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Mini DNA barcodes reveal the details of the foraging ecology of the largehead hairtail, *Trichiurus lepturus* (Scombriformes: Trichiuridae), from São Paulo, Brazil

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The largehead hairtail, *Trichiurus lepturus*, is an opportunistic, voracious, and piscivorous predator. Studies of fish feeding behavior based on the analysis of stomach contents are limited by the potential for the visual identification of the ingesta. However, molecular tools, in particular DNA barcoding, have been used successfully to identify stomach contents. When morphological analyses are not possible, molecular tools can precisely identify the components of the diet of a fish based on its stomach contents. This study used mini barcoding to identify food items ingested by *T. lepturus* off the northern coast of São Paulo State, Brazil. Forty-six sequences were obtained and were diagnosed as belonging to six different fish species: *Pimelodus maculatus*, *Paralonchurus brasiliensis*, *Isopisthus parvipinnis*, *Opisthonema oglinum*, *Harengula clupeola*, and *Pellona harroweri* or as belonging to the genera *Lycengraulis* and *Sardinella*. *Trichiurus lepturus* is an opportunistic predator that will exploit an available prey of an appropriate size. The results indicate that these fish migrate to warmer waters, such as those found in estuarine environments, at certain times of the year, where they exploit prey species that reproduce in this environment. One example was *Pimelodus maculatus*, which was the prey species most exploited based on the analysis of the material collected.

Keywords: Largehead hairtail, COI gene, Mini-barcode, Molecular tool.

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O peixe-espada, *Trichiurus lepturus*, é um predador oportunista, voraz e piscívoro. Os estudos do comportamento alimentar dos peixes com base na análise do conteúdo estomacal são limitados pelo potencial de identificação visual do material ingerido. No entanto, ferramentas moleculares, em particular o DNA barcode, têm sido utilizadas com sucesso para identificar o conteúdo do estômago. Quando as análises morfológicas não são possíveis, essas ferramentas moleculares podem identificar com precisão os componentes da dieta de um peixe com base em seu conteúdo estomacal. Este estudo utilizou o mini barcode (uma sequencia parcial do gene COI do DNA mitocondrial) para identificar alimentos ingeridos por *T. lepturus* no litoral Norte do estado de São Paulo, Brasil. Quarenta e seis sequências foram obtidas e combinadas com seis espécies diferentes de peixes: *Pimelodus maculatus*, *Paralonchurus brasiliensis*, *Isopisthus parvipinnis*, *Opisthonema oglinum*, *Harengula clupeola* e *Pellona harroweri* ou como pertencente aos gêneros *Lycengraulis* e *Sardinella*. *Trichiurus lepturus* é um predador oportunista que explora qualquer presa disponível que possua tamanho apropriado. Os resultados indicam que esses peixes migram para águas mais quentes em determinadas épocas do ano, como as encontradas em ambientes estuarinos, onde exploram espécies que se reproduzem neste ambiente. Um exemplo foi *Pimelodus maculatus*, sendo a espécie mais explorada por *T. lepturus*, a partir da análise do material coletado.

Palavras-chave: Peixe espada, Gene COI, Mini-barcode, Ferramenta molecular.

INTRODUCTION

The family Trichiuridae of the order Scombriformes is composed of 10 genera and 44 species of mostly large-sized fishes that have an elongated and laterally-compressed body, reduced or absent pelvic fin, and sharp, triangular teeth (Randall, 1967; Nelson *et al*., 2016). These fish represent an important group of predators with diurnal habits and highly selective feeding behavior (Ros Pichs, Castillo, 1978; Pardo-Rodríguez *et al*., 2003). *Trichiurus* Linnaeus, 1758, has at least 10 commercially-important species that are targeted by fisheries around the world (Nakamura, Parin, 1993), although catches are often grouped in a single category of fish (Tzeng, Chiu, 2012). The largehead hairtail, *Trichiurus lepturus* Linnaeus, 1758, is a single circumglobal species, which is an important fishery resource, the eleventh most exploited fish species in the world, with a catch of 1.151.000 tons in 2018 (FAO, 2020).

Despite its commercial importance, few data are available on the ecology of *T. lepturus*, which is classified as Least Concern (LC) in Brazil (ICMBio, 2018). *Trichiurus lepturus* is a benthopelagic species, which is typically found on the continental shelf, ranging from inshore waters to depths of approximately 350 m and occurs in dense schools. This fish is a marine predator (Costa *et al*., 2009) that reaches a body length of approximately 160 cm (Meriem *et al*., 2011), occupies a relatively high level in the marine food chain, feeding on a variety of prey, including fishes (*Stolephorus device*, *Sardinella longiceps*, *Saurida tumbil*, and other fishes) besides juveniles of *T. lepturus*,

indicating cannibalistic behaviour, squids (*Loligo* sp., *Octopus* sp., *Sepia* sp.), and crustaceans (*Acetes* sp., *Oratosquila nepa* and unidentified shrimps) (Randall, 1995; Chiou *et al*., 2006; Rohit *et al*., 2015). It is a prey species of elasmobranchs and small cetaceans (Costa *et al*., 2009).

The collection of detailed data on the composition of the diet of an animal species, can provide important insights into a range of questions, from the understanding of the biology of the species to the trophic flows and functioning of ecosystems. In the specific case of fish, feeding habits and trophic levels have traditionally been defined primarily through the quantification of stomach contents (Buckland *et al*., 2017). In this approach, the composition of the prey of carnivorous species is determined through the visual inspection of the ingesta, with the items encountered being identified taxonomically, although this approach is often hampered by the digestion of the prey, which may leave only partial fragments of the animals ingested (Arroyave, Stiassny, 2014).

The DNA barcode can be as a molecular tool to identify stomach contents, based on a sequence of approximately 650 base pairs (bps) of the mitochondrial DNA Cytochrome c Oxidase subunit I (COI) gene, which can be used to identify even minuscule fragments of the prey with great precision (Hebert *et al*., 2003). This tool can be extremely useful when the food item cannot be identified using morphological criteria, due to advanced digestion (Zeale *et al*., 2011), or when the diet cannot be deduced by observing feeding behavior (Deagle *et al*., 2005; Passmore *et al*., 2006). This DNA based method permits the discrimination and identification of food items, often at species level, even from partially digested tissue fragments (Arroyave, Stiassny, 2014; Xing *et al*., 2020). In a recent review, Sousa *et al.* (2019), referred to this field of research as dDNA (dietary DNA) and discussed its importance when associated with eDNA (environmental DNA).

Meusnier *et al.* (2008) developed universal primers known as mini-barcodes to optimize the DNA barcoding of processed samples or avoid the difficulty of analyzing degraded material. These primers amplify short target regions of the full COI barcode (*e.g*., 100–300 bps), and provide an ideal amplicon for the analysis of stomach contents. The efficiency of the mini-barcode technique has been tested in several different types of animals, including mammals (Rodrigues *et al*., 2020), fish (Dhar, Ghosh, 2017), crustaceans (Govender *et al*., 2019), snakes (Dubey *et al*., 2011), birds, and insects (see Meusnier *et al*., 2008). The mini-barcode approach has been used to resolve a range of problems resulting from the fragmentation of the DNA of highly processed samples. Studies have included the monitoring of populations of invasive rabbits using degraded fecal DNA (Rodrigues *et al*., 2020), the identification of the prey species of the long-eared bat (Alberdi *et al*., 2012) and birds (Joo, Park, 2012), and the identification of the species used to produce shark fin soup (Fields *et al*., 2015) and other processed fishery products (Sultana *et al*., 2018). This was possible due to a comparative analysis of sequences, with species being identified based on the presence or absence of specific nucleotide sequences.

The present study applied the DNA mini-barcode tool to identify prey species of the carnivorous fish *Trichiurus lepturus*, based on the analysis of stomach contents. This species has economic importance, being widely consumed, and occupies a high level in the marine food chain, as a carnivorous species, and thus the correct knowledge of

its preys can allow the identification of species that carries undesirable products, like heavy metals and plastic, to humans. Additionally, this study can be used as further model to the investigation of stomach content in fishes.

MATERIAL AND METHODS

A total of 246 specimens of largehead hairtail *Trichiurus lepturus* were collected off the northern coast of São Paulo State, in the municipality of São Sebastião, southeastern Brazil (23°49'25"S 45°32'11"W). The samples were collected by local fishers in May, June, July, August, September, October, November, and December 20116 (Tab. **S1**). The fish were measured, and their stomachs were removed, weighed, the contents were separated, and the specimens were fixed in ethanol 96%. The specimens were deposited in the collection of the Laboratório Biologia e Genética de Peixes, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Botucatu, São Paulo, Brazil.

The total DNA was extracted from the stomach contents following the protocol proposed by Ivanova *et al*. (2006). Partial sequences of approximately 250 base pairs (bps) of the COI gene were obtained by PCR amplification using Meusnier *et al*., (2008) primers Minibarcode F1 (5'-TCCACTAATCACAARGATATTGGT-3') and Minibarcode R1 (3'- GAAAATCATAATGAAGGCATGAGC-5'). The PCR reactions were performed using the following temperature cycle (Veriti®erititiitThermal Cycler, BiosystemsTM Applied or Mastercycler®aEPGradient, Eppendorf): 2 min at 95°C, 1 min at 95°C, 1min at 46°C and 30s at 68°C for five cycles, followed by 30 cycles of 1 min at 95°C, 1 min at 52°C, and 30s at 68°C, with a final extension of 5 min at 68°C. The PCR mix contained: 8.55 µL of ultrapure water (milli-Q); 1.25 µL of 10X buffer; 0.5 µL of $MgCl_2$ (50mM), 0.5 µL of dNTPs (2mM); 0.25 µL of each primer (10 mM); 0.2 µL of 5U/µL *Taq* DNA polymerase; 50–100 ng of PHT (Phoneutria Biotecnologia e Serviços Ltda, Brazil), and 1 µL of the DNA template. The results of the PCR were confirmed by electrophoresis in 1% agarose gel using Blue Green Loading Dye I (LGC Biotecnologia).

The amplified PCR products were purified with ExoSap-IT® solution (USB Corporation) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems). The reaction solution contained: 3.9 µl of ultrapure water; 1.05 µl of 5X buffer; 0.7µl of BigDye Terminator mix; 0.35µl of the Uni Minibar F1 or Uni Minibar R1 primers (10 mM), and 1.0µl of the purified PCR product (50 ng/µl). The cycle was 2 min at 96°C, followed by 35 cycles of 30s at 96°C, 15s at 54°C, and 4 min at 60°C. The purified PCR products were then precipitated in 125nm EDTA/sodium acetate/alcohol and the samples were sequenced automatically using an ABI 3130X1 sequencer (Applied Biosystems™).

The original sequences obtained here were analyzed using Geneious 4.8.5 (Kearse *et al*., 2012) to construct consensus sequences for each sample and then submitted to GenBank at the National Center for Biotechnology Information, NCBI, using the BLASTn tool (Johnson *et al*., 2008), to check the identity of the sequences.

RESULTS

A total of 246 largehead hairtail specimens were analyzed. These specimens ranged in total length from 234 mm to 1.430 mm and weighed between 139.2g and 1.424.81g. Around a third (75) of the specimens had some material in the stomach, with between 29 and 33 samples of stomach contents being collected per month (excluding May and October, when all stomachs were empty). A total of 139 prey items were obtained from these samples, including partially digested specimens that consisted of body parts, such as scales, tissue fragments and bones. None of these prey items could be morphologically identified to the species level prior to the DNA barcoding.

It was possible to obtain COI mini-barcode sequences from 46 of these 140 prey items. These sequences had between 73 and 247 base pairs, although 68% had at least 150 bps. No indels or stop codons were detected in any of the sequences. The partial COI sequences of the study specimens had a mean nucleotide content of A = 27.0%, G = 23.6%, C = 23.7%, and T = 26.7%.

All the sequences obtained in the present study matched the NCBI reference sequences, with a similarity from 90% to 100%, samples with match values between 90 and 97.9% were listed as genus and samples with values below 90% were discarded (Tab. **S2**). The DNA mini-barcode permit to identified specimens from three fish orders, Siluriformes, Acanthuriformes, and Clupeiformes, including elements of the five families Pimelodidae, Sciaenidae, Clupeidae, and Pristigasteridae, representing six species of six genera (Tab. 1).

The most frequent prey species identified in the stomach content samples (Fig. 1) was *Pimelodus maculatus* (n = 12 sequences), followed by *Paralonchurus brasiliensis* (n = 8), *Opisthonema oglinum* (n = 2), *Harengula clupeola* (n = 2), *Pellona harroweri* (n = 1), and *Isopisthus parvipinnis* (n = 1).

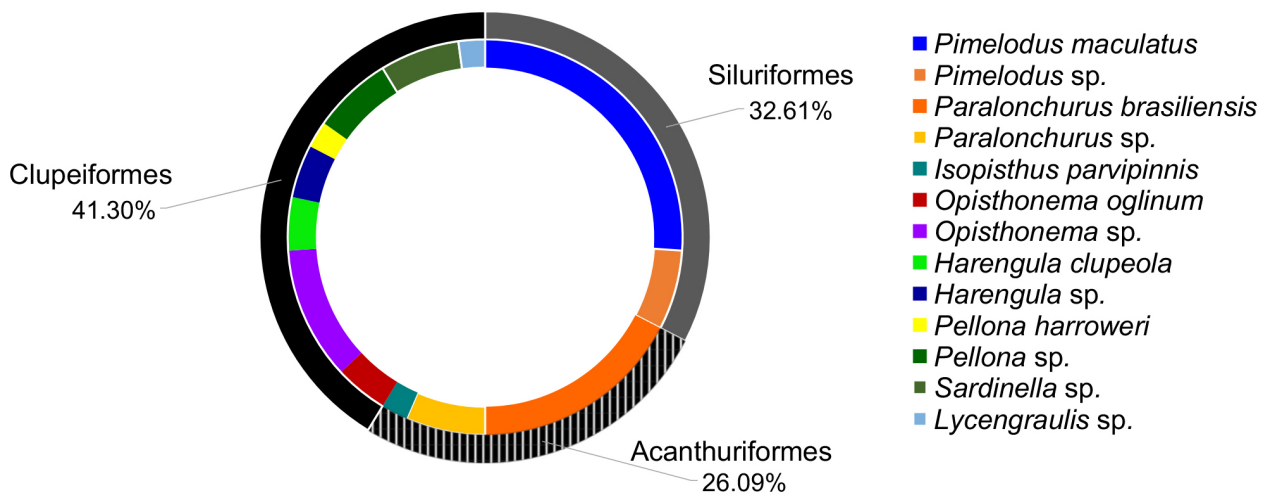

FIGURE 1 | Diagram showing the taxonomic composition of the prey items identified in the stomach of the largehead hairtail, *Trichiurus lepturus*, collected off the coast of São Paulo state in southeastern Brazil.

TABLE 1 | List of the prey fish species encountered in the stomach contents of the largehead longtail *Trichiurus lepturus*, identified by DNA mini-barcoding analysis.

Order	Family	Species	Common name in Brazil	Number of samples	Conservation status	Similarity
Siluriformes	Pimelodidae	*Pimelodus maculatus*	Bagre	12	LC	98%
Acanthuriformes	Sciaenidae	*Paralonchurus brasiliensis*	Maria-luíza	8	LC	98%
		Isopisthus parvipinnis	Pescadinha	1	LC	100%
Clupeiformes	Clupeidae	*Opisthonema oglinum*	Sardinha-laje	2	LC	98%
		Harengula clupeola	Savelha-cascuda	2	LC	99%
	Pristigasteridae	*Pellona harroweri*	Sardinha-branca	1	LC	100%

DISCUSSION

The present study is the first report of the application of the DNA mini-barcode technique to the diagnosis of the composition of the prey of the largehead hairtail *Trichiurus lepturus* in the South Atlantic Ocean, off the coast of southeastern Brazil. The study demonstrated the effectiveness of this molecular marker for the identification of the prey items of this species. The results of the present study reinforce the findings of other recent studies, which have shown that sequences of the standard COI barcoding region that contain at least 100 bases can distinguish 91–94% of the species of different taxonomic groups (Hajibabaei *et al*., 2006; Meusnier *et al*., 2008; Virgilio *et al*., 2010; Shokralla *et al*., 2011; Nagy *et al*., 2012).

In the present study only approximately one third (32.85%) of the samples were sequenced successfully, which may reflect certain limitations of the technique or the quality of the samples. This may, in turn, have been related to the inadequate storage of the material during transportation to the laboratory for processing. Many studies have shown that the DNA mini-barcode can be effective for the analysis of degraded samples, however. These studies include dietary analyses (Valdez-Moreno *et al*., 2012; Dahl, Ghosh, 2017; Pavan-Kumar *et al*., 2020), the identification of fish species traded illegally in markets and restaurants, and even processed fishery products, as shown by Xing *et al*. (2020), who analyzed samples of fish sold in Taiwanese markets and obtained reliable identifications.

The fish species *Stolephorus device* and *Sardinella longiceps* (Clupeiformes – Indian Ocean), and *Saurida tumbil* (Aulopiformes Indo-West Pacific) were found in the stomach content of *T. lepturus* (Randall, 1995; Chiou *et al*., 2006; Rohit *et al*., 2015). These species do not occur in Brazil and here *T. lepturus* predate other species. *Trichiurus lepturus* is predominantly carnivorous, and although cannibalism has been reported in this genus, no evidence of this behavior was found in the present study. The diet of the species was varied and composed mostly of marine fish, although it is interesting to note that the most common prey species, the siluriform *Pimelodus maculatus* (identified

in 13 samples) is a freshwater species. This curious finding in consistent with the findings of Martins *et al.* (2005), who showed that *T. lepturus* has a flexible diet and is able to enter estuarine environments in search of food. Lima *et al.* (2019) recorded the presence of *P. maculatus* in estuaries.

Prey belonging to three acanthuriform species were identified in the present study, all representing the family Scianidae, which is a common family in the coastal and estuarine waters of the southwestern Atlantic (Hoff *et al*., 2020). The most common species were *P. brasiliensis* and *I. parvipinnis*. *Paralonchurus brasiliensis* is distributed throughout the western Atlantic, being commonly found in sandy-mud substrates, and despite not having an estuarine phase *per se*, it is often found in the proximity of estuarine areas. *P. brasiliensis* has benthic demersal habits and is an opportunistic predator with a diverse diet (Sedrez *et al*., 2021), this fact may explain the common occurrence of this species in the stomach contents of *T. lepturus*. While often harvested by fisheries, *P. brasiliensis* is of little commercial value, and is often discarded as bycatch at sea (Robert *et al*., 2007), which means that it is not included in catch data. This lack of data may contribute to the misinterpretation of the conservation status of the species, which is currently listed as Least Concern (LC) in Brazil (ICMBio, 2018).

Isopisthus parvipinnis, a euryhaline sciaenid distributed widely in the warm and shallow waters of the western Atlantic, was identified in only one *T. lepturus* stomach. This fish occurs in coastal marine waters at depths of up to 50 m, and in shallow estuarine waters no more than 15 m in depth and is usually associated with sandy and muddy bottoms. It is one of the principal sciaenids taken as bycatch by the commercial shrimping fleets that operate in the coastal waters off southern and southeastern Brazil (Hoff *et al*., 2020). This species is classified as Least Concern (LC) in Brazil (ICMBio, 2018).

Five of the species identified in the *T. lepturus* stomach contents – *O. oglinum*, *H. clupeola*, *P. harroweri*, *Sardinella* sp., and *Lycengraulis grossidens* – are clupeiforms. The order Clupeiformes is large and complex, although many species are known simply as sardines and are typically not identified by their scientific names, which hampers the collection of reliable fishery statistics. While many clupeiforms are economically valuable (Ferreira-Araújo *et al*., 2021), and are exploited commercially on a large scale, the species are classified as Least Concern (LC) or Data Deficient (DD) in Brazil (ICMBio, 2018). The imprecise classification of the species of this group is due to its taxonomic complexity, which requires more detailed research.

Trichiurus lepturus is an opportunistic predator, feeding on any available prey of appropriate size. Many of its prey species are targeted by commercial fisheries, and are also exploited by other fish, dolphins, sharks, marine birds, and commercially important fish species, such as tuna (Chiou *et al*., 2006; Ferreira-Araújo *et al*., 2021). This emphasizes the need for further monitoring. At certain times of the year, *T. lepturus* migrates to warmer waters, such as those found typically in estuarine environments, where they coexist with other species, such as the pimelodid *P. maculatus*, which was exploited relatively extensively by *T. lepturus*.

Some species found in this study have already been reported in the diet of *T. lepturus* in other studies, *L. grossidens* and *P. harroweri* (Bittar *et al*., 2008, 2012), other species of the genus *Sardinella* (Bakhoum, 2007), *P. brasiliensis* (Bittar *et al*., 2008) and the other species are being reported for the first time in the diet of *T. lepturus*.

The results of the present study confirm the diagnostic potential of the DNA mini-barcode, even for the identification of degraded material, such as the partially digested contents of fish stomachs. This molecular tool permitted the identification of prey items to the species level, even when the material was so degraded that morphological identification was impossible and can thus be considered an extremely valuable approach for the determination of species diversity. Xie *et al.* (2021) recently used the DNA mini-barcode to identify pangolins (Pholidota), comparing the mini-barcode primers with the universal primers of the COI and Cytb genes, and finding a higher amplification rate, of up to 100%, which confirms the effectiveness of the mini-barcode as an effective and accurate method for the identification of species, which should be applied in future research.

Some limitations were found in this methodology, such as the difficulty in obtaining sequences that allow the identification of samples at the species level due to the degradation of the samples, making it difficult to amplification of regions that are sufficiently variable for identification at the species level.

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AUTHORS' CONTRIBUTION

Beatriz R. Boza: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing-original draft, Writing-review and editing.

Vanessa P. Cruz: Conceptualization, Data curation, Writing-original draft, Writing-review and editing.

Gustavo Stabile: Data curation, Methodology.

Matheus M. Rotundo: Data curation, Writing-original draft, Writing-review and editing.

Fausto Foresti: Funding acquisition, Project administration, Supervision, Writing-original draft, Writing-review and editing.

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ETHICAL STATEMENT

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COMPETING INTERESTS

The authors declare no competing interests.

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