Molecular barcoding reveals patterns of egg predation in small pelagic fish

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ABSTRACT: Cannibalism and intraguild predation occur in a vast number of small pelagic fish (SPF) species. Egg and larval predation can have important consequences on mortality, and its accurate assessment is important to estimate the impact on recruitment strength and population dynamics of predators and prey. Such assessments are hampered by limitations in visual species identification of many fish eggs and larvae in the predators' stomachs. European sardine *Sardina pilchardus* and Atlantic chub mackerel *Scomber colias*, the dominant species of the pelagic food web off the Canary Current Upwelling ecosystem, are major predators of fish eggs. Egg predation by these SPF species is particularly high on sardine and anchovy eggs, but many preyed fish eggs are not amenable to visual identification. This study provides a proof-of-concept application of molecular identification of diverse fish eggs from SPF stomach contents not suitable for visual identification, as a way to improve our understanding of the impact of intraguild predation on fish population dynamics. Results show a high diversity of fish species in the eggs ingested by sardines and chub mackerel (18 and 15 families, respectively), mostly comprising locally abundant coastal taxa. Sardine ingested predominantly anchovy, sardine and sparid eggs, while chub mackerel ingested predominantly sparid eggs, followed by serranid (*Serranus* spp.) and sardine eggs. Sardines also showed higher variability in prey composition compared to chub mackerel. Exploratory analyses also suggested variability in prey composition with sampling area, season and maturity stage for sardine and chub mackerel, highlighting the need for dedicated follow-up studies.

KEY WORDS: DNA barcoding · Metabarcoding · Fish eggs · Intraguild predation

1. INTRODUCTION

Fish egg mortality is closely linked to interannual variability in recruitment and is likely mostly driven by predation (Houde 2002). Small pelagic fish (SPF) species are known to predate fish eggs and larvae, both their own (cannibalism) and those of co-occurring pelagic species, which is known as intraguild predation (Garrido & van der Lingen 2014). An accurate assessment of the impact of cannibalism and intraguild predation is extremely important in estimating mortality and fluctuations in year-class dynamics for a given species (Smith & Reay 1991). However, esti-mates of egg mortality due to cannibalism or intra-guild predation vary greatly, both between species at a given location as well as within species among different sites (Hunter & Kimbrell 1980, Alheit 1987, Valdés et al. 1987, Valdés Szeinfeld 1991, Gennotte et al. 2007, Garrido et al. 2008).

Fish eggs are frequently found in the stomachs of European sardines *Sardina pilchardus* and Atlantic chub mackerel *Scomber colias* in eastern Atlantic waters, both identified as the major predators of fish eggs (Silva 1954, Varela et al. 1988, Garrido 2003, Bachiller 2012, Garrido et al. 2015, Fonseca et al. 2022). Indeed, sardines were shown to select fish eggs

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when offered a variety of prey types such as phytoplankton cells, copepods and decapods, and use particulate-feeding to prey on eggs with very high ingestion rates (Garrido et al. 2007a). Fish eggs are recognized as one of the most important prey items for sardines in this area, particularly when zooplankton availability is low (Garrido et al. 2008, 2015, Fonseca et al. 2022). Given the relationship between the nutritional condition of the females and both the fecundity and viability of the larvae, the availability of fish eggs as prey can have a great impact in the recruitment of sardines as predators (Kjesbu 1989, Garrido et al. 2007b, Beldade et al. 2012). In turn, chub mackerel is a sub-tropical species whose habitat has been expanding northwards likely as a result of climate change (Martins et al. 2013). This species can have a double impact as a direct competitor but also as a predator on the eggs of other coastal pelagic species that inhabit the areas it is expanding to.

In previous studies, fish eggs in the stomachs of sardines and chub mackerel were identified as 'sardine eggs', 'anchovy eggs' and 'other fish eggs', the first 2 having specific characteristics that allow visual identification, by size and shape, as opposed to the last (Russell 1976, Garrido & van der Lingen 2014). Given the high biomass of sardine and chub mackerel in the pelagic food webs when compared to other coastal pelagic species, and the high numbers and frequency of occurrence of fish eggs of species other than sardine and anchovy in the stomachs, it is very important to identify the species that may be strongly impacted by these very abundant predators.

Molecular markers can help identify taxa where diagnostic morphological traits of eggs are missing, are unknown or impaired by digestion in stomach contents. Moreover, molecular genetics can identify prey after longer digestion times when compared to visual methods (Carreon-Martinez et al. 2011). Of particular interest to this topic is DNA barcoding, whereby a specific variable region of the mitochondrial or the nuclear genome of a specimen is amplified via PCR and subsequently sequenced (Ward et al. 2009). The resulting nucleotide sequence is then compared against available reference sequence databases of the putative target taxa to find the best match using stringent criteria, leading to a molecular identification. Indeed, DNA barcoding has greatly aided and validated the identification of fish eggs and larvae (Lewis et al. 2016), although most efforts are surprisingly recent (<20 yr; reviewed by Lira et al. 2023). However, its successful application has elucidated many topics relevant to fisheries science and management such as delimiting spawning activity in time and space (Harada

et al. 2015, Ahern et al. 2018, Burrows et al. 2019), understanding ichthyoplankton dynamics (Burghart et al. 2014, Kerr et al. 2020) or detecting fish eggs and larvae predation (Rosel & Kocher 2002, Albaina et al. 2015, Lutz et al. 2020, Allan et al. 2021).

Here, we present a proof-of-concept for the molecular identification of fish eggs from stomach contents of 2 SPF species, sardine and chub mackerel, combining DNA barcoding of single egg samples (1 egg per stomach) with DNA metabarcoding of mixed egg samples (e.g. all unidentified eggs per stomach). We focus particularly on samples showing higher prevalence of 'other fish eggs' (i.e. visually unidentified) in the stomachs of both predator species. Based on these data, we aimed to (1) assess the diversity of fish eggs ingested by sardines and chub mackerel and (2) compare the species composition of prey taxa between predator species to begin elucidating the prey preferences and potential impacts of egg predation exerted by these SPF species. Last, we provide some considerations on the different molecular genetic approaches used here, and list some variables that should be considered in future studies of molecular identification of fish eggs and larvae.

2. MATERIALS AND METHODS

2.1. Field sampling and fish processing

European sardine and Atlantic chub mackerel were collected during the spring and fall of 2018 (Table 1), during 3 annual acoustic surveys, mostly by pelagic trawl. The PELAGO18 survey took place during

Table 1. Sample collections used in the molecular identification of the preyed eggs according to predator species. Sample collections compared at the intraspecific level are in **bold**

	Adults	Juveniles	Total
Sardina pilchardus	15	40	48
Fall			10
South coast		1	1
West coast	5	8	9
Spring			38
South coast	2	**13**	13
West coast	8	**18**	25
Scomber colias	24	50	71
Fall			14
South coast	3		2
West coast	6	7	12
Spring			57
South coast	**15**	**42**	56
West coast		1	1

spring (April and May) and covered the coastal waters from Galicia to the Gulf of Cadiz; the ECOCADIZ-Reclutas18 survey occurred during fall (October), and covered the southern Iberian coast from Cape S. Vicente (Portugal) to Cape Trafalgar (Spain), including the Gulf of Cadiz; the IBERAS18 survey took place during fall (November) along the western Iberian coast from Galicia to Cape S. Vicente. The samples were grouped under 2 distinct areas, the west coast (defined by the ICES sub-divisions 9aCN and 9aCS) and the south coast (defined by the ICES sub-division 9aS).

Sardine and chub mackerel were classified as juvenile or adult fish (Table 1) by comparing their total length (in cm) to the mean size at first maturity (L50), following the method described by Fonseca et al. (2022). The stomachs were removed from individual fish and frozen at −20°C until processing. The stomach contents were analyzed individually, and the observed fish eggs were sorted under a stereo microscope (Olympus SZX10 magnification 90×), visually identified as anchovy, sardine or 'other fish eggs', and conserved in individual vials in 96% ethanol. For more details on the sampling procedure of the sardines and Atlantic chub mackerel, see Fonseca et al. (2022).

Eggs from stomach contents of sardine and chub mackerel labeled under the category 'other fish eggs' were processed for molecular identification using 2 independent approaches (Fig. 1). One approach used a single egg per sardine or chub mackerel stomach randomly sampled from the group of 'other fish eggs'. Each egg was processed independently to obtain nucleotide sequences for 3 mitochondrial gene markers (i.e. DNA barcodes) using Sanger sequencing. This was performed as a first step to assess the success of molecular identification of fish eggs retrieved from stomach contents, as well as to provide a baseline for the expected species diversity in preyed eggs. The second approach used all eggs under the category 'other fish eggs' isolated from single stomachs (3−26 eggs per stomach, including stomachs analyzed for single eggs, as described above). The group of unidentified eggs per stomach was processed together as a single mixed egg sample (i.e. each stomach equates to a sample) to obtain nucleotide sequences for the different eggs in the sample (i.e. DNA metabarcodes) for a single molecular marker using high-throughput sequencing. DNA metabarcoding is a more cost-efficient approach than DNA barcoding of large sample sizes, as it allows multiple samples to be sequenced simultaneously. DNA barcoding of single egg samples was used in a total of 118 eggs (1 per stomach), while DNA metabarcoding of mixed egg samples was used in a total of 568 eggs from 72 stomachs.

2.2. Molecular barcoding of single egg samples

DNA extractions from single egg samples were performed with the EasySpin Genomic DNA Tissue kit (Citomed) following the manufacturer's recommendations except for a final elution of 50 µl with EB buffer. Molecular identification of single eggs was based on PCR amplification and Sanger sequencing of 3 mitochondrial markers commonly used in
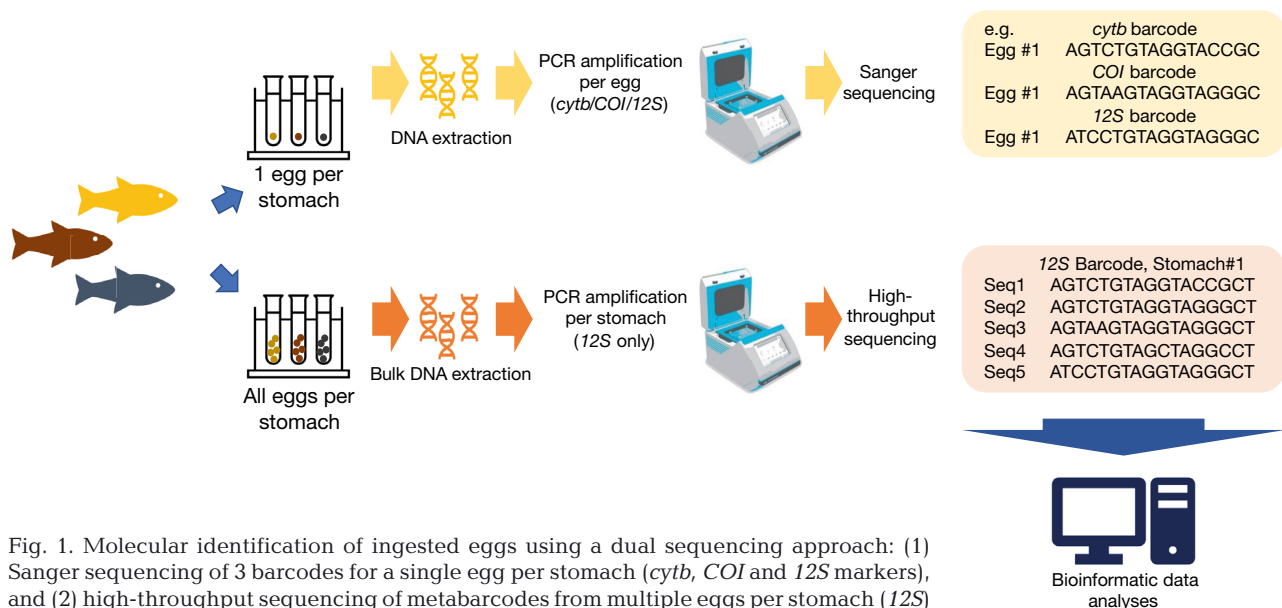

Fig. 1. Molecular identification of ingested eggs using a dual sequencing approach: (1) Sanger sequencing of 3 barcodes for a single egg per stomach (*cytb*, *COI* and *12S* markers), and (2) high-throughput sequencing of metabarcodes from multiple eggs per stomach (*12S*)

molecular identification of fish samples/specimens: cytochrome b (*cytb*, ~450 bp) using primers Gludg-L (Palumbi et al. 1991) and H15915 (Irwin et al. 1991); cytochrome oxidase subunit I (*COI*, ~300 bp) using primers mlCOIintF-XT (Wangensteen et al. 2018) and jgHCOI2198 (Leray et al. 2013); and 12S ribosomal RNA (*12S*, ~170 bp) using primers MiFish-U F and R (Miya et al. 2015). PCR amplification was performed in 10 µl reactions including 5 µl of MyTaq master mix, 0.5 µl of each primer (10 µM), 3 µl of extracted DNA and 1 µl of autoclaved water. The PCR temperature profile for *cytb* included an initial denaturation step of 15 min at 95°C; followed by 17 cycles of 95°C for 30 s, decreasing annealing temperatures of 0.5°C per cycle starting from 56°C for 30 s, and 72°C for 30 s; plus 23 cycles of 95°C for 30 s, 48°C for 30 s, 72°C for 30 s; and a final extension step at 60°C for 10 min. The temperature profile for the *COI* marker included an initial denaturation step of 3 min at 95°C; followed by a touch-down PCR of 16 cycles of 95°C for 30 s, 62°C (−1°C per cycle) for 30 s, 72°C for 1 min; followed by 24 cycles of 95°C for 30 s, 46°C for 30 s, 72°C for 1 min; and a final extension step at 60°C for 10 min. The temperature profile for the *12S* marker included an initial denaturation step of 3 min at 95°C; followed by a touchdown PCR of 11 cycles of 95°C for 20 s, 62°C (−0.5°C per cycle) for 20 s, 72°C for 15 s; followed by 29 cycles of 98°C for 20 s, 60°C for 15 s, 72°C for 15 s; and a final extension step at 72°C for 5 min. Success of PCR amplifications was checked by electrophoresis of 2% agarose gels stained with GelRed® and run in 0.5× TAE buffer at 300 V. Successful amplifications were cleaned of excess primers and dNTPs using 1 µl of ExoSap-IT™ (Thermo Fisher) and processed for Sanger sequencing in the forward and reverse directions using the Big-Dye™ Terminator v3.1 Cycle Sequencing Kit following the manufacturer's instructions. The resulting nucleotide sequences were checked for quality in Geneious Prime® 2020.1.2, and the forward and reverse reads were aligned per sample to obtain the resulting consensus sequence for each egg. The final consensus sequences were used in the molecular identification of the eggs.

2.3. Molecular metabarcoding of mixed egg samples

DNA extraction and amplification of multiple egg samples per individual stomach were performed in ultra-clean laboratories dedicated to non-invasive DNA samples to minimize contaminant carry-over during laboratory work. DNA extractions were per-

formed with the Qiagen DNeasy Blood and Tissue kit according to the manufacturer's instructions, except for a final elution in 50 µl of EB buffer.

PCR amplification was performed for all samples using the smaller *12S* barcode (~170 bp), the MiFish-U and the 2-step approach described by Miya et al. (2015). This marker was chosen based on the consistent molecular identifications across *cytb*, *COI* and *12S* (described in Section 3), and on the higher amplification success of *12S* in the single egg samples compared to *cytb* and *COI* (Table S1 in the Supplement at www.int-res.com/articles/suppl/m741p113_supp.pdf), thus making *12S* the most cost-efficient marker maximizing egg identification. The first round of PCR reactions was performed in 10 µl reactions including 5 µl of Qiagen Multiplex master mix, 2 µl of autoclaved water, 0.5 µl of each primer (5 µM) and 2 µl of eDNA (10 ng µl^{-1}). The temperature conditions of the first PCR were a touch-down profile 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 with a decrease of 0.5°C per cycle, and 72°C for 15 s; followed by 29 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 blanks). The second round of PCR served for adding sample-specific (and replicate-specific, where applicable) indexes and Illumina-compatible barcodes as described by Miya et al. (2015). Each sample was run in triplicate, and all PCR reactions were checked for positive amplification on a 2% agarose gel stained with Gel Red Nucleic Acid Gel Stain (Biotium) and run at 300 V in 0.5× TAE buffer. PCR triplicates were quantified and pooled equimolar per sample. Eight samples were chosen at random, and the 3 PCR replicates and the corresponding pool were sequenced independently to assess among-PCR replicate consistency and consistency between the pool and the corresponding replicates per sample. Extraction and PCR blanks were processed along with samples to assess putative contamination during the laboratory work.

The indexed libraries were cleaned using Ampure Beads (0.6×), quantified and pooled equimolar into a single library. Insert size was checked on the Tape-Station (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 10 pM, and sequenced on an Illumina MiSeq using the MiSeq Reagent Kit v2 (500 cycles) aiming at 50 000 reads per sample.

Raw reads from metabarcoding of mixed egg samples were quality filtered (−q 30), trimmed of adaptors and primers, and discarded if shorter than 100 bp using 'cutadapt' (Martin 2011). Cleaned reads were processed with 'dada2' (Callahan et al. 2016) as follows. Reads were trimmed to 150 and 100 bp, for forward and reverse reads, respectively, due to decreased quality at the 3′-end of the reads, discarded if the maximum expected errors was ⩾2 and if ambiguous bases (i.e. N) were present. Forward and reverse reads were dereplicated and subsequently aligned allowing for no mismatches (maxMismatch = 0). The resulting aligned sequences, designated amplicon sequence variants (ASVs), were inspected for and filtered from artifacts (i.e. sequences resulting from 2 or more biological sequences that were incorrectly aligned) using the method 'consensus'. The final cleaned ASVs were used in generating the final read count table across samples.

2.4. Molecular identification of egg samples

Molecular identification of the consensus sequences obtained for single eggs by Sanger sequencing was performed using BLAST searches ('megablast' algorithm) against the full nucleotide (nt) database on NCBI (accessed on 23 October 2021 for *cytb*, on 2 June 2021 for *COI* and on 11 September 2023 for *12S*), using default parameters and a maximum of 10 hits per search. BLAST results were filtered to select the hit with the highest percent identity per sample per marker, for a query coverage >97%. Identification at different taxonomic levels was performed according to percent identity of the top BLAST hit, namely at the species level: >98% identity; at the genus level: >95%; and at the family level: >90%. Given the shorter length of the *12S* barcode (mean ± SD: 167.9 ± 12.6 bp), more 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. Identifications with <95% identity for *12S* and <90% for *COI* and *cytb* were discarded from further analysis.

Consistency between visual and molecular identification of anchovy and sardine eggs was confirmed in the lab. Briefly, 4 eggs visually identified as anchovy were processed for Sanger sequencing as described above, and 12 samples of sardine eggs (n = 1−10 eggs per sample) extracted from 8 chub mackerel and 4 sardine stomachs were processed for metabarcoding as described above. Molecular identification of eggs as either anchovy or sardine showed 100% concordance with visual identifications.

Molecular identification of the final ASVs obtained from the metabarcoding of mixed egg samples was performed using BLAST searches as described above (NCBI nt database accessed on 4 November 2021). Read counts from different ASVs with identical taxon identification were summed on a per sample basis. Further filtering of the metabarcoding dataset included removal of all ASVs matching terrestrial or freshwater taxa, or marine taxa with no egg stages, as they were considered external contaminants. Finally, 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).

Regardless of the sequencing approach, in cases when a given nucleotide sequence produced multiple top hits with the same percent query coverage and identity for different taxa, molecular identification was performed at the genus level where multiple hits refer to congeners, or at the family level where multiple hits refer to different genera of the same family.

2.5. Statistical data analyses

The data on prey composition obtained with the visual identification of sardine and anchovy eggs and the molecular identification of the 'other fish eggs' (using the 2 technical approaches) were merged to obtain a final matrix of presence/absence of prey taxa per individual stomach. All downstream statistical analyses were performed after combining the different prey taxa into their corresponding taxonomic family. Differences in prey composition between predator species was tested with a permutational multivariate analysis of variance (PERMANOVA) and Jaccard's index of dissimilarity (Jaccard 1900) as implemented in the functions 'adonis2' and 'vegdist' of the 'vegan' R package (Oksanen et al. 2022). Multivariate homogeneity of group dispersions (variances), with groups corresponding to predator species, was also tested using the function 'betadisper' in 'vegan'. We also performed exploratory, unconstrained ordination analysis by means of principal coordinate analysis (PCoA) for each predator species independently, using Jaccard's index of dissimilarity (Jaccard 1900), as implemented in the function 'wcmdscale' of 'vegan'. In addition, we calculated the regression of the variables season, area and maturity stage to the PCoA ordination axes using the 'envfit' function of the 'vegan' R package, to help the interpretation of results.

3. RESULTS

3.1. Barcoding of individual eggs

Molecular identification of single egg samples using Sanger sequencing was successful in 102 samples (87%), with 16 samples failing PCR amplification and not producing sequences for either marker (Table S1; nucleotide sequences for *COI, cytb* and *12S* markers are available at https://data.mendeley.com/datasets/jkj4k7wgd4/1). The vast majority of the resulting nucleotide sequences (>80% across markers) matched available reference sequences on the NCBI GenBank nt database meeting the species-level identification criteria, while 4 sequences were identified at the genus level and 18 sequences at the family level (Table S2).

The proportion of identified eggs varied among markers, with *COI* performing similarly to *cytb* (67 and 62% eggs identified, respectively), but both performing considerably worse than *12S* (84% eggs identified; Table S1). In terms of the total number of families and species for which nucleotide sequences were obtained, the diversity detected across markers was very similar (18, 17 and 14 families, and 30, 31 and 28 species, for *12S, COI* and *cytb*, respectively; Table S2). All molecular identifications across the 3 markers were fully congruent at the family level, except for a single case (egg #46; Table S2). Discrepancies among markers (32 eggs) were due to multiple top hits with equal query coverage and percent identity in 1 or more markers, although in most cases, all markers had top hits for 1 species in common (Table S2). Fish eggs within the families Mugilidae and Sparidae were more challenging to identify at the species level, regardless of the molecular marker used.

Single egg samples from chub mackerel stomachs had higher identification success compared to those from sardines (91 vs. 81%), irrespective of molecular marker (Table S1). In total, 25 species from 18 different families were identified from 102 samples (Table S2), with similar numbers of species being detected in eggs from chub mackerel and sardine stomachs (n = 14 and 16 species, respectively), although more prey families were detected in the latter (n = 10 and 15 families, respectively).

3.2. Metabarcoding of multiple eggs per stomach

Upon filtering of the raw reads with our bioinformatic pipeline, the average read count per sample (excluding blanks) was 7330.6 (min.—max.: 373—62904). All PCR blanks had no reads, suggesting no contamination at this step of the protocol; thus, they were removed from further analyses. The DNA extraction blank had 1 ASV detected (i.e. ASV#12, *Lepidotrigla* sp.) with 268 reads, corresponding to <2% of reads of this ASV overall.

Our technical approach of sequencing the pooled PCR replicates per sample showed no bias in terms of the ASVs recovered and their relative frequency when compared to sequencing each PCR replicate separately. The ASVs recovered in the 8 samples for which PCR replicates and their corresponding pools were run separately showed 100% consistency (Table S3). The variance in % read count per ASV detected across pools and replicates was low (1—2%); however, there was higher variance in replicates with lower read counts (<1000). The congruence between PCR replicates and their respective pools supports the use of the latter as a cost-efficient approach to detect species in heterogeneous DNA samples from bulk egg samples.

A total of 89 ASVs were found over all samples (ASVs available at https://data.mendeley.com/datasets/jkj4k7wgd4/1) but were subsequently filtered as follows: (1) 15 ASVs were discarded (comprising 11% of the total read count) as they had <95% identity to their best BLAST hit, and (2) 12 ASVs were discarded (comprising 0.4% of the total read count) as they resulted in terrestrial, freshwater or marine contaminants. From the remaining 62 ASVs, 34 were identified at the species level (n = 23 species), 17 at the genus level (n = 8 genera) and 11 at the family level (n = 8 families). Upon the final filtering step, a total of 22 species and 20 families (some families had no species-level identifications) were detected with the metabarcoding approach. Out of the 72 bulk egg samples analyzed, only 2 samples failed sequencing. Eggs from sardine and Atlantic chub mackerel stomachs were identified as belonging to 14 and 13 different families, respectively, using the metabarcoding approach (Table 2).

Based on the above results, molecular identification of preyed fish eggs was generally consistent between sequencing approaches (Fig. 2, Table 2). Some families, genera or species were detected only with 1 approach, but these were represented by only 1 or 2 samples (egg or stomach, depending on the sequencing approach; Table 2). These inconsistencies may be due to the small sample sizes and/or lower occurrence of some prey families in the diet of the 2 predators.

3.3. Diversity of eggs ingested by sardine and Atlantic chub mackerel

The combination of visual (i.e. sardine and anchovy) and molecular identification (all taxa) of

Table 2. Molecular taxon identification of the preyed eggs and number of stomachs with eggs of each identified taxon according to predator (*Sardina pilchardus* and *Scomber colias*) and approach used: metabarcoding or single barcoding ('X' indicates taxon detection). Taxon identification was performed at species, genus and family levels, as described in Section 2

Taxon	Meta-barcoding	Bar-coding	Sardine	Chub mackerel	Taxon	Meta-barcoding	Bar-coding	Sardine	Chub mackerel
Callionymidae					**Scombridae**				
Callionymus lyra	X	X	2		*Scomber japonicus*	X			3
Carangidae					*Scomber scombrus*		X		1
Trachurus trachurus	X	X		16	**Scophthalmidae**				
Clupeidae					*Zeugopterus regius*	X			1
Sardina pilchardus	X	X	8	5	**Serranidae**				
Engraulidae					Serranidae	X		1	18
Engraulis encrasicolus	X	X	8	1	*Serranus cabrilla*	X	X		9
Gadidae					*Serranus hepatus*		X	1	4
Trisopterus luscus	X	X	5		**Soleidae**				
Gaidropsaridae					*Buglossidium luteum*	X		1	
Ciliata mustela	X	X	4		*Microchirus azevia*		X		1
Gaidropsaridae	X		3	1	*Solea senegalensis*	X		3	
Gaidropsarus sp.		X	1		Soleidae	X	X	6	1
Haemulidae					**Sparidae**				
Parapristipoma sp.		X		1	*Boops boops*	X	X	5	46
Pomadasys incisus		X		1	*Dentex dentex*		X		1
Labridae					*Dentex gibbosus*	X	X	1	1
Ctenolabrus rupestris	X	X	2		*Diplodus* sp.	X	X	2	5
Merlucciidae					*Diplodus vulgaris*		X		2
Merluccius merluccius	X			1	*Evynnis* sp.	X			2
Mugilidae					*Pagellus acarne*	X		2	22
Chelon labrosus	X			4	*Pagellus erythrinus*	X	X	3	13
Chelon sp.		X		2	*Pagellus* sp.	X	X	1	1
Mugilidae		X	3	3	*Pagrus pagrus*	X	X		2
Mullidae					Sparidae	X	X	8	17
Mullus surmuletus	X		1		**Tetraodontidae**				
Phycidae					*Arothron* sp.	X		1	1
Phycis phycis		X	2		**Trachinidae**				
Pleuronectidae					*Echiichthys vipera*	X	X	5	
Pleuronectidae	X		1		*Trachinus draco*		X	1	
Scianidae					**Trichiuridae**				
Scianidae		X	1		*Lepidopus caudatus*		X	1	
Umbrina canariensis		X	1		**Triglidae**				
Scomberosocidae					*Chelidonichthys lastoviza*	X	X	1	1
Cololabis sp.	X			2	*Chelidonichthys lucerna*		X	1	
					Chelidonichthys sp.	X			4
					Lepidotrigla sp.	X	X	1	13

egg samples from stomach contents show that sardines and chub mackerel prey on a large diversity of fish families (24 in total; Fig. 3). Moreover, sardines exhibited slightly higher prey diversity compared to chub mackerel at the family level (18 vs. 15 families, respectively; Fig. 3) but not at the species level (21 and 20 prey species, respectively; Table 2). The data also show similarities in the most frequent prey families between predator species despite differences in their corresponding percent frequency of occurrence (% FO) (Fig. 3a): specifically, fish eggs in sardine stomachs belonged pre-

dominantly to anchovy (60%) followed by sardine and sparids (33 and 29% FO), while chub mackerel showed a marked predominance of sparid eggs (76% FO), with important contributions from serranids (*Serranus* spp.), anchovy and sardine (36, 27 and 28%, respectively).

In line with the above results, we found significant differences in prey composition between predator species, although the R^2 value was low (Adonis test $R^2 = 0.09$, $F = 12.30$, p < 0.001). We also found small but significant differences in dispersion between species, with slightly larger dispersion, and thus broader prey
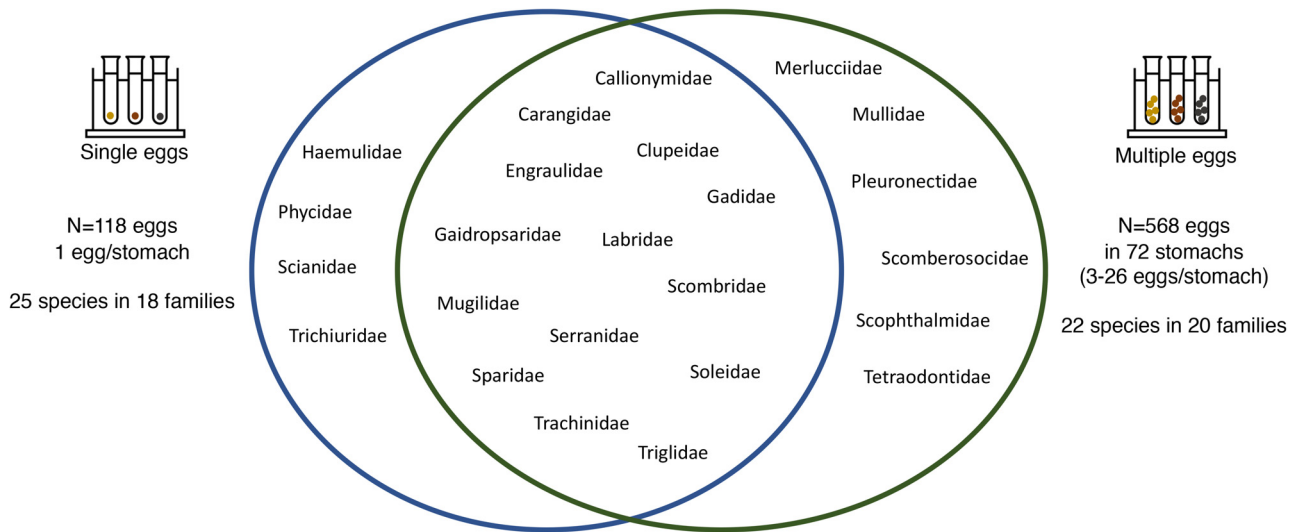
Fig. 2. Prey diversity detected with the 2 different sequencing approaches (see Fig. 1). Families detected by a single approach occurred in only 1 or 2 samples
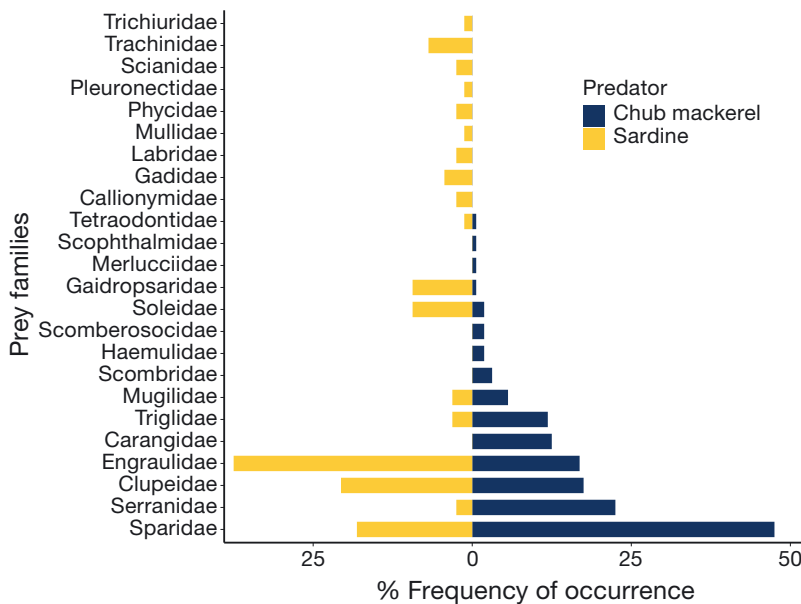

Fig. 3. Percent frequency of occurrence of each prey family per predator species, based on visual and molecular identification of fish eggs in stomachs

maturity stage were significantly correlated (p < 0.05) with PCoA axis 1 and 2 in both sardines and chub mackerel (explaining 50 and 52% of total inertia, respectively). Season had a stronger association with prey diversity in both predators compared to area and maturity stage (Table 3). Nevertheless, traits were highly correlated among variables and require further validation; for instance, most chub mackerel fall samples were from the south coast, while most spring samples were from the west coast (Table 1).

4. DISCUSSION

Predation is generally thought to be the main cause of pelagic fish egg mortality (Houde 2002). However, species identification at the egg stage is often challenging using visual microscopy. Here, we provide a proof-of-concept on molecular identification of fish eggs from stomach contents that were not amenable to visual identification due to the lack of diagnostic morphological traits. Our methodological protocols of barcoding of single eggs or metabarcoding of mixed egg samples showed high consistency in the prey taxa detected. Moreover, the results show that the molecular identification of the previously 'unidentified fish eggs' in the stomachs of 2 SPF spe-

variability, in sardines compared to chub mackerel (average distance to centroid: sardine = 0.579, chub mackerel = 0.523; $F = 5.152$, p < 0.05). Since group dispersion overlapped between predator species (Fig. 4), and slightly larger variance was observed for the smaller-sized group (i.e. sardines), the PERMANOVA results should be interpreted with caution and require further validation with larger and more balanced sample sizes (Anderson & Walsh 2013). Exploratory PCoA on prey species composition performed for each predator independently showed that season, area and
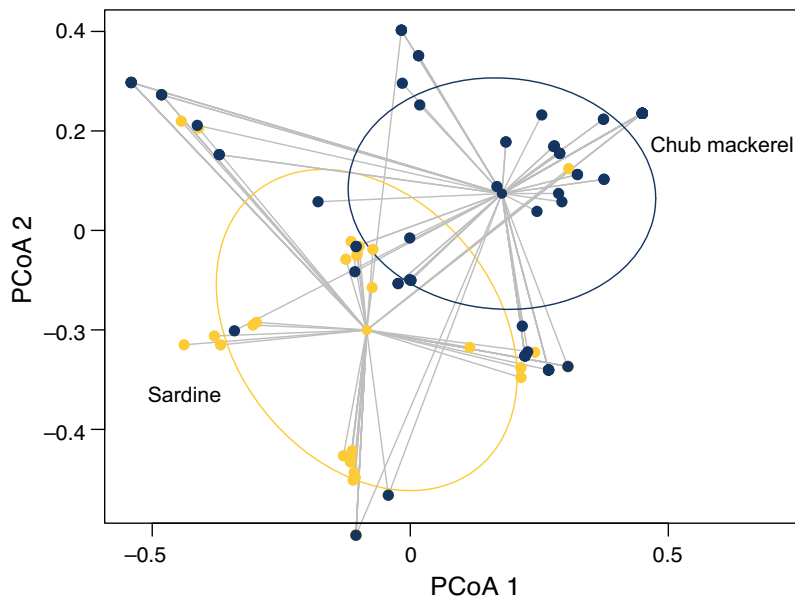
Fig. 4. Principal coordinate analysis of non-Euclidean distances between prey items and group (predator) centers. Data points correspond to individuals, and may overlap between sardine (yellow) and chub mackerel (blue). Ellipses refer to 1 SD from the group median

Table 3. Goodness of fit (r^2) of sampling season, sampling area and maturity stage onto ordination axis 1 and 2 of a principal coordinate analysis on the prey composition of European sardine and chub mackerel. Significance values are $^*p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$

Variable	Sardine	Chub mackerel
Season	0.3944***	0.2482***
Maturity	0.1193**	0.1269***
Area	0.0644*	0.152***

cies, sardine and chub mackerel, uncovered a high diversity of prey taxa (Figs. 2 & 3). Most prey families comprise regionally abundant pelagic and demersal coastal fish off Atlantic Iberian waters that produce pelagic eggs upon spawning (Froese & Pauly 2022).

Despite the conspicuous diagnostic visual features of sardine and anchovy eggs (Russell 1976, Garrido & van der Lingen 2014), only a small fraction of the eggs identified using molecular markers belonged to these 2 species (13 and 9%, respectively). This suggests that visual identification may not be 100% accurate and/or that diagnostic morphological traits may deteriorate during digestion. Contamination of unidentified egg samples with DNA from sardine and anchovy is less likely since molecular identification of sardine and anchovy eggs was mostly based on single egg samples (Table 2; Table S2), where the DNA from the prey species is expected to be at a higher concentration than any potential contaminant DNA.

Most of the prey families detected here (19 out of 24) are also commercially important fish resources locally (Martins & Carneiro 2018). Given the preference and high abundance of fish eggs in sardine and chub mackerel stomachs, these SPF species likely play a major role in egg mortality of a large number of species with which they co-occur. In particular, the impact of egg predation by SPF species may be significant not only for anchovy and sardine, as described previously (Fonseca et al. 2022), but also for other commercially valued taxa like sparids and serranids. Indeed, our results also indicate that intraguild predation is predominant in these SPF species, but it is not exclusive. The most abundant fish eggs in the sampled stomachs belonged to pelagic species such as anchovy, sardine, horse mackerel and bogue (Table 2); however, piscivorous demersal fishes also provide important contributions to sardine and chub mackerel diets, namely serranids and sparids (except bogue).

Multi-species interactions that focus on predator—prey relationships are of increasing interest, especially when fishing pressure on one species may influence the abundance of other species (Bailey & Houde 1989). For instance, molecular identification of eggs in the stomachs of 2 other SPF species, herring and sprat, found that they were likely the main predators of plaice eggs and larvae in the Irish Sea, with more than 90% of herring stomachs testing positive for plaice (Fox et al. 2012). This demonstrates that the impact of intraguild predation on egg mortality should not be disregarded when modeling coastal pelagic fish recruitment variability. In the case of Atlantic Iberian waters, ichthyoplankton communities are highly diverse, and the most abundant species, particularly sardine, have a broad distribution over the continental shelf (Garrido et al. 2009). Therefore, eggs from sardine and other fish species have high spatial overlap with predators (sardine and chub mackerel). Given the high abundance of sardine when compared to most other coastal pelagic fish species, their impact on egg mortality is likely to be very large. The impact of egg predation by SPFs may differ between predator species. Our results show significant differences in prey composition between sar-

dines and chub mackerel, although many fish families are predated by both species. The species-specific difference in prey taxa may be further influenced by location, season and maturity stage of the predator (Table 3), highlighting the need for assessments of variability in egg predation in each predator species with larger and more balanced sample sizes.

While food web models already take into account egg predation by sardine on its own eggs, the same is not true in the case of intraguild predation of other species (Szalaj et al. 2021). For instance, in contrast to sardine, anchovy eggs have a limited distribution area off the Iberian coast, occurring mostly at the north-western Portuguese coast and off the Gulf of Cadiz. This distribution is in line with the higher occurrence of anchovy eggs in the stomachs of sardine, with which they have a broader spatial overlap compared to chub mackerel that is mostly distributed in the south (despite some inter-annual variability). This means that anchovy is particularly vulnerable to sardine predation when sardine abundance is high, and less vulnerable to chub mackerel. On the other hand, we demonstrate that chub mackerel is a predator of horse mackerel eggs, and its impact on horse mackerel recruitment should be further investigated and taken into account. In contrast, we did not identify any horse mackerel eggs in sardine stomachs, probably due to differences in distribution where horse mackerel occurs more offshore than sardine.

Our work shows that molecular identification of fish eggs in stomach contents is feasible and can complement and confirm visual identification of prey items in the diet. This approach can also be extended to bulk stomach content samples aiming at identification and quantification of predation on fish eggs and larvae (Hunter et al. 2012). The consumption of eggs by SPF is more frequently described than the consumption of larvae (Smith & Reay 1991), and it is still unknown if this reflects higher predation of eggs and/or higher digestibility of larvae. Fish eggs have a chorion that is more resistant to digestion, while fish larvae may be digested more rapidly (Legler et al. 2010). Thus, fish larvae may be an important part of the diet not detected in visual analyses of stomach contents. Given the potential double impact of trophic interactions on coastal fish species that act as competitors but also as predators of eggs and larvae, population dynamics of these species should also take these interactions into account.

Additional insights into the biology of the prey taxa can be gained from the current data. Given the broad taxonomic prey diversity of fish eggs retrieved from SPF stomachs, these species can act as natural monitors of the spawning events of their prey. In fact, the reported spawning season of prey species matches the sampling season in which their respective eggs were found in the stomachs of sardine and chub mackerel (Table S4). Importantly, these data can also inform on the spawning season and location of understudied species. For instance, there are no published data on the spawning season of the comber *Serranus cabrilla* or of the brown comber *S. hepatus* for Atlantic Iberian waters, but egg stages of these species were detected in stomach contents of SPFs in the south coast mostly during spring, implying recent spawning activity (Table S4).

4.1. Technical considerations for molecular identification of fish eggs

Application of molecular genetics to diet analysis dates back to the 1990s (reviewed by Symondson 2002) and has become a powerful and useful complementary approach to visual methods (e.g. reviewed by Alberdi et al. 2019; Amundsen & Sánchez-Hernández 2019, Sousa et al. 2019). Its major advantage is the ability to identify prey taxa that are not amenable to visual identification, coupled with increased sensitivity of detection and a higher resolution of taxonomic identifications (reviewed by King et al. 2008). Furthermore, high-throughput sequencing technologies now allow simultaneous sequencing of hundreds of mixed prey samples in a single run, making it a very cost-efficient approach when compared to labor-intensive visual prey identification and DNA barcoding of individual samples. DNA-based prey identification can be performed using different approaches ranging from single species detection using specific probes to multiple species detection from bulk sample analysis of stomach contents (reviewed by Traugott et al. 2021).

For the purpose of assessing egg predation using DNA-based methods, previous studies have generally applied species-specific assays to detect specific target species in stomach contents (e.g. Albaina et al. 2015, Schreier et al. 2016, Lutz et al. 2020, Allan et al. 2021). Here, we successfully used 2 different DNA-based approaches to identify ingested fish eggs belonging to multiple unknown prey species: barcoding of single eggs and metabarcoding of mixed egg samples. However, a few aspects should be considered when choosing the most suited technical approach for molecular identification of ingested eggs (or other prey items), notably (1) sequencing success, (2) cost-efficiency and (3) quantitative data collection (reviewed by Alberdi et al. 2019). Regarding sequencing success, and as men-

tioned earlier, Sanger sequencing of single egg samples had variable success rates depending on the genetic marker used, with higher PCR failure for the longer barcodes (Table S1). DNA quality and quantity of single egg extractions were generally low, which may have resulted in high stochasticity of PCR amplification success. In addition, DNA extracted from feces and stomach contents is often highly degraded due to digestion and exposure to other environmental factors (King et al. 2008 and references therein). Thus, shorter barcodes (<300 bp; e.g. our *12S* marker) often perform better than longer ones (e.g. our *cytb* and *COI* markers), and may provide higher sequencing success in molecular identification of fish eggs retrieved from stomach contents (King et al. 2008). In accordance with the above, out of the 72 bulk egg samples processed using the short *12S* marker, only 2 failed to produce sufficient reads for analysis upon filtering.

In contrast, cost-efficiency is an important aspect to consider: processing single eggs rather than bulk egg samples implies higher labor and consumable costs on a per-stomach basis, particularly when processing hundreds or thousands of eggs and/or stomachs. In such cases, using bulk DNA extractions of multiple eggs per stomach, as used here, coupled with metabarcoding and high-throughput sequencing can greatly reduce the overall costs. The above considerations make metabarcoding of bulk egg samples a more cost-efficient approach aiming at the molecular identification of prey taxa, compared to barcoding of single specimens (Nobile et al. 2019).

Regarding quantification of prey items, the 2 approaches used here differ considerably in their outcomes. While analyses of single eggs allow for direct quantification of the number of eggs per prey taxon per stomach, metabarcoding can provide (at best) a relative quantification based on the percentage of total reads assigned to a given taxon on a per-stomach basis. Such results still need to be interpreted with caution since other factors, e.g. primer-bias amplification and variable DNA content per egg across prey taxa, may affect the final distribution of reads (Duke & Burton 2020). Dedicated laboratory trials may be needed to ascertain the source and level of bias in the detection and quantification of the different prey in these types of samples and for any given molecular barcode.

Regardless of the sequencing approach chosen, the choice of the molecular barcode used should be based on (1) the probability of accurate taxon detection and (2) the taxonomic resolution offered by the barcode. Different barcodes can vary in their primer-binding affinity across the target taxa, resulting in PCR ampli-

fication bias, i.e. some taxa may be preferentially amplified to the detriment of others (Krehenwinkel et al. 2017, van der Loos & Nijland 2021). In the event of successful PCR amplification, taxon detection may still be compromised by incomplete taxonomic coverage of the reference sequence database used, whereby missing taxa will result in no hits of a given query sequence and thus lead to false negatives. These issues may be reduced by using multiple barcodes on a given sample aiming at maximizing taxon detection and identification accuracy. However, additional sequencing of individual species may be needed to ensure completeness of the reference sequence database. Finally, genes used as barcodes may evolve at variable rates and, thus, may have different diagnostic power in terms of the taxonomic resolution of their identifications (e.g. Table S2), e.g. some barcodes may not be able to distinguish closely related species (Costa et al. 2012, van der Loos & Nijland 2021). Both *in silico* and *in vitro* PCR trials can be made to choose the best combination of barcodes aimed at molecular identifications at the desired taxonomic level, prior to routine implementation (Ficetola et al. 2010).

4.2. Conclusions

Molecular identification of stomach contents can provide important complementary information to traditional diet studies based on visual prey identification, particularly when visual identification is not feasible. Here, we successfully identified fish eggs from stomachs of 2 abundant and commercially important SPF species using molecular genetic markers. Our results show that SPF species prey on the eggs of a high diversity of coastal pelagic and demersal fish, most of which are of commercial interest, and call for additional in-depth studies estimating the impact of intraguild predation in both predator and prey species dynamics. The molecular protocols used here can be applied to other predators of fish eggs, although the choice of single egg or mixed egg analyses should be made considering the available budget, the number of samples to be processed and whether quantification of prey specimens is required.

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