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Feeding ecology of the invasive *Cynoscion regalis* on the Portuguese coast: Insights from a molecular approach

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Abstract

Invasive species can profoundly disrupt native ecosystems, as their self-sustaining populations alter niche occupation, increase competition, and exert predation pressure on indigenous species. Predation by invasive species, particularly on vulnerable life stages such as larvae and juveniles, can lead to population declines, loss of genetic diversity, disruption of food webs, extinction of keystone species, impaired ecosystem services, and delayed recovery of indigenous populations. *Cynoscion regalis* (Bloch and Schneider, 1801), commonly known as the weakfish, is a sciaenid species native to the eastern coast of North America, where it acts as a generalist predator. Since 2009, its presence has also been documented in European waters, prompting concerns about its potential impact on local fish communities. This study presents the first molecular analysis of the feeding ecology of *C. regalis* outside its native range. Using molecular barcoding of prey items coupled with metabarcoding of gastric fluids, this research provides insights into the weakfish's diet in Portuguese waters (southwestern Europe) beyond those obtained in studies relying solely on visual stomach content identification. This underscores the value of molecular techniques in dietary analysis. Findings reveal that *C. regalis* exhibits

cannibalistic tendencies, with conspecifics forming a substantial part of its diet. Other dietary species include *Atherina presbyter*, *Diplodus annularis*, *Dicentrarchus punctatus*, *Engraulis encrasicolus*, *Sardina pilchardus*, and *Spondyliosoma cantharus*. In addition, other prey were identified at the family level as Sparidae and Scorpaenidae. The presence of these species, many of which are common in estuarine habitats, particularly during juvenile stages, supports the establishment of weakfish populations in nursery areas, potentially resulting in adverse ecological impacts. This study contributes to understanding the dynamics of invasive species, highlighting the ecological interactions and disturbances they may cause. Effective monitoring and management of weakfish populations outside their native range are critical to mitigating potential ecological consequences.

Keywords: barcoding of stomach contents, invasive species, metabarcoding, weakfish

Introduction

Non-native species can have profound and far-reaching impacts on local ecosystems by forming stable populations and exerting pressure on native species through ecosystem disturbance. Recent studies predict a global increase of invasive species in the next decades, leading to an inevitable cascade of impacts like native species richness reduction and native population declines, ecosystem changes, and even consequences on human health, among several other effects (e.g., Seebens et al. 2020; IPBES 2023).

The weakfish, *Cynoscion regalis* (Bloch and Schneider, 1801), is a marine fish species believed to have been introduced to European waters from the Americas via ballast water (Morais and Teodósio 2016; Bañón et al. 2017a, 2017b). Its native distribution spans the western Atlantic, from southern Florida to Nova Scotia (Froese and Pauly 2025). The first documented occurrence in Europe was in 2009, with the capture of a juvenile in Belgium's Scheldt estuary (ANB 2011). Subsequent records followed in Spain’s Gulf of Cadiz in 2011 and Portugal’s Sado, Tagus, and Mira estuaries between 2013 and 2016 (Morais and Teodósio 2016; Bañón et al. 2017a; Morais et al. 2017). The increasing presence of *C. regalis* in European waters is corroborated by fishing data, which report landings of hundreds of kilograms in recent years (Bañón et al. 2017a, 2017b). Once considered an incidental bycatch, the species has gained commercial interest and targeted fishing has

been suggested as a dual-purpose strategy, serving both as a population control method and as a means of developing a new fishery resource (Bañón et al. 2017a, 2017b; Béarez et al. 2016).

Concerning its ecology, *Cynoscion regalis* is a gregarious demersal fish species that inhabits coastal and estuarine ecosystems, typically preferring depths of 10-26 meters over sandy or muddy substrates (Froese and Pauly 2025). Known as a generalist predator, its diet includes fish, molluscs, and crustaceans, showcasing its adaptability to diverse food sources (e.g., Wilk 1979; Schultz 2004). A study examining the feeding strategy of *C. regalis* outside its native range through stomach content analysis conducted in Portugal's Sado Estuary (Cerveira et al. 2021) confirmed that the species retains its generalist feeding behaviour. It also revealed a significant trophic overlap with the meagre (*Argyrosomus regius*), suggesting potential competition for food and habitat resources, which may adversely affect native fish populations.

This study presents the first molecular analysis of the feeding ecology of *C. regalis* in its non-native range, focusing on its fish prey in Portuguese waters. Using barcoding of prey items and metabarcoding of gastric fluids, our research provides a more detailed perspective of the weakfish's diet compared to visual stomach content analyses. This approach highlights the utility of molecular techniques in dietary studies, offering insights into the ecological interactions and potential disturbances caused by an invasive species in the native fish populations in the same area. By improving our understanding of the feeding ecology and potential ecological impact of *C. regalis*, this study underscores the importance of effective monitoring and management strategies of non-native species to mitigate potential ecological consequences in recipient ecosystems.

Materials and methods

Sample collection

Thirty individuals of *C. regalis* from the Tagus estuary were collected, labelled, measured (total length), weighed (total weight) and carefully dissected to extract the digestive tracts, which were examined for stomach content analysis. Semi-digested fish prey items were selected for molecular identification via molecular barcoding, and small muscle tissue

samples were carefully extracted, preserved in 96% ethanol and stored in individually labelled Eppendorf tubes. The stomachs and their contents were also preserved in 96% ethanol to facilitate metabarcoding analysis of gastric fluids. Vouchers for all specimens and tissue samples were deposited in the MARE-Ispa tissue collection, ensuring proper documentation and accessibility for future reference.

Laboratory procedures

Genomic DNA of the undigested contents was extracted using the REDExtract-N-Amp Tissue PCR kit from Sigma-Aldrich, following the manufacturer's instructions. For the DNA-barcoding of undigested tissue, the mitochondrial DNA cytochrome c oxidase I (COI) gene was used. PCR amplification of COI was conducted using primers COI-F1 (5'-TCAACCACCCACAAAGACATTGGCAC-3') and COI-R2 (5'-ACTTCAGGGTGACCGAAGAATCAGAA-3') (Ward et al. 2005). PCR conditions used were: 2 min initial denaturation at 95 °C, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min, and final extension of 10 min at 72 °C. PCR products were purified and sequenced in STABVIDA (Sanger sequencing, https://www.stabvida.net/).

Concerning metabarcoding of gastric fluids, four samples plus a blank were extracted with the Qiagen DNeasy Blood and Tissue kit following the manufacturer´s instructions, including two elutions of 100 µl each with EB buffer. Metabarcoding was performed using two-rounds of amplification via the polymerase chain reaction (PCR) with the MiFish-U primer pair (Miya et al. 2015) plus a newly designed, species-specific reverse blocking primer Creg-MiFishUR-blockCCCAGTTTGTGTCATAGCTTTCGTGGGTTCAAAGTTG(C3spacer). The first round of amplification was performed in 10 µl reactions including 5 µl of Qiagen Multiplex Mastermix, 0.5 µl of each of the MiFish-U primers (10 µM), 2.5 µl of Creg_MiFish-UR_block (100 µM; 50x final concentration) and 1.5 µl of DNA from stomach content extractions. The temperature profile followed a touch-down PCR consisting in an initial denaturing step of 95 °C for 15 min; 11 cycles of 95 °C for 20 s, 65 °C for 15 s (-0.5 °C/cycle), and 72 °C for 15 s; followed by 19 cycles of 98 °C for 20 s, 60 °C for 15 s and 72 °C for 15 s; and a final extension step of 72 °C for 5 min. All PCRs were performed in triplicate for each sample (including the extraction blank) and a

PCR blank was run per batch of PCR replicates. The second round of PCR added sample- and replicate-specific indexes and Illumina-compatible barcodes as described in Miya et al. (2015). Indexed libraries were cleaned using Ampure Beads (0.6X), quantified and pooled equimolar. Final insert size was checked on the TapeStation (Agilent Technologies) using the DNA D1000 High Sensitivity assay kit (Agilent Technologies). The final pooled library was validated on a real-time PCR machine using the KAPA Library Quantification Kit (KAPA BIOSYSTEM) for Illumina platforms, adjusted to 12 pM, and sequenced on an Illumina MiSeq using the MiSeq Reagent Nano Kit (500 cycles) aiming at 20000 reads per sample.

DNA sequence data analyses

The sequences obtained from individual fish prey items were visually inspected and edited with Codon Code Aligner (Codon Code Corporation, https://www.codoncode.com/). Molecular identification was performed by comparing sequences against those available in GenBank in the nucleotide (nt) database (online, accessed on 01.06.2024), using megablast in BLAST (Basic Local Alignment Search Tool). Species identification was confirmed if the sequence exhibited at least 97% query coverage and ≥99% identity at the species level (e.g., Hebert et al. 2003; Wong and Hanner 2008).

In metabarcoding analysis, raw reads were filtered for quality (–q 30), trimmed of adaptors and primers, and discarded if shorter than 100 bp using ‘cutadapt’ (Martin 2011). The cleaned reads were subsequently filtered with ‘dada2’ (Callahan et al. 2016) if the maximum expected errors ≥2 and if ambiguous bases (i.e., N) were present. Forward and reverse reads were dereplicated and merged, allowing no mismatches (maxMismatch=0) to reconstruct the corresponding amplicon sequence variants (ASVs). The set of ASVs was inspected for and filtered from chimeric sequences (i.e., sequences resulting from 2 or more biological sequences that were incorrectly aligned) using the method ‘consensus’. The final set of ASVs was screened across samples and read counts were estimated per sample. Molecular identification of the final ASVs was performed using BLAST searches (megablast algorithm) against the nucleotide (nt) database on NCBI (accessed on 27.12. 2022), using default parameters and a maximum of 10 hits per search. Given the short length of the MiFish-U barcode (~170 bp), stringent cut-offs were considered for taxon-

level identifications, namely: ≥97% query coverage and ≥99% identity at the species level, ≥97% at the genus level and ≥95% at the family level. ASVs with read counts <0.03% of total read count per sample were considered as false positives and excluded from each sample's taxon list (Calderón-Sanou et al. 2020).

Results

The *C. regalis* individuals sampled (N=30) had an average total length of 439.9 mm ± 39.7 mm (max: 540 mm; min: 370 mm) and an average total weight of 893.9 ± 243.34 g (max: 1567.6 g; min: 498.1 g).

Visual inspection of stomach contents enabled the identification of crustaceans (Fig. 1A) in the majority of the examined individuals. Pieces of fish muscle and nearly intact preyed fish were also observed (Fig. 1B). A total of 20 fish prey specimens, originating from the stomach contents of 13 weakfish individuals were visually identified and/or barcoded through tissue analysis. The remaining fishes had only digested material or crustaceans.

Figure 1. A Undigested crustacean in the stomach of *Cynoscion regalis* from the Tagus estuary (left) **B** *Cynoscion regalis* from the Tagus estuary and respective semi-digested fish prey found in stomach contents (right).

The COI barcodes identified 5 species belonging to 4 families. Among the sampled prey, half were barcoded as *C. regalis* (N=10), while the remaining were attributed to the European anchovy *Engraulis encrasicolus* (Eugraulidae, N=7), the annular seabream *Diplodus annularis* (Sparidae, N=1), the black seabream *Spondyliosoma cantharus* (Sparidae, N=1), and the sand smelt *Atherina presbyter* (Atherinidae, N=1).

Metabarcoding of the gastric fluids revealed the presence of the following species: *Cynoscion regalis*, *Engraulis encrasicolus*, *Sardina pilchardus* and *Dicentrarchus punctatus*. In addition, other preys were identified at family-level as Sparidae and Scorpaenidae. This adds at least Clupeidae, Moronidae and Scorpaenidae to the list of families preyed on by the weakfish.

Discussion

The molecular approach employed in this study allowed the identification of the following prey species of *C. regalis*: *Atherina presbyter*, *Diplodus annularis*, *Spondyliosoma cantharus*, *Engraulis encrasicolus*, *Sardina pilchardus* and *Dicentrarchus punctatus*. Sparidae and Scorpaenidae were also detected at family-level, adding Clupeidae, Moronidae and Scorpaenidae to the list of families preyed on by the weakfish outside its native area. It also confirmed the cannibalistic nature of *C. regalis* already documented in previous studies and its preference for pelagic preys (e.g., Merriner 1975; Cerveira 2019, 2021) such as *Atherina presbyter*, *Engraulis encrasicolus* and *Sardina pilchardus*, which are common pelagic fish in the Portuguese estuaries.

Considering the prey species listed, they are all found in estuaries, at least in some part of their life cycle (for details on the species occurring in the Tagus estuary, see Cabral et al. 2001; Brandão 2021). These findings from the Tagus estuary align with the study by Cerveira et al. (2021) in Sado, further supporting evidence of trophic niche maintenance in non-native habitats. Cerveira et al. (2021) reported the presence of the black goby *Gobius niger* and horse mackerel *Trachurus trachurus*, absent from this study, but failed to identify 3% of the fish species due to the advanced state of digestion, where molecular methods offer a powerful tool.

Taken together, these results underscore the adaptability of *C. regalis* in exploiting available resources, which may amplify its ecological impact on estuarine ecosystems, critical as feeding grounds and nurseries. The large size the adults can attain, and the high fecundity of this species raise additional concerns about the potential effects of *C. regalis*' presence on local biodiversity (Bañón et al. 2017a, 2017b).

This study has limitations that should be considered when interpreting the findings. The relatively small sample size limits the representativeness of the results for the broader population, while collecting samples from a single estuary (Tagus) at a specific time introduces potential temporal and spatial biases, neglecting seasonal or geographical variations in diet. The reliance on a single genetic marker (COI for tissue sequencing and 12S for metabarcoding) may not fully resolve taxonomic ambiguities, especially in closely related species, and the metabarcoding approach may favour the detection of species with less degraded or more abundant DNA. Additionally, the exclusion of amplicon sequence variants (ASVs) with low read counts (<0.03%) could result in the omission of rare prey items, which might play ecologically significant roles. The study's focus on dietary analysis excludes broader ecological interactions, such as prey availability, competition, and predator-prey dynamics, and it lacks complementary methods, such as stable isotope analysis, which could provide insights into long-term dietary trends. Furthermore, the primers used in metabarcoding may not amplify all prey taxa equally, leading to potential biases in diet composition, and the selection of semi-digested prey for tissue sequencing might overlook prey that is too degraded to yield usable DNA. These limitations and the need to include other prey items, like crustaceans, in future molecular approaches to *C. regalis* diet highlight the need for more comprehensive studies.

Despite these caveats, this study contributes to a deeper understanding of the ecological dynamics of invasive species. By incorporating molecular methods into the assessment of feeding ecology, we have demonstrated their value in providing detailed insights into dietary patterns, which are critical for understanding the ecological impacts of invasive species. Future monitoring and management of *C. regalis* populations in Portuguese waters are essential to mitigate unforeseen ecological consequences and preserve biodiversity in estuaries.

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