00244-010-9489-2.

Perović, D.J. et al. 2018. Managing biological control services through multi-trophic trait interactions: review and guidelines for implementation at local and landscape scales. *Biological Reviews* 93(1), pp. 306–321. doi: 10.1111/brv.12346.

Persson, A. and Hansson, L.-A. 1999. Diet shift in fish following competitive release. *Canadian Journal of Fisheries and Aquatic Sciences* 56(1), pp. 70–78. doi: 10.1139/cjfas-56-1-70.

Petchey, O.L. et al. 2004. Species loss and the structure and functioning of multitrophic aquatic systems. *OIKOS* 104, pp. 467–478.

Peterson, B.J., Howarth, R.W. and Garritt, R.H. 1985. Multiple Stable Isotopes Used to Trace the Flow of Organic Matter in Estuarine Food Webs. *Science* 227(4692), pp. 1361–1363. Available at: https://www.science.org.

Petrosky, A.L., Rowsey, D.M. and Heaney, L.R. 2021. Molecular assessment of dietary niche partitioning in an endemic island radiation of tropical mammals. *Molecular Ecology* 30(22), pp. 5858–5873. doi: 10.1111/mec.16158.

Phillips, D.L. 2012. Converting isotope values to diet composition: The use of mixing models. *Journal of Mammalogy* 93(2), pp. 342–352. doi: 10.1644/11-MAMM-S-158.1.

Phillips, D.L. et al. 2014a. Best practices for use of stable isotope mixing models in food-web studies. *Canadian Journal of Zoology* 92(10), pp. 823–835. doi: 10.1139/cjz-2014-0127.

Phillips, R.D., Scaccabarozzi, D., Retter, B.A., Hayes, C., Brown, G.R., Dixon, K.W. and Peakall, R. 2014b. Caught in the act: pollination of sexually deceptive trap-flowers by fungus gnats in Pterostylis (Orchidaceae). *Annals of Botany* 113(4), pp. 629–641. doi: 10.1093/aob/mct295.

Piñol, J., Senar, M.A. and Symondson, W.O.C. 2019. The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. *Molecular Ecology* 28(2), pp. 407–419. doi: 10.1111/mec.14776.

Pocock, M.J.O., evans, D.M. and Memmott, J. 2012. The Robustness and Restoration of a Network of Ecological Networks. *Science* 335(6071), pp. 970–973. doi: 10.1126/science.1215156.

Pompanon, F., Deagle, B.E., Symondson, W.O.C., Brown, D.S., Jarman, S.N. and Taberlet, P. 2012. Who is eating what: Diet assessment using next generation sequencing. *Molecular Ecology* 21(8), pp. 1931–1950. doi: 10.1111/j.1365-294X.2011.05403.x.

Post, D.M. 2002. USING STABLE ISOTOPES TO ESTIMATE TROPHIC POSITION: MODELS, METHODS, AND ASSUMPTIONS. *Ecology* 83(3), pp. 703–718.

Post, D.M., Layman, C.A., Arrington, D.A., Takimoto, G., Quattrochi, J. and Montaña, C.G. 2007. Getting to the fat of the matter: Models, methods and assumptions for dealing with lipids in stable isotope analyses. *Oecologia* 152(1), pp. 179–189. doi: 10.1007/s00442-006-0630-x.

Poulakis, G.R. et al. 2017. Sympatric elasmobranchs and fecal samples provide insight into the trophic ecology of the smalltooth sawfish. *Endangered Species Research* 32, pp. 491–506. doi: 10.3354/ESR00824.

Quast, C. et al. 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research* 41(D1). doi: 10.1093/nar/gks1219.

R Core Team. 2022. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rani, V., Sreelakshmi, C., Nandan, S.B., Santu, K.S. and Preethy, C.M. 2023. Feeding ecology of Parasesarma plicatum and its relation to carbon structuring in mangrove ecosystem. *Hydrobiologia*. doi: 10.1007/s10750-022-05133-y.

Ratnasingham, S. and Hebert, P.D.N. 2007. BOLD: The Barcode of Life Data System: Barcoding. *Molecular Ecology Notes* 7(3), pp. 355–364. doi: 10.1111/j.1471-8286.2007.01678.x.

Rau, G.H., Sweeney, R.E., Kaplan, I.R., Mearns, A.J. and Young, D.R. 1981. Notes and Discussions Differences in Animal 13C, 15N and D Abundance Between a Polluted and an Unpolluted Coastal Site: Likely Indicators of Sewage Uptake by a Marine Food Web. *Estuarine, Coastal and Shelf Science* 13, pp. 701–707.

Richards, D.R. and Friess, D.A. 2016. Rates and drivers of mangrove deforestation in Southeast Asia, 2000-2012. *Proceedings of the National Academy of Sciences of the United States of America* 113(2), pp. 344–349. doi: 10.1073/pnas.1510272113.

Richardson, D.S., Jury, F.L., Blaakmeer, K., Komdeur, J. and Burke, T. 2001. Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (Acrocephalus sechellensis). *Molecular Ecology* 10(9), pp. 2263–2273. doi: 10.1046/j.0962-1083.2001.01355.x.

Riebesell, U. 2004. Effects of CO2 Enrichment on Marine Phytoplankton. Journal of *Oceanography* 60, pp. 719–729.

Rivera-Hutinel, A., Bustamante, R.O., Marín, V.H., Medel, R., Bustamante, K.O., Marín, V.H. and Medel2', R. 2012. Effects of sampling completeness on the structure of plant-pollinator networks.

Robinson, B.W. and Wilson, D.S. 1998. Optimal foraging, specialization, and a solution to Liem's paradox. *American Naturalist* 151(3), pp. 223–235. doi: 10.1086/286113.

Robinson, M.D., McCarthy, D.J. and Smyth, G.K. 2009. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1), pp. 139–140. doi: 10.1093/bioinformatics/btp616.

Rog, S.M., Clarke, R.H. and Cook, C.N. 2017. More than marine: revealing the critical importance of mangrove ecosystems for terrestrial vertebrates. *Diversity and Distributions* 23(2), pp. 221–230. doi: 10.1111/ddi.12514.

Rolton, A. et al. 2015. Susceptibility of gametes and embryos of the eastern oyster, Crassostrea virginica, to Karenia brevis and its toxins. *Toxicon* 99, pp. 6–15. doi: 10.1016/j.toxicon.2015.03.002.

Romanuk, T.N., Hayward, A. and Hutchings, J.A. 2011. Trophic level scales positively with body size in fishes. *Global Ecology and Biogeography* 20(2), pp. 231–240. doi: 10.1111/j.1466-8238.2010.00579.x.

Rombouts, I. et al. 2013. Food web indicators under the Marine Strategy Framework Directive: From complexity to simplicity? *Ecological Indicators* 29, pp. 246–254. doi: 10.1016/j.ecolind.2012.12.021.

Roshith, C.M. et al. 2018. Phytoplankton community structure of the Gangetic (Hooghly-Matla) estuary: Status and ecological implications in relation to eco-climatic variability. Flora: Morphology, Distribution, Functional Ecology of Plants 240, pp. 133–143. doi: 10.1016/j.flora.2018.01.001.

Roslin, T. and Majaneva, S. 2016. The use of DNA barcodes in food web construction-terrestrial and aquatic ecologists unite! *Genome* 59(9), pp. 603–628. doi: 10.1139/gen-2015-0229.

Roslin, T., Traugott, M., Jonsson, M., Stone, G.N., Creer, S. and Symondson, W.O.C. 2019. Introduction: Special issue on species interactions, ecological networks and community dynamics – Untangling the entangled bank using molecular techniques. In: Molecular Ecology. Blackwell Publishing Ltd, pp. 157–164. doi: 10.1111/mec.14974.

Rountree, R.A. and Able, K.W. 1992. Foraging Habits, Growth, and Temporal Patterns of Salt-Marsh Creek Habitat Use by Young-of-Year Summer Flounder in New Jersey. *Transactions of the American Fisheries Society* 121(6), pp. 765–776. doi: 10.1577/1548-8659(1992)121<0765:fhgatp>2.3.co;2.

Rowney, F.M. et al. 2021. Environmental DNA reveals links between abundance and composition of airborne grass pollen and respiratory health. *Current Biology* 31(9), pp. 1995-2003.e4. doi: 10.1016/j.cub.2021.02.019.

Roy, K., Ewing, C.P. and Price, D.K. 2021. Diet Analysis of Hawai,i Island's Blackburnia hawaiiensis (Coleoptera: Carabidae) using Stable Isotopes and High-Throughput Sequencing. *Pacific Science* 74(3), pp. 245–256. doi: 10.2984/74.3.3.

Rozas, L.P. and Lasalle, M.W. 1990. A Comparison of the Diets of Gulf Entering and Leaving a Mississippi Brackish Marsh. *Estuaries* 13(3), pp. 332–336.

RStudio Team. 2020. RStudio: Integrated Development for R. Available at: http://www.rstudio.com/ [Accessed: 31 August 2022].

Rueckert, S., Simdyanov, T.G., Aleoshin, V. V. and Leander, B.S. 2011. Identification of a divergent environmental DNA sequence clade using the phylogeny of gregarine parasites (apicomplexa) from crustacean hosts. *PLoS ONE* 6(3). doi: 10.1371/journal.pone.0018163.

Rydene, D.A. and Matheson, R.E. 2003. Diurnal fish density in relation to seagrass and drift algae cover in Tampa Bay, Florida. *Gulf of Mexico Science* 21(1), pp. 35–58. doi: 10.18785/goms.2101.04.

Salomon, A.K., Gaichas, S.K., Shears, N.T., Smith, J.E., Madin, E.M.P. and Gaines, S.D. 2010. Atributos clave y dependencia del contexto de cascadas tróficas inducidas por pesquerías. *Conservation Biology* 24(2), pp. 382–394. doi: 10.1111/j.1523-1739.2009.01436.x.

Salzburger, W. 2009. The interaction of sexually and naturally selected traits in the adaptive radiations of cichlid fishes. *Molecular Ecology* 18(2), pp. 169–185. doi: 10.1111/j.1365-294X.2008.03981.x.

Sánchez-Hernández, J., Nunn, A.D., Adams, C.E. and Amundsen, P.A. 2019. Causes and consequences of ontogenetic dietary shifts: a global synthesis using fish models. *Biological Reviews* 94(2), pp. 539–554. doi: 10.1111/brv.12468.

Sato, J.J., Shimada, T., Kyogoku, D., Komura, T., Uemura, S., Saitoh, T. and Isagi, Y. 2018. Dietary niche partitioning between sympatric wood mouse species (Muridae: Apodemus) revealed by DNA meta-barcoding analysis. *Journal of Mammalogy* 99(4), pp. 952–964. doi: 10.1093/jmammal/gyy063.

Schlacher, T.A. and Connolly, R.M. 2014. Effects of acid treatment on carbon and nitrogen stable isotope ratios in ecological samples: a review and synthesis. *Methods in Ecology and Evolution* 5(6), pp. 541–550. doi: 10.1111/2041-210X.12183.

Schluter, D. 2000. The Ecology of Adaptive Radiation. OUP Oxford.

Schoch, C.L. et al. 2020. NCBI Taxonomy: a comprehensive update on curation, resources and tools. *Database* 2020. doi: 10.1093/database/baaa062.

Scopel, L.C., Diamond, A.W., Kress, S.W., Hards, A.R. and Shannon, P. 2018. Seabird diets as bioindicators of atlantic herring recruitment and stock size: A new tool for ecosystem-based fisheries management. *Canadian Journal of Fisheries and Aquatic Sciences* 75(8), pp. 1215–1229. doi: 10.1139/cjfas-2017-0140.

SECOORA. 2022. Meet the Winners of the Harmful Algal Bloom Request for Proposals. Available at: Meet the Winners of the Harmful Algal Bloom Request for Proposals [Accessed: 12 April 2023].

Seehausen, O. 2004. Hybridization and adaptive radiation. *Trends in Ecology and Evolution* 19(4), pp. 198–207. doi: 10.1016/j.tree.2004.01.003.

Seehausen, O., Witte, F., van Alphen, J.J.M. and Bouton, N. 1998. Direct mate choice maintains diversity among sympatric cichlids in Lake Victoria. *Journal of Fish Biology*, pp. 37–55.

Selleslagh, J., Lobry, J., Amara, R., Brylinski, J.M. and Boët, P. 2012. Trophic functioning of coastal ecosystems along an anthropogenic pressure gradient: A French case study with emphasis on a small and low impacted estuary. *Estuarine, Coastal and Shelf Science* 112, pp. 73–85. doi: 10.1016/j.ecss.2011.08.004.

Serafy, J.E., Faunce, C.H. and Lorenz, J.J. 2003. MANGROVE SHORELINE FISHES OF BISCAYNE BAY, FLORIDA. *BULLETIN OF MARINE SCIENCE* 72(1), pp. 161–180. Available at: https://www.researchgate.net/publication/233515960.

Serrano, X., Grosell, M. and Serafy, J.E. 2010. Salinity selection and preference of the grey snapper Lutjanus griseus: Field and laboratory observations. *Journal of Fish Biology* 76(7), pp. 1592–1608. doi: 10.1111/j.1095-8649.2010.02585.x.

Shearer, C.A. et al. 2007. Fungal biodiversity in aquatic habitats. *Biodiversity and Conservation* 16(1), pp. 49–67. doi: 10.1007/s10531-006-9120-z.

Sheaves, M. 2005. Nature and consequences of biological connectivity in mangrove systems. *Marine Ecology Progress Series* 302, pp. 293–305.

Sheaves, M. 2009. Consequences of ecological connectivity: The coastal ecosystem mosaic. *Marine Ecology Progress Series* 391, pp. 107–115. doi: 10.3354/meps08121.
Sheaves, M., Baker, R., Nagelkerken, I. and Connolly, R.M. 2015. True Value of Estuarine and Coastal Nurseries for Fish: Incorporating Complexity and Dynamics. *Estuaries and Coasts* 38(2), pp. 401–414. doi: 10.1007/s12237-014-9846-x.

Sheaves, M., Johnston, R. and Connolly, R.M. 2010. Temporal dynamics of fish assemblages of natural and artificial tropical estuaries. *Marine Ecology Progress Series* 410, pp. 143–157. doi: 10.3354/meps08655.

Sheaves, M. and Molony, B. 2000. Short-circuit in the mangrove food chain. *MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser* 199, pp. 97–109.

Sheppard, S.K. and Harwood, J.D. 2005. Advances in molecular ecology: Tracking trophic links through predator-prey food-webs. *Functional Ecology* 19(5), pp. 751–762. doi: 10.1111/j.1365-2435.2005.01041.x.

Shepta, E., Perkin, J., Mayes, K.B. and Montaña, C.G. 2021. Insights on the establishment of the invasive Sheepshead minnow (Cyprinodon variegatus) in the Brazos River.

Sheridan, P. and Hays, C. 2003. ARE MANGROVES NURSERY HABITAT FOR TRANSIENT FISHES AND DECAPODS? Available at: http://www.csa.com/.

Sheridan, P.F. 1978. Food Habits of the Bay Anchovy, Anchoa mitchilli, in Apalachicola Bay, Florida. *Northeast Gulf Science* 2(2). doi: 10.18785/negs.0202.06.

Sigee, D.C. 2005. Freshwater microbiology : biodiversity and dynamic interactions of microorganisms in the aquatic environment. J. Wiley.

Skov, M.W. and Hartnoll, R.G. 2002. Paradoxical selective feeding on a low-nutrient diet: Why do mangrove crabs eat leaves? *Oecologia* 131(1), pp. 1–7. doi: 10.1007/s00442-001-0847-7.

Soininen, E.M. et al. 2014. Sources of variation in small rodent trophic niche: New insights from DNA metabarcoding and stable isotope analysis. *Isotopes in Environmental and Health Studies* 50(3), pp. 361–381. doi: 10.1080/10256016.2014.915824.

de Sousa, L.L., Silva, S.M. and Xavier, R. 2019. DNA metabarcoding in diet studies: Unveiling ecological aspects in aquatic and terrestrial ecosystems. *Environmental DNA* 1(3), pp. 199–214. doi: 10.1002/edn3.27.

Spence, A.R., Wilson Rankin, E.E. and Tingley, M.W. 2022. DNA metabarcoding reveals broadly overlapping diets in three sympatric North American hummingbirds. *Ornithology* 139(1). doi: 10.1093/ornithology/ukab074.

Spens, J. et al. 2017. Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in Ecology and Evolution* 8(5), pp. 635–645. doi: 10.1111/2041-210X.12683.

Spies, R.B., Kruger, H., Ireland, R. and Rice, D.W. 1989. Stable isotope ratios and contaminant concentrations in a sewage-distorted food web. *Marine Ecology Progress Series* 54, pp. 157–170.

Staples, D.J.;, Vance, D.J.; and Heales, D.S. 1984. Habitat requirements of juvenile penaeid prawns and their relationship to offshore fisheries. In: Rothlisberg, P. C. , Hill, B. J. , S., and taples, D. J. eds. 2nd Australian National Prawn Seminar. Kooralbyn, Queensland. : CSIRO, Australia, pp. 47–54.

Stapleton, T.E., Weinstein, S.B., Greenhalgh, R. and Dearing, M.D. 2022. Successes and limitations of quantitative diet metabarcoding in a small, herbivorous mammal. *Molecular Ecology Resources* 22(7), pp. 2573–2586. doi: 10.1111/1755-0998.13643.

Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M.D.M., Breiner, H.W. and Richards, T.A. 2010. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. *Molecular Ecology* 19(SUPPL. 1), pp. 21–31.
doi: 10.1111/j.1365-294X.2009.04480.x.

Stoner, A.W. 1980. Feeding Ecology of Lagondon Rhomboides (Pices:Sparidae): Variation and FUnctional Responses. Fishery Bulletin.

Strayer, D.L. 2009. Benthic Invertebrate Fauna, Lakes and Reservoirs. In: Encyclopedia of Inland Waters. Elsevier, pp. 191–204. doi: 10.1016/B978-012370626-3.00161-7.

Streelman, J.T. and Albertson, R.C. 2006. Evolution of novelty in the cichlid dentition. Journal of Experimental Zoology Part B: *Molecular and Developmental Evolution* 306(3), pp. 216–226. doi: 10.1002/jez.b.21101.

Summers, R.W. 1980. The Diet and Feeding Behaviour of the Flounder Platichthys flesus (L.) in the Ythan Estuary, Aberdeenshire, Scotland. *Estuarine and Coastal Marine Science* 11, pp. 217–232.

Svardal, H., Salzburger, W. and Malinsky, M. 2021. Genetic Variation and Hybridization in Evolutionary Radiations of Cichlid Fishes Evolutionary radiations: periods of significant acceleration in the rate of species diversification in particular lineages. *Annual Reviews of Animal Biosciences* 9, pp. 55–79. Available at: https://doi.org/10.1146/annurev-animal-061220-.

Swanson, B.O., Gibb, A.C., Marks, J.C. and Hendrickson, D.A. 2003. Trophic Polymorphism and Behavioral Differences Decrease Intraspecific Competition in a Cichlid, Herichthys minckleyi. *Ecology* 84(6), pp. 1441–1446.

Swanson, B.O., Gibb, A.C., Marks, J.C. and Hendrickson, D.A. 2008. Variation in foraging behavior facilitates resource partitioning in a polymorphic cichlid, Herichthys minckleyi. *Environmental Biology of Fishes* 83(2), pp. 147–154. doi: 10.1007/s10641-007-9307-5.

Symonds, M.R.E. and Moussalli, A. 2011. A brief guide to model selection, multimodel inference and model averaging in behavioural ecology using Akaike's information criterion. *Behavioral Ecology and Sociobiology* 65(1), pp. 13–21. doi: 10.1007/s00265-010-1037-6.

Taberlet, P., Bonin, A., Zinger, L. and Coissac, E. 2018. Environmental DNA: for Biodiversity research and monitoring. Oxford University Press.

Taguchi, T., Miura, Y., Krueger, D. and Sugiura, S. 2014. Utilizing stomach content and faecal DNA analysis techniques to assess the feeding behaviour of largemouth bass Micropterus salmoides and bluegill Lepomis macrochirus. *Journal of Fish Biology* 84(5), pp. 1271–1288. doi: 10.1111/jfb.12341.

Takahashi, M., DiBattista, J.D., Jarman, S., Newman, S.J., Wakefield, C.B., Harvey, E.S. and Bunce, M. 2020. Partitioning of diet between species and life history stages of sympatric and cryptic snappers (Lutjanidae) based on DNA metabarcoding. *Scientific Reports* 10(1). doi: 10.1038/s41598-020-60779-9.

Tam, J.C. et al. 2017. Towards ecosystem-based management: Identifying operational food-web indicators for marine ecosystems. In: ICES Journal of Marine Science. Oxford University Press, pp. 2040–2052. doi: 10.1093/icesjms/fsw230.

Tanentzap, A.J. et al. 2017. Terrestrial support of lake food webs: Synthesis reveals controls over cross-ecosystem resource use. Science Advances.

Tercel, M.P.T.G., Symondson, W.O.C. and Cuff, J.P. 2021. The problem of omnivory: A synthesis on omnivory and DNA metabarcoding. *Molecular ecology* 30(10), pp. 2199–2206. doi: 10.1111/mec.15903.

Terry, C. 2018. Package "bipartiteD3" Type Package Title Interactive Bipartite Graphs. Available at: https://bl.ocks.org/NPashaP.

Teunisse, H.G.M. 2022. Fantaxtic: fantaxtic plots from Phyloseq data. R package version 0.1.0. 2018.

Thevenon, F., Williamson, D., Vincens, A., Taieb, M., Merdaci, O., Decobert, M. and Buchet, G. 2003. A late-Holocene charcoal record from Lake Masoko, SW Tanzania: climatic and anthropologic implications. *The Holocene* 13, pp. 785–792.

Thomas, D. and Rumbold, D. 2006. Coastal Bays and Barrier Islands Conceptual Ecological Model. Coastal Bays & Barrier Islands CEM.

Thompson, R.M., Hemberg, M., Starzomski, B.M. and Shurin, J.B. 2007. Trophic levels and trophic tangles: The prevalence of omnivory in real food webs. *Ecology* 88(3), pp. 612–617. doi: 10.1890/05-1454.

Thorpe, R.S., Surget-Groba, Y. and Johansson, H. 2008. The relative importance of ecology and geographic isolation for speciation in anoles. *Philosophical Transactions of the Royal Society B: Biological Science*s 363(1506), pp. 3071–3081. doi: 10.1098/rstb.2008.0077.

Thrush, S.F. and Dayton, P.K. 2010. What can ecology contribute to ecosystem-based management? *Annual Review of Marine Science* 2(1), pp. 419–441. doi: 10.1146/annurev-marine-120308-081129.

Tixier, P., Giménez, J., Reisinger, R.R., Méndez-Fernandez, P., Arnould, J.P.Y., Cherel, Y. and Guinet, C. 2019. Importance of toothfish in the diet of generalist subantarctic killer whales: implications for fisheries interactions. *Marine Ecology Progress Series* 613, pp. 197–210. doi: 10.3354/meps12894.

Tolan, J.M., Holt, S.A. and Onuf, C.P. 1997. Distribution and Community Structure of Ichthyoplankton in Laguna Madre Seagrass Meadows: Potential Impact of Seagrass Species Change. *Estuaries* 20(2), pp. 450–464.

Tolley, S.G., Volety, A.K. and Savarese, M. 2005. Influence of salinity on the habitat use of oyster reefs in three Southwest Florida estuaries. *Journal of Shellfish Research* 24(1), pp. 127–137. doi: 10.2983/0730-8000(2005)24[127:IOSOTH]2.0.CO;2.

Touchette, B.W. and Burkholder, J.M. 2000. Overview of the physiological ecology of carbon metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology* 250, pp. 169–205. Available at: www.elsevier.nl/locate/jembe.

Traugott, M., Thalinger, B., Wallinger, C. and Sint, D. 2021. Fish as predators and prey: DNA-based assessment of their role in food webs. *Journal of Fish Biology* 98(2), pp. 367–382. doi: 10.1111/jfb.14400.

Traveset, A. et al. 2015. Bird-flower visitation networks in the Galápagos unveil a widespread interaction release. *Nature Communications* 6. doi: 10.1038/ncomms7376.

Trifonova, N., Kenny, A., Maxwell, D., Duplisea, D., Fernandes, J. and Tucker, A. 2015. Spatio-temporal Bayesian network models with latent variables for revealing trophic dynamics and functional networks in fisheries ecology. *Ecological Informatics* 30, pp. 142–158. doi: 10.1016/j.ecoinf.2015.10.003.

Trueman, C.N., Mackenzie, K.M. and Palmer, M.R. 2012. Identifying migrations in marine fishes through stable-isotope analysis. *Journal of Fish Biology* 81(2), pp. 826–847. doi: 10.1111/j.1095-8649.2012.03361.x.

Tse, P., Nip, T.H.M. and Wong, C.K. 2008. Nursery function of mangrove: A comparison with mudflat in terms of fish species composition and fish diet. *Estuarine, Coastal and Shelf Science* 80(2), pp. 235–242. doi: 10.1016/j.ecss.2008.08.002.

Turner, G. 2007. Adaptive radiation of cichlid fish. *Current Biology*.

Turner, G.F., Seehausen, O., Knight, M.E., Allender, C.J. and Robinson, R.L. 2001. How many species of cichlid fishes are there in African lakes? *Molecular Ecology* 10, pp. 793–806.

Turner, G.F., Turner, G.F., Ngatunga, B.P. and Genner, M.J. 2019. The Natural History of the Satellite Lakes of Lake Malawi. *EcoEvoRxiv*.

Turner, R.E. 1977. Intertidal Vegetation and Commercial Yields of Penaeid Shrimp. *Transactions of the American Fisheries Society* 106(5), pp. 411–416. doi: 10.1577/1548-8659(1977)106<411:IVACYO>2.0.CO;2.

Tyers, A. 2013. Divergence and speciation of East African haplochromine cichlid fish. Bangor University.

Tyers, A.M. and Turner, G.F. 2013. Signal and preference divergence among populations of the non-endemic basal Lake Malawi cichlid fish Astatotilapia calliptera (Perciformes: Cichlidae). *Biological Association of Linnean Society* 110, pp. 180–188. Available at: https://academic.oup.com/biolinnean/article/110/1/180/2415659.

Umaña-Villalobos, G. 2010. Temporal variation of phytoplankton in a small tropical crater lake, Costa Rica. *Rev. Biol. Trop*. 58(4), pp. 1405–1419.

Urakawa, H., Martens-Habbena, W. and Stahl, D.A. 2010. High abundance of ammonia-oxidizing archaea in coastal waters, determined using a modified DNA extraction method. *Applied and Environmental Microbiology* 76(7), pp. 2129–2135. doi: 10.1128/AEM.02692-09.

Vanhove, M.P.M., Hablützel, P.I., Pariselle, A., Šimková, A., Huyse, T. and Raeymaekers, J.A.M. 2016. Cichlids: A Host of Opportunities for Evolutionary Parasitology. *Trends in Parasitology* 32(10), pp. 820–832. doi: 10.1016/j.pt.2016.07.002.

de Vargas, C. et al. 2015. Eukaryotic plankton diversity in the sunlit ocean. *Science* 348(6237). doi: 10.1126/science.1261605.

Vasconcellos, R.M., Gomes-Gonçalves, R.S., Santos, J.N.S., Cruz Filho, A.G. and Araújo, F.G. 2018. Do closely related species share of feeding niche along growth? Diets of three sympatric species of the mojarras (Actinopterygii: Gerreidae) in a tropical bay in southeastern Brazil. *Environmental Biology of Fishes* 101(6), pp. 949–962. doi: 10.1007/s10641-018-0750-2.

Vaslet, A., Phillips, D.L., France, C., Feller, I.C. and Baldwin, C.C. 2012. The relative importance of mangroves and seagrass beds as feeding areas for resident and transient fishes among different mangrove habitats in Florida and Belize: Evidence from dietary and stable-isotope analyses. *Journal of Experimental Marine Biology and Ecology* 434–435, pp. 81–93. doi: 10.1016/j.jembe.2012.07.024.

Vega, M.P. 1999. Life-Stage Differences in the Diet of Parabroteas sarsi (DADAY) (Copepoda, Calanoida): A Field Study. *Limnologica*, pp. 186–190.

Walters, B.B. et al. 2008. Ethnobiology, socio-economics and management of mangrove forests: A review. *Aquatic Botany* 89(2), pp. 220–236. doi: 10.1016/j.aquabot.2008.02.009.

Wang, G. and Wang, Y. 1997. Frequency of Formation of Chimeric Molecules as a Consequence of PCR Coamplification of 16S rRNA Genes from Mixed Bacterial Genomes. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 63(12), pp. 4645–4650. Available at: https://journals.asm.org/journal/aem.

Wanless, S., Harris, M.P., Redman, P. and Speakman, J.R. 2005. Low energy values of fish as a probable cause of a major seabird failure in the North Sea. *Marine Ecology Progress Series* 294, pp. 1–8.

Ward, R.D., Hanner, R. and Hebert, P.D.N. 2009. The campaign to DNA barcode all fishes, FISH-BOL. *Journal of Fish Biology* 74(2), pp. 329–356. doi: 10.1111/j.1095-8649.2008.02080.x.

Wasno, R.M., Toll, R.B. and Volety, A. 2020. Investigation of Trophic Transfer from Oyster Reefs to Predatory Fish in Southwest Florida. *Journal of Shellfish Research* 39(2), pp. 257–268. doi: 10.2983/035.039.0207.

Watanabe, K., Monaghan, M.T., Takemon, Y. and Omura, T. 2008. Biodilution of heavy metals in a stream macroinvertebrate food web: Evidence from stable isotope analysis. *Science of the Total Environment 394*(1), pp. 57–67. doi: 10.1016/j.scitotenv.2008.01.006.

Wei, C., Wang, H., Ma, M., Hu, Q. and Gong, Y. 2020. Factors Affecting the Mixotrophic Flagellate Poterioochromonas malhamensis Grazing on Chlorella Cells. *Journal of Eukaryotic Microbiology* 67(2), pp. 190–202. doi: 10.1111/jeu.12770.

Wei, L., Yan, C., Ye, B. and Guo, X. 2008. Effects of salinity on leaf δ13C in three dominant mangrove species along salinity gradients in an estuarine wetland, southeast China. *Journal of Coastal Research* 24(1), pp. 267–272. doi: 10.2112/06-0765.1.

Weiss, S. et al. 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* 5(1). doi: 10.1186/s40168-017-0237-y.

Wells, B.K. et al. 2020. Implementing Ecosystem-Based Management Principles in the Design of a Salmon Ocean Ecology Program. *Frontiers in Marine Science* 7. doi: 10.3389/fmars.2020.00342.

Whitaker, M.R.L., Baker, C.C.M., Salzman, S.M., Martins, D.J. and Pierce, N.E. 2019. Combining stable isotope analysis with DNA metabarcoding improves inferences of trophic ecology. *PLoS ONE* 14(7). doi: 10.1371/journal.pone.0219070.

Whitfield, A.K. 2015. Why are there so few freshwater fish species in most estuaries? *Journal of Fish Biology* 86(4), pp. 1227–1250. doi: 10.1111/jfb.12641.

Whittall, J.B. and Hodges, S.A. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* 447(7145), pp. 706–709. doi: 10.1038/nature05857.

Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis.

Winemiller, K.O. 1990. Spatial and Temporal Variation in Tropical Fish Trophic Networks. *Ecological Monographs* 60(3), pp. 331–367. doi: 10.2307/1943061.

Woodland, R.J., Rodríguez, M.A., Magnan, P., Glémet, H. and Cabana, G. 2012. Incorporating temporally dynamic baselines in isotopic mixing models. *Ecology* 93(1), pp. 131–144. doi: 10.1890/11-0505.1.

Woodward, G. 2009a. Biodiversity, ecosystem functioning and food webs in fresh waters: Assembling the jigsaw puzzle. *Freshwater Biology* 54(10), pp. 2171–2187. doi: 10.1111/j.1365-2427.2008.02081.x.

Woodward, G. 2009b. Biodiversity, ecosystem functioning and food webs in fresh waters: Assembling the jigsaw puzzle. *Freshwater Biology* 54(10), pp. 2171–2187. doi: 10.1111/j.1365-2427.2008.02081.x.

Woodward, G., Ebenman, B., Emmerson, M., Montoya, J.M., Olesen, J.M., Valido, A. and Warren, P.H. 2005. Body size in ecological networks. *Trends in Ecology and Evolution* 20(7), pp. 402–409. doi: 10.1016/j.tree.2005.04.005.

Woodward, G. and Hildrew, A.G. 2002. Food web structure in riverine landscapes. *Freshwater Biology* 47, pp. 777–798.

Wootton, K.L. 2017. Omnivory and stability in freshwater habitats: Does theory match reality? *Freshwater Biology* 62(5), pp. 821–832. doi: 10.1111/fwb.12908.

Wordley, C., Slate, J. and Stapley, J. 2011. Mining online genomic resources in Anolis carolinensis facilitates rapid and inexpensive development of cross-species microsatellite markers for the Anolis lizard genus. *Molecular Ecology Resources* 11(1), pp. 126–133. doi: 10.1111/j.1755-0998.2010.02863.x.

Yeager, L.A. and Layman, C.A. 2011. Energy flow to two abundant consumers in a subtropical oyster reef food web. *Aquatic Ecology* 45(2), pp. 267–277. doi: 10.1007/s10452-011-9352-1.

Yeager, L.A., Layman, C.A. and Hammerschlag-Peyer, C.M. 2014. Diet variation of a generalist fish predator, grey snapper Lutjanus griseus, across an estuarine gradient: Trade-offs of quantity for quality? *Journal of Fish Biology* 85(2), pp. 264–277. doi: 10.1111/jfb.12416.

Yvon-Durocher, G. et al. 2011a. Across ecosystem comparisons of size structure: Methods, approaches and prospects. *Oikos* 120(4), pp. 550–563. doi: 10.1111/j.1600-0706.2010.18863.x.

Yvon-Durocher, G., Montoya, J.M., Trimmer, M. and Woodward, G. 2011b. Warming alters the size spectrum and shifts the distribution of biomass in freshwater ecosystems. *Global Change Biology* 17(4), pp. 1681–1694. doi: 10.1111/j.1365-2486.2010.02321.x.

vander Zanden, Jake.M., Shuter, B.J., Lester, N.P. and Rasmussen, J.B. 2000. Within-and among-population variation in the trophic position of a pelagic predator, lake trout (Salvelinus namaycush).

Vander Zanden, M.J., Hulshof, M., Ridgway, M.S. and Rasmussen, J.B. 1998. Application of Stable Isotope Techniques to Trophic Studies of Age-0 Smallmouth Bass. *Transactions of the American Fisheries Society* 127(5), pp. 729–739. doi: 10.1577/1548-8659(1998)127<0729:aositt>2.0.co;2.

vander Zanden, M.J. and Rasmussen, J.B. 1999. Primary Consumer δ 13C and δ 15N and the Trophic Position of Aquatic Consumers. *Ecology* 80(4), pp. 1395–1404.

Vander Zanden, M.J. and Vadeboncoeur, Y. 2002. Fishes as integrators of benthic and pelagic food webs in lakes. *Ecology* 83(8), pp. 2152–2161. doi: 10.1890/0012-9658(2002)083[2152:FAIOBA]2.0.CO;2.

Zhang, G.K., Chain, F.J.J., Abbott, C.L. and Cristescu, M.E. 2018. Metabarcoding using multiplexed markers increases species detection in complex zooplankton communities. *Evolutionary Applications* 11(10), pp. 1901–1914. doi: 10.1111/eva.12694.

Zhang, Z., Mai, Y. and Yang, M. 2022. Basic and Advanced Statistical Power Analysis.

zu Ermgassen, P.S.E. et al. 2020. Fishers who rely on mangroves: Modelling and mapping the global intensity of mangrove-associated fisheries. *Estuarine, Coastal and Shelf Science* 247. doi: 10.1016/j.ecss.2020.106975.