Dietary specialisation in a Critically Endangered pipefish revealed by faecal eDNA metabarcoding

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Abstract

The estuarine pipefish, *Syngnathus watermeyeri*, is one of the rarest animals in Africa and occurs in only two South African estuaries. The species was declared provisionally extinct in 1994, but was later rediscovered and is currently listed by the IUCN as Critically Endangered. A conservation programme was launched in 2017, with the re-introduction of captive-bred individuals into estuaries where this species was recorded historically was the main aims. Successful captive breeding requires knowledge of the species' dietary requirements. In the present study, we used metabarcoding of faecal DNA to identify prey species consumed by wild-captured *S. watermeyeri* from one of the two surviving populations. We compared the diet of the estuarine pipefish with that of the longsnout pipefish, *S. temminckii*, in the same estuary, to determine whether these two species compete for the same prey items. Both species occupy similar estuarine habitats, but *S. temminckii* has a much wider distribution and also occurs in the marine environment. Our results show that even though both pipefish species prey on three major invertebrate classes (Gastropoda, Malacostraca and Maxillopoda), the relative proportions differ. *Syngnathus watermeyeri* primarily targets Maxillopoda, with a single species of calanoid copepod constituting >95% of the Amplicon Sequence Variants (ASVs) identified from its faecal DNA, whereas the diet of *S. temminckii* mostly comprises snail and decapod crustacean larvae. Our finding supports the hypothesis that population declines and localised extirpations of *S. watermeyeri* during previous decades may have been the result of reductions in the abundance of calanoid copepods. Calanoids rely on freshwater pulses to thrive, but such events have become rare in the two estuaries

inhabited by *S. watermeyeri* due to excessive freshwater abstraction for urban and agricultural use.

Introduction

Estuaries are semi-closed bodies of water with simultaneous connections to both rivers and the ocean (Costalago et al., 2014). The resulting fluctuations in salinity and temperature (James & Harrison, 2010) make estuaries unique and challenging habitats, inhabited only by species that can tolerate such conditions (Harrison & Whitfield, 2012). In addition to subsets of riverine and marine species being present, at least temporarily, these systems also harbour species that are endemic to estuaries (Teske & Wooldridge, 2003; Turpie et al., 2002).

Estuaries are amongst the most threatened aquatic habitats in South Africa, and many have become functionally degraded as a result of anthropogenic pressures (Kajee et al., 2018; Kaselowski & Adams, 2013; Turpie et al., 2002). These include water abstraction for agricultural activities, pollution, and urban development (Morant & Quinn, 1999). Nutrients provided by freshwater input are a primary determinant in the trophic structure of estuarine ecosystems by contributing to planktonic productivity (Allanson et al., 2000) and to the functioning of pelagic food webs in particular (Grange et al., 2000; Mbandzi et al., 2018). However, some catchments in South Africa now retain more than 50% of the freshwater that the estuaries would receive under natural conditions (Wooldridge & Callahan, 2000).

Several endemic estuarine species in South Africa are threatened, including the Endangered Knysna seahorse*, Hippocampus capensis* (Lockyear et al., 2006; Mkare et al., 2017), the Critically Endangered limpet *Siphonaria compressa* (Allanson & Herbert, 2005) and the Critically Endangered estuarine pipefish, *Syngnathus watermeyeri* (Whitfield, 1995). All three species are associated with submerged macrophyte beds that are dominated by the eelgrass *Zostera capensis*, which is itself listed as vulnerable by the IUCN because it is sensitive to anthropogenic pressure, and experiences widespread degradation as a result of increased coastal development (Adams, 2016; Payne et al., 1998).

The estuarine pipefish was the first African fish species to be declared extinct (Groombridge, 1994) after it had not been recorded for several decades following its original description in 1963 (Bruton, 1995). The species was re-discovered in 2006 (Vorwerk et al., 2007) but remains listed as Critically Endangerd on the IUCN's Red List (Pollom, 2017). This is largely as a result of significant reductions in river flow and nutrients entering certain estuaries leading to a collapse in pelagic productivity (Grange et al., 2000), the loss of submerged plant habitat (Riddin & Adams, 2012), and the small population size resulting from being restricted to a few estuaries (Mwale et al., 2014; Whitfield et al., 2017).

Historically, the estuarine pipefish was present in five estuaries on South Africa's eastern south coast, but was recorded in only two of these viz. the Kariega and Bushmans estuaries during recent surveys. Both are marine-dominated, permanently open systems (Mwale et al., 2014), a type of estuary that is

comparatively rare in South Africa (Whitfield & Baliwe, 2013). Salinity in these estuaries is similar to that of the ocean (Teske & Wooldridge, 2003), but hypersaline conditions may develop during episodic droughts because of evaporative loss (Froneman & Vorwerk, 2013; Grange et al., 2000).

The estuarine pipefish shares its habitat with the longsnout pipefish, *S. temminckii*, which is also endemic to southern Africa but is not currently listed by the IUCN (the "Least Concern" status sometimes attributed to this species is a result of previous synonymy with the European *S. acus*). It should be noted that it likely has the same status as *S. acus* because it is both abundant and widespread, and it is not restricted to estuaries but also occurs in the ocean (Heemstra & Heemstra, 2004).

The two species are readily distinguishable by snout morphology, with *S. watermeyeri* having a much smaller and shorter snout (Mwale, 2005). Pipefishes use their narrow, pipe-like snouts for suction feeding, with longer snouts facilitating prey capture at a greater distance (Kendrick & Hyndes, 2005; van Wassenbergh et al., 2011). Because of the similarity in the mode of foraging, it is possible that dietary competition exists between *S. watermeyeri* and *S. temminckii*, and that the Critically Endangered estuarine pipefish is outcompeted by its larger and more abundant congener (Whitfield et al., 2017).

There is an urgent need for conservation action to safeguard the long-term survival of the estuarine pipefish. Effective conservation requires a thorough understanding of its life history, but the implementation of a conservation plan has been hindered by the paucity of information on the species' biology (Mwale et al., 2014). In 2017, a conservation programme was launched that includes captive breeding and release into estuaries where the species was present historically as major objectives. An important starting point for this initiative is an improved knowledge about the species' dietary requirements. This information is important, not only to offer suitable prey items for estuarine pipefishes in captivity, but also to release captive-bred individuals at sites where, or during periods when, their preferred food is abundant. In this study, we used metabarcoding of faecal DNA to determine the dietary preference of the Critically Endangered *S. watermeyeri*, and we compared this information with corresponding data from *S. temminckii*. Dietary overlap would indicate food competition between the two species, making the presence of *S. temminckii* a factor that reduces the survival potential of *S. watermeyeri*.

Materials and Methods

Sample collection

Sampling was conducted under permit RES2020/77 from the Department of Environmental Affairs, which allowed the capture of up to six pipefishes per species, and ethical clearance was granted by the SAIAB Animal Ethics Committee (REF#:

25/4/1/7/5_2018-07). Five pipefishes per species were captured from a site in the lower reaches of the Bushmans Estuary (33°40′23″S, 26°38′50″S) by repeated deployment of a seine net (5 × 2 m, with 5 mm stretch mesh) through eelgrass beds over a period of ~2 hours. Captured individuals were transferred to small, transparent plastic aquaria containing ~5 litres of estuarine water, which were placed in a shaded area. The aquaria were aerated by means of portable air pumps, and the water was replaced with estuarine water approximately every 30 min. Each aquarium contained one or two individuals of the same species. The pipefish were left in these containers until they defecated (1-3 h), whereupon they were released at their location of capture. Faecal pellets were collected from the aquaria using a different sterile medicine dropper for each species, briefly dried on a paper towel, and placed into 2 ml screw cap microcentrifuge tubes containing RNAlater stabilisation and storage reagent (QIAGEN, Hilden, Germany). The tubes containing the faecal pellets were stored at -70°C until extraction.

DNA extraction and amplification

The faecal pellets in RNAlater were thawed by leaving them to stand at room temperature. Once thawed, the pellets were transferred to 1.5 ml Eppendorf tubes, cut into smaller pieces, and left to dry in a heat block at 37°C for two hours. Once all liquid had evaporated, the DNA was extracted using the CTAB protocol (Doyle & Doyle, 1987). DNA extractions were assessed for degradation and quantity by means of agarose gel electrophoresis and Qubit 2.0 fluorometry (Thermofisher), respectively. All samples passed quality screening, and equimolar concentrations of all the samples from a particular pipefish species were pooled for downstream reactions.

The extracted DNA was amplified using Polymerase Chain Reaction (PCR), targeting the mitochondrial cytochrome oxidase *c* subunit I (COI) gene. This marker was amplified using forward primer mlCOIintF and reverse primer jgHCO2198 (Leray et al., 2013) as described in Ntuli et al. (2020). The PCR products were purified using the AMPure XP system (Beckman Coulter), and a NEBNext Ultra DNA Library Prep Kit (New England BioLabs, United States) was used for the preparation of genomic libraries. The resulting libraries were screened for size distribution using a 2100 Bioanalyzer (Agilent), and quantified using real-time PCR. They were then sequenced on an Illumina HiSeq 4000 platform (San Diego California, United States) at Novogene (Hong Kong), using 250 bp paired-end chemistry according to the manufacturer's instructions.

Sequence assembly and analysis

The raw reads were processed using a modified version of the Anacapa Toolkit (Curd et al., 2019). Cutadapt (Martin, 2011) was used to remove NEBNext adaptors and COI barcoding primer sequences from the reads. Low quality sequences (Phred-score <25) were then removed using the FASTX-Toolkit

(https://github.com/agordon/fastx_toolkit). The same program was used to remove low quality sequences at the 3' ends of forward and reverse reads. To compensate for the comparatively lower quality of the reverse sequences, as is typical for Illumina sequencing, 50 bp and 20 bp were removed from 3' of the reverse and forward sequences, respectively.

All quality-filtered sequences were dereplicated, denoised and, when possible, merged into unique non-chimeric Amplicon Sequence Variants (ASVs, i.e., higher resolution analogues of traditional Operational Taxonomic Units, or OTUs), using the DADA2 R package (Callahan et al., 2016). In DADA2, the maximum error rate for the forward reads was set to 2, but for the lower quality reverse sequences, this value was increased to 5. A combination of the short-read aligner Bowtie2 (Langmead & Salzberg, 2012) and a specific Bayesian Least Common Ancestor method (BLCA) (https://github.com/qunfengdong/BLCA), applied within the Anacapa distribution, was used to assign a taxonomic rank to each ASV. In this script, the lowest common ancestor for each ASV was reported based on the similarity to the known species in a pre-made CRUX reference database (https://drive.google.com/drive/folders/0BycoA83WF7aNOEFFV2Z6bC1GM1E) and a confidence value for assigned taxonomy ranks was calculated using bootstrapping (Gao et al., 2017).

The resulting taxonomy tables were visualized in the R package ranacapa (Kandlikar et al., 2018). Amplicon Sequence Variant counts for each species were agglomerated into the taxonomic rank of class, and were subsequently normalised relative to the total ASV count estimated for each species using the phyloseq R package (McMurdie & Holmes, 2013).

The most important prey species found in the pipefish faeces were identified based on each prey species' number of ASVs counts constituting at least 0.1% of the total number of ASVs generated from the faeces of each pipefish species. As many of the invertebrate species present in the range of *S. watermeyeri* have not yet been barcoded, which resulted in uncertain taxon assignments, South African species that may be closely related to the species identified with Anacapa were identified phylogenetically using the neighbour-joining method (Curd et al., 2019) in MEGA version 6 (Tamura et al., 2013), with nodal support calculated using 1000 non-parametric bootstrap replications (Felsenstein, 1985). In the case of the Malacostraca, resolution was highest using amino acid sequences with the Poisson model (Bishop & Friday, 1987) specified, whereas nucleotide data and the Kimura 2-parameter model (Kimura, 1980) were used for the Maxillopoda.

Results

The sequencing runs produced 3 221 385 raw reads for *S. watermeyeri* and 2 904 779 reads for *S. temminckii*. The Anacapa pipeline assembled a total of 114 527 and 976 304 ASVs for *S. watermeyeri* and *S. temminckii*, respectively.

Six major animal classes were identified, namely Arachnida, Gastropoda, Hydrozoa, Insecta, Malacostraca and Maxillopoda. Numerous ASVs were assigned to other classes, but these were excluded from subsequent analyses because they collectively constituted 0.01% and 0.02% of the total number of animal ASVs obtained from the faecal DNA of *S. watermeyeri* and *S. temminckii*, respectively. Of the remaining six classes, the Gastropoda, Malacostraca and Maxillopoda were represented by the largest number of ASVs (Fig. 1). Amplicon Sequence Variants originating from the Maxillopoda were dominant in the faeces of *S. watermeyeri*, Gastropoda and Malacostraca were comparatively rare, the number of ASVs from the Insecta were negligible (0.05%), and no ASVs from the Arachnida and Hydrozoa were present. The same three classes that were most common in the faeces of *S. watermeyeri* were also important in *S. temminckii*, but their proportions differed considerably. Amplicon Sequence Variants that originated from the Gastropoda and Malacostraca were the most common, and those from the Maxillopoda were comparatively rare. The combined counts assigned to ASVs originating from the classes Arachnida, Hydrozoa and Insecta together comprised only 0.11% of total counts.

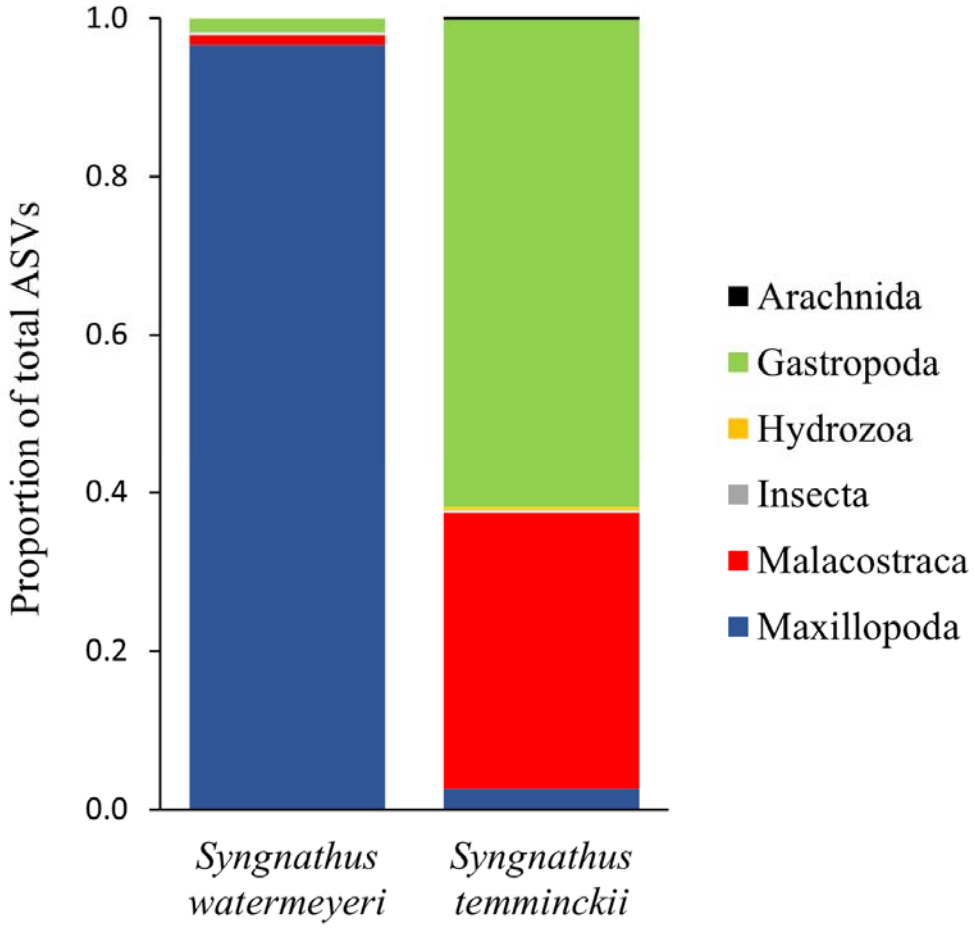

Fig. 1. Proportion of Amplicon Sequence Variants (ASVs) assigned to six prey animal classes found in the faeces of the two pipefish species. The ASVs of three of these (Gastropoda, Malacostraca and Maxillopoda) were considerably more common than those of the Arachnida, Hydrozoa and Insecta, which are barely visible in this figure.

The potential for dietary competition was investigated further by comparing individual prey species. While the COI ASVs proved useful to identify prey classes and assess their relative importance, they failed to reliably identify species and even genera in many cases (Table 1). This is not a shortcoming of the method but rather reflects the lack of published sequence data for South African estuarine invertebrate species. We were, however, able to identify the following macroinvertebrates to species level based on available COI sequences: the snails *Assimenia capensis* and *Hydrobia knysnaensis*, and the decapod crustacean *Palaemon peringueyi*. A fourth species,

the calanoid copepod *Pseudodiaptomus hessei*, was added to the BOLD database (BIN identifier: XXX) when it was found that the ASVs identified as either *Labidocera rotunda* or *Sinocalanus sinensis* matched an unpublished COI sequence of this species.

The remaining species were identified phylogenetically but, in most cases, identifications remain tentative. The Malacostraca ASVs found in the faeces of *S. temminckii* that matched with *Caridina multidentata* and *Pandalus montagui* are likely from the same species (Supplementary Information, Fig. S1), which remains unidentified. The ASVs in the faeces of *S. watermeyeri* that matched the isopod *Spherillo dorsalis* were recovered as a sister lineage of *Cirolana harfordi* in the phylogenetic tree and may represent the South African congener *C. fluviatilis*. The ASVs that matched with *P. hessei* clustered among other species of the genus *Pseudodiaptomus* (Supplementary Information, Fig. S2). As *P. hessei* is the only representative of this genus in South Africa and is one of the most common copepod species in the region's estuaries (Jerling & Wooldridge, 1995), this identification is unmistakeable. The two other copepod species (both of which were rare) remain unidentified.

Of the most common prey species, only the calanoid copepod *P. hessei* and the snail *H. knysnaensis* were present in the faeces of both species. While DNA from *P. hessei* was most common in the faeces of *S. watermeyeri* and rare in those of *S. temminckii*, the inverse was true for *H. knysnaensis*.

Table 1. Closest matches of species whose DNA was common in the faeces of *Syngnathus watermeyeri* and *S. temminckii* on the basis of their total number of ASVs constituting at least 0.1% of the total number of reads per faecal sample (exact percentages for each species are shown in brackets). Many of these are not found in the estuaries inhabited by the two pipefishes. South African prey taxa from which the faecal DNA potentially originated, but for which no DNA barcoding records exist yet, are indicated with question marks.

Class	Closest matches with published COI sequences		Prey species
	S. watermeyeri faeces	*S. temminckii* faeces	
Gastropoda	*Assimenia capensis* (1.78%)		*Assimenia capensis*
	Hydrobia knysnaensis (0.29%)	*Hydrobia knysnaensis* (62.45%)	*Hydrobia knysnaensis*
Malacostraca		*Caridina multidentata*[1] (10.85%)	Caridea sp.?
		Pandalus montagui[1] (2.2%)	Caridea sp.?
		Palaemon peringueyi (21.76%)	*Palaemon peringueyi*
	Spherillo dorsalis (2.5%)		*Cirolana fluviatilis*?
Maxillopoda		*Caudoeuraphia caudata* (0.21%)	*Nitokra* sp.?
	Harpacticoida sp. (0.78%)		Harpacticoida sp.
	Labidocera rotunda[2] (0.44%)		*Pseudodiaptomus hessei*
	Sinocalanus sinensis[2] (95.36%)	*Sinocalanus sinensis*[2] (2.37%)	*Pseudodiaptomus hessei*

Superscript numbers: different ASVs of what are likely the same species (see Supplementary Information, Figs S1 and S2).

Discussion

This study used faecal DNA metabarcoding to assess dietary preferences in the Critically Endangered estuarine pipefish, *Syngnathus watermeyeri*, and compared these to the preferences of its more abundant congener *S. temminckii*. The aim of the research was to provide practical information that contributes towards improving the conservation management of this threatened species, particularly in terms of captive breeding and releases of captive-bred progeny into estuaries within the historical range.

The metabarcoding results showed clear differences in dietary preferences between the two pipefish species. Our results provide only a short-term snapshot of prey consumption given that the faecal samples originated from relatively few individuals because of the high conservation status of *S. watermeyeri*. Nonetheless, the fact that the two species were caught on the same day and in the same seagrass bed (where they have access to the same prey items) rejects the idea that the differences in dietary preferences found here could be an artefact of small sample sizes, or spatial and temporal separation of the captured individuals.

While it is possible that species from the largely terrestrial classes Arachnida and Insecta (few of which have larvae that can survive in estuarine water), as well as small hydrozoans are opportunistically consumed, particularly by the more generalist *S. temminckii*, only three invertebrate classes (Gastropoda, Malacostraca and Maxillopoda) were found to be important in the diet of both pipefish species. However, the relative abundance of these prey classes differed, with *S. watermeyeri* showing a clear preference for species in the class Maxillopoda, as well as what were likely veliger larvae of snails and mancae (small post-larvae) of an isopod, and *S. temminckii* preferring snail veligers and the larvae of decapod crustaceans. As with all PCR-based approaches, amplification bias may have affected our results (Krehenwinkel et al. 2017). However, the primers used here have a higher success rate in amplifying the DNA of metazoan species present in faecal samples than any other primers currently in use (Lemey et al. 2013), and amplification bias cannot account for the clear difference in the number of ASVs amplified for prey species whose DNA was present in the faeces of both pipefish species.

Differences in prey selection between the two pipefish species is likely a function of several factors that may include gape size and the length of the snout, as well as differences in hunting strategies. The smaller snout size of *S. watermeyeri* compared to *S. temminckii* may limit the size of prey items on which this species can feed, and this suggests that copepods are preferred primarily because of their small body size. The position of the eyes of *S. watermeyeri* closer to the snout tip (where the mouth is situated) may also allow this species to detect small zooplankton such as copepods better than *S. temminckii*, which has a much greater distance between the eyes and the snout tip. In addition to stronger suction and great gape size, *S. temminckii* has been observed to actively hunt its prey, whereas *S. watermeyeri* is more passive and waits for prey animals to swim within reach (Sven-Erick Weiss, pers. obs.). Together,

these factors allow *S. temminckii* to stalk prey that might otherwise escape, although it does not solely rely on these prey items and also consumes copepods.

The finding that *S. watermeyeri* relies to a large extent on relatively small zooplankters (copepods) supports the hypothesis that significant reductions in zooplankton abundance in response to reduced freshwater influx (Grange et al., 2000) can result in estuarine pipefish population declines (Whitfield et al., 2017). Excessive freshwater abstraction has transformed both estuaries inhabited by *S. watermeyeri* from systems with well-developed salinity gradients to homogeneously marine-dominated systems. The Bushmans River, for example, has approximately 30 impoundments in its upper reaches that have significantly reduced freshwater inflow (Bornman & Klages, 2004). While this would have resulted in a decrease in phytoplankton biomass (and, by extension, zooplankton biomass) (Hilmer & Bate, 1990), the resulting increase in water clarity would also have facilitated the formation of the current extensive submerged macrophyte beds (Bornman & Klages, 2004). Because of this contradiction (reduced food availability but increased habitat availability), it cannot be ruled out that the two estuaries in their current marine-dominated state have a higher carrying capacity for *S. watermeyeri* than they would have had under natural conditions, at least during periods of rainfall. In addition, periodic flooding that can negatively affect populations of endemic estuarine fish species, including the Endangered Knysna seahorse (Lockyear et al., 2006), no longer occurs in the Bushmans Estuary (Lubke & Webb 2016), making it a long-term stable habitat for the estuarine pipefish.

The large number of ASVs from the calanoid copepod *P. hessei* in the faeces of *S. watermeyeri* was clearly linked to low freshwater inflow into this estuary during the study period. The zooplankton community of the Bushmans Estuary has been poorly studied, but during low or zero river flow periods in the Kariega Estuary, *P. hessei* dominates the zooplankton community (up to 76% by number). This can change following river flooding when another calanoid, *Acartia longipatella*, becomes more abundant (Froneman & Vorwerk, 2013). Possible dietary switches by *S. watermeyeri* according to changes in copepod species composition in the zooplankton community have yet to be determined.

Conclusion

This study is the first to confirm the hypothesis proposed by Whitfield (1995) that the Critically Endangered estuarine pipefish, *S. watermeyeri*, is a specialist feeder that preys mostly on smaller zooplankton species, with a single calanoid copepod species, *P. hessei*, as the preferred prey item. This highly specialised feeding behaviour puts its survival at risk because zooplankton stocks in estuaries decline in response to low freshwater input (Wooldridge, 2010; Montoya-Maya & Strydom, 2009). The population declines of the estuarine pipefish that resulted in it being declared extinct in the previous century were likely linked to reductions in zooplankton abundance (Whitfield, 1995; Grange et al., 2000).

Although *S. watermeyeri* accepts other prey items in captivity, e.g. *Artemia* (Sven-Erick Weiss, pers. obs.), it is advised that captive breeding programmes establish cultures of *P. hessei*, or other calanoid copepods whose adults are of similar size. This is not only important to ensure that the ideal combination of nutrients is provided, but also to prepare captive-bred progeny for the prey items they will encounter when released back into the estuaries in the native range where *S. watermeyeri* has become extinct, should this be adopted as a suitable conservation strategy.

In contrast, *S. temminckii* primarily preys on the early life history stages of several macrobenthic species. These become particularly important to estuarine food webs during times when zooplankton abundance declines (Whitfield, 2005), making this pipefish less likely to experience population declines. Although some dietary competition exists between the two pipefish species and thus contributes towards decreasing the fitness of *S. watermeyeri*, the specialised diet of this Critically Endangered species is likely a significantly more important factor putting the survival of this species at risk.

The efficiency of metabarcoding to identify species relies on wide coverage and high quality of taxon reference records in the DNA repositories (Hestetun et al., 2020; Leite et al., 2020). Our study highlights the fact that a more comprehensive reference database for South African estuarine macroinvertebrates is required to unequivocally identify the prey items consumed by the two pipefish species studied here. This issue is presently being addressed by an initiative aimed at generating DNA barcodes for all eastern South African estuarine macroinvertebrates, which was initiated as a direct result of the research gaps identified in the present study.

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Supplementary Information

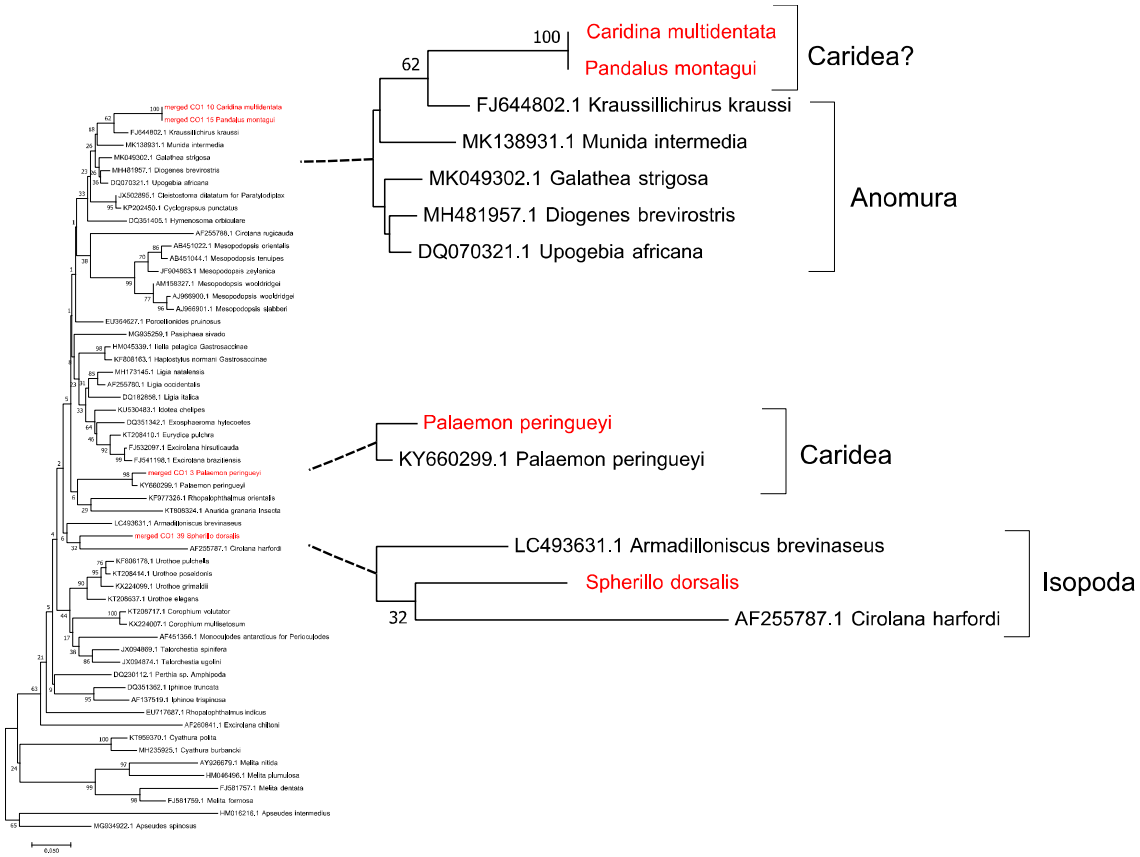

Fig. S1. Neighbour-joining bootstrap tree reconstructed using amino acid COI sequences of representative samples of all Malacostraca genera that have been reported from South African estuaries and for which DNA barcodes (local or from elsewhere) are available. The reads that matched with *Caridina multidentata* and *Pandalus montagui* are likely from same species, but even though both are Caridea, they did not cluster with other Caridea (e.g. *Palaemon peringueyi*), but with Anomura. The read that matched *Spherillo dorsalis* is sister to *Cirolana harfordi*, and may represent the only South African congener of this species present in the study area, *C. fluviatilis*. Nodes above some branches are bootstrap values (1000 replications).

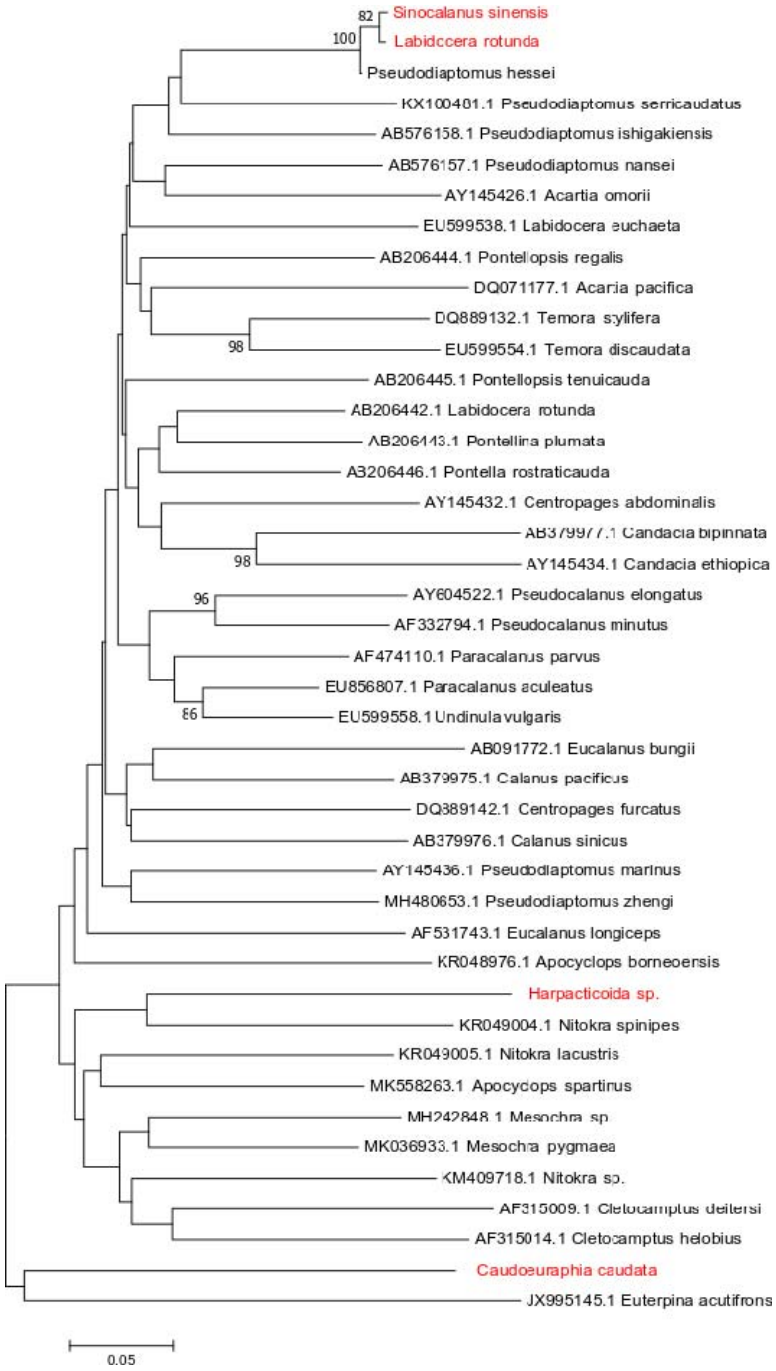

Fig. S2. Neighbour-joining bootstrap tree reconstructed using COI sequences of all Maxillopoda genera that have been reported from South African estuaries and for which DNA barcodes (local or from elsewhere) are available. The four “species” identified in the diet of *Synganthus* spp. are highlighted in red. Nodes above some branches are bootstrap values (1000 replications).