Automated image analysis suggests the consumption of herring eggs by red mullet in the English Channel

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Abstract :

Consumption of fish eggs is a major component of the trophodynamics of several marine ecosystems. Nevertheless, demonstrating the consumption by stomach content analysis is complex, notably due to the high digestibility of eggs. Atlantic herring (Clupea harengus) of the Downs substock migrate from the North Sea to spawn in the Eastern English Channel during winter. On the basis of an opportunistic sampling of 5 red mullets (Mullus surmuletus) displaying herring eggs in their stomachs, this paper was the opportunity to document for the first time the trophic importance of herring eggs in the English Channel, by combining automated analysis of prey images with stable isotope and C:N ratios measurement. Eggs represented more than 90% of the prey items observed in the stomachs. In addition, low δ13C and high δ15N and C:N ratios demonstrated that this observation does not reflect occasional consumption of eggs, but that eggs are a common prey all through the entire spawning season. Automated image analysis demonstrated efficiency to produce semi-quantitative indicators of the diet, but manual post processing should be implemented if accuracy is needed. This work was nevertheless the opportunity to start the development of a learning set adapted to benthic organisms and available for further similar applications. This work also demonstrated the importance of a holistic approach of trophic interactions to manage exploited species.

Résumé :

L'analyse automatisée d'images d'estomacs de rougets suggère la consommation d'œufs de harengs en Manche

La consommation d'œufs joue un rôle clé dans le fonctionnement trophique de nombreux écosystèmes marins. Cependant, démontrer cette consommation par une analyse des contenus stomacaux est complexe, entre autres du fait de la digestion rapide des œufs. Le hareng (Clupea harengus) du sous stock des Downs migre en hiver depuis la mer du Nord pour venir pondre en Manche orientale. La capture de 5 rougets (Mullus surmuletus) dans lesquels des œufs de harengs ont été observés est une opportunité de documenter pour la première fois l'importance trophique des œufs de harengs, par une approche couplant analyse semi automatisée des images des proies dans les contenus stomacaux, et analyse des traceurs trophique intégrés (isotopes stables du C et du N et rapport C:N). Les œufs de harengs représentaient 90% des proies dans les estomacs. De plus, les valeurs faibles pour le δ13C et

élevées pour le δ15N étaient cohérentes avec une consommation régulière d'œufs de hareng. L'analyse d'image automatisée appliquée aux estomacs est efficace pour produire des indicateurs semi-quantitatifs de l'alimentation des poissons, mais requière un post-traitement manuel si des indicateurs quantitatifs précis sont nécessaires. Ce travail a été l'occasion d'initier le premier développement d'un jeu d'apprentissage adapté aux organismes benthiques, qui pourrait par exemple servir à évaluer la consommation d'œufs de harengs par d'autres espèces de poissons benthiques. Ce travail a aussi confirmé l'importance d'adopter une approche plus holistique des interactions trophiques pour mieux gérer les espèces exploitées.

INTRODUCTION

As marine ecosystems are intrinsically connected and dynamic, pulsed inputs of trophic resources are major drivers of their functioning (Varpe *et al.*, 2005; McMeans *et al.*, 2015; Gounand *et al.*, 2018; Nielsen *et al.*, 2019; van Denderen *et al.*, 2021). Pulsed inputs are highly diverse: they can involve nutrients or organic matter flowing between terrestrial and costal ecosystems (Darnaude *et al.*, 2004; Liénart *et al.*, 2021, 2017) or between pelagic and benthic ecosystems (Duffill Telsnig *et al.*, 2019; Cresson *et al.*, 2020). They can also result from organism migrations, for example between shallow and deep areas (Trueman *et al.*, 2014; McMeans *et al.*, 2015) or between remote ecosystems. Spawning migrations are iconic examples of this type of pulsed inputs. Massive aggregation of top-predators produce high-impact images largely broadcasted in public media, and these inputs also drive the trophodynamics of the system (Hocking and Reimchen, 2002; Varpe *et al.*, 2005; Willson and Womble, 2006; Mourier *et al.*, 2016; Nielsen *et al.*, 2019). Varpe *et al.* (2005) estimated for example that the migration realized by Norwegian herring to spawn represent a transfer of $1.3 \ 10^6$ tons of organic matter (*i.e.* ~ 25% of the spawning stock biomass) between feeding and spawning areas. Impact of these pulsed inputs is even more important when spawning occurs in resource-limited areas or seasons (Kotterba *et al.*, 2014; Nielsen *et al.*, 2019). Eggs represent a "*boon*" for consumers, as they provide large amounts of essential fatty acids, but do not request searching, chasing or handling time, and are usually not defended after being laid notably for species spawning numerous eggs like herring (Fuiman *et al.*, 2015; Fuiman, 2018). Eggs could thus represent a major trophic input for consumers. Fuiman *et al.* (2015) estimated that more than 90% of the eggs enter the food webs by direct consumption. Impact of egg consumption on predator biomass and on herring's population survival was documented by the identification of eggs in stomach content, resulting whether from natural consumption (Kotterba *et al.*, 2017; Wiegleb *et al.*, 2018) or feeding trials (Kotterba *et al.*, 2014; Wiegleb *et al.*, 2018) and by numerical analyses (Varpe *et al.*, 2005; Richardson *et al.*, 2011; Fuiman, 2018; Nielsen *et al.*, 2019).

Visual identification of preys in stomach content is a long-standing approach in trophic ecology. Several decade of uses of the technique have demonstrated its utility to elucidate fish diet, notably by its ability to provide an accurate taxonomic identification of preys and also quantitative indicators of their relative importance (Baker *et al.*, 2014; Saikia, 2016; Amundsen and Sánchez-Hernández, 2019). Nevertheless, one of the limitations of this method lies in the digestibility of the preys. Stomach content only informs on the last consumed preys, and make it difficult or impossible to observe preys with soft tissues, like fish eggs, that are quickly

digested. Experimental studies demonstrated that the number of eggs in stomachs decrease logarithmically, with half of the eggs digested in 3 hours, and all eggs in ~10 hours (Kotterba et al., 2014; Wiegleb et al., 2018). In addition, stomach content analysis is a time-consuming method. Identifying preys in stomach could literally take "*guts*" (Amundsen and Sánchez-Hernández, 2019). New methodological developments, which can limit the processing time of stomach without degrading the quality of the data produced, have thus to be investigated. Automated image analysis could represent a promising avenue to explore, notably as it can allow visual identification and numbering of the preys but request lower treatment time than classical analysis. In addition, automated estimation of object size could also represent a major breakthrough for the analysis of trophic relationships, as relative size is a major driver of predator-prey interactions (Scharf et al., 2000) and a key parameter in ecosystem models design (Travers-Trolet et al., 2019).

Zooscan was initially developed to analyse zooplankton samples (Benfield et al., 2007; Gorsky et al., 2010; Grandremy et al., 2023; Grosjean et al., 2004; Irisson et al., 2022). After a digitization of the samples, automated classification of zooplankton images (hereafter vignettes) allows detection, identification and numeration of all digitized objects, by comparison with an *a-priori* prepared learning set, *i.e.* a set of images previously identified by trained scientists (Gorsky *et al.*, 2010; Irisson *et al.*, 2022). As reviewed by Irisson *et al.* (2022), the use of imagery for zooplankton samples treatment has massively increased during the last years: as zooplankton is largely affected by climate change, number of samples collected to monitor changes dramatically increased. Automated analyses have allowed diminishing the time requested to process plankton samples. This technique is also powerful to identify fish eggs, as it provide similar results than traditional microscopy methods (Lelièvre *et al.*, 2014). Nevertheless, application of this technique to stomach content samples is very limited. It is not listed in the most comprehensive and recent review about it so far (Irisson *et al.*, 2022). To the best of our knowledge, it has only been applied once, to understand the importance of zooplankton in the diet of *Coregonus* sp. from a German lake (Vogelmann *et al.*, 2022). This application remain thus close to the initial aim of the tool, as it was aimed at identifying zooplankton and benefited from the learning set of zooplankton samples. However, applying routinely this tool to the analysis of preys in the stomachs of benthic-feeding fish (*i. e.* degraded benthic preys), and notably using the predicted results without individual validation of all vignettes, would require the development of a new learning set, specifically designed to this aim.

Stomach content analysis has also proven useful to be used in combination with stable isotopes, as both methods are largely complementary (Vander Zanden *et al.*, 1998; Cresson *et al.*, 2014; Pethybridge *et al.*, 2018, Timmerman *et al.*, 2020). As preys are eventually digested, stomach content gives a snapshot of the diet over a short period after food intake. On the contrary, stable isotopes do not inform precisely on the preys consumed, but provide an integrated vision of the trophic behaviour of a consumer. In the English Channel, previous studies notably applied stable isotopes analyses to identify the relative importance of benthic and pelagic pathways at the base of food webs. These works were based on the isotopic difference between benthic and pelagic pathways. As pelagic production exhibits lower δ^{13}C values, fish species feeding on this pathway, such as herring, usually have lower δ^{13}C values than benthic species (Kopp *et al.*, 2015; Giraldo *et al.*, 2017; Cresson *et al.*, 2020; Timmerman *et al.*, 2021).

Atlantic herring *Clupea harengus* Linnaeus, 1758 has a central ecological, economic and patrimonial importance in the North Sea. Herring is traditionally a key species for fisheries in this area, with annual landings ranging between 360 and 600 kt between 2012 and 2021 (ICES, 2022), *i.e.* ~20% of the total landings and half of the pelagic landings in the area (Eurostat, 2020). This importance of herring consumption can be tracked back to 10th century (Barrett *et al.*, 2004). As zooplankton-feeding fish species, like herring and sprat, are predominant in the biomass of the fish assemblage in the North Sea, they exert a wasp-waist control on the functioning of the North Sea, both by predating zooplankton, and by being a prey for piscivores fish, mammals and birds (Fauchald *et al.*, 2011). The species is separated between four different substocks that spawn in different areas off North Sea and English Channel coasts (Fässler et al., 2010). The currently most important herring population for fisheries in the North Sea is considered an autumn spawning population (August to January). Among them, herring from the Downs component migrates to spawn in the Eastern English Channel between November to January. Adults lay patches of adhesive eggs on coastal and shallow bottom. Eggs hatch after several days to three weeks depending of the temperature (Maucorps, 1969). First steps of herring life-cycle are thus largely driven by environmental factors, explaining why global warming is a major threat for this population (Denis *et al.*, 2016; Joly *et al.*, 2021; Toomey *et al.*, 2023). Warming is notably suspected to be the cause of the shortening of the reproduction period (1.5 month less) since the beginning of the 21st century (P. Marchal, comm. pers.). This massive arrival of migrating adult is also an opportunity for local fisheries, illustrated in the cultural events: numerous coastal cities in the North of France organize "*Fêtes*

du hareng" (herring festivals) in winter, to celebrate the return of herring and large catches. This massive migration is also expected to represent a key trophic input for the community.

Surprisingly, there are few data about the trophic role of herring locally. There are for instance no records of herring diet in the English Channel in the DAPSTOM database of stomach contents (Pinnegar, 2014). The only available data are from modelling outputs: EEC-OSMOSE model (Travers-Trolet *et al.*, 2019) estimated that herring is consumed by several piscivores species (cod, whiting and squids notably). Similarly, the importance of herring as a forage species was confirmed by outputs of the Atlantis model (Girardin *et al.*, 2018; Ito *et al.*, 2023) or by statistical models based on isotopic ratios (Giraldo *et al.*, 2017). Nevertheless, the resolution of the two latter was not sufficient to separate herring from all other zooplankton-feeding species. To the best of our knowledge, the only empirical data from stomach contents are for large (> 20 cm) whiting *Merlangius merlangus*, that integrate adult herring as an important part of their diet (Timmerman *et al.*, 2020). This study also proposed that unexpectedly high $\delta^{15}N$ ratio in small whiting in winter could be related to the consumption of herring eggs. This hypothesis could not be confirmed by stomach content analyses, potentially as eggs are quickly digested. In other systems, spawning migration of herring generates changes in the diet, recruitment or catches of birds and crustaceans (Skov *et al.*, 2000; Varpe *et al.*, 2005; Willson and Womble, 2006). Such a predation was also suspected to be a potential risk of decline for herring population (Richardson *et al.*, 2011; Kotterba *et al.*, 2014; Wiegleb *et al.*, 2018). Nevertheless, how consumption of herring – whether migrating adults or eggs and larvae afterwards – affects ecology of predators and sustainability of Downs herring population has never been investigated in the English Channel.

During the IBTS 2021 survey, fish eggs were opportunistically observed in the stomach content of a red mullet *Mullus surmuletus* Linnaeus, 1758 caught in the Eastern English Channel. Red mullet has become an important species for the fisheries locally during the last years (Bourdaud et al., 2019; Pinto et al., 2019). Quick visual observation of some eggs by specialist on-board confirmed that this was herring eggs. Four other red mullets caught during this haul were collected for an analysis of their diet. This opportunistic sampling allowed investigating the possible consumption of herring eggs. From a methodological point of view, the limited sample size (5 stomachs) is an opportunity to initiate the application of automated image analysis to stomach content analysis and the generation of a learning set adapted to identify benthic degraded items, but also to test of the limits and strengths of the tool. Then, it could also provide a first vision of the trophic importance of herring eggs in the diet of benthic fish species like *M. sumuletus*, notably by addressing the importance of eggs in the diet of the

individuals collected in 2021, but also by comparing their isotopic and elemental ratios, with values previously measured in the same area.

MATERIAL AND METHODS

Five red mullet (*Mullus surmuletus* Linnaeus, 1758) individuals were collected on January 21, 2022 at 10:40 GMT in a shallow station (31 m depth) during the International Bottom Trawl Survey (IBTS) near the Dover Strait, at the limit between the Eastern English Channel and the North Sea (Station A1028; 50.8693°N, 1.3476°E). Red mullets were observed at all stations but two during this survey, and with abundances (whether determined or estimated) ranging between 2 and 242 (Fig. 1). Logistical reasons on-board precluded from sampling a larger number of individuals. IBTS is an ecosystemic survey aimed at estimating fisheries indices by bottom trawling and at providing an integrated picture of the ecosystem functioning, by sampling all components of the system from plankton to top predators (Cresson *et al.*, 2020; Auber and Lazard, 2022). After the opportunistic observation of eggs in the stomach of one individual, all mullets sampled on the concerned haul were collected, measured (total length, to the nearest mm), weighed (total mass, to the nearest g) and dissected on-board. During dissection, stomach content and muscle were sampled for diet and stable isotopes analyses, respectively. Sex and maturity were determined on-board by visual examination of the gonads, following the IBTS routine protocol. Stomachs were immediately placed in a –70°C freezer for 4 h to stop digestion. All samples were then stored frozen (–20°C) on-board and then at the laboratory. Muscle tissues were freeze-dried and grinded before being analysed for C and N stable isotopes.
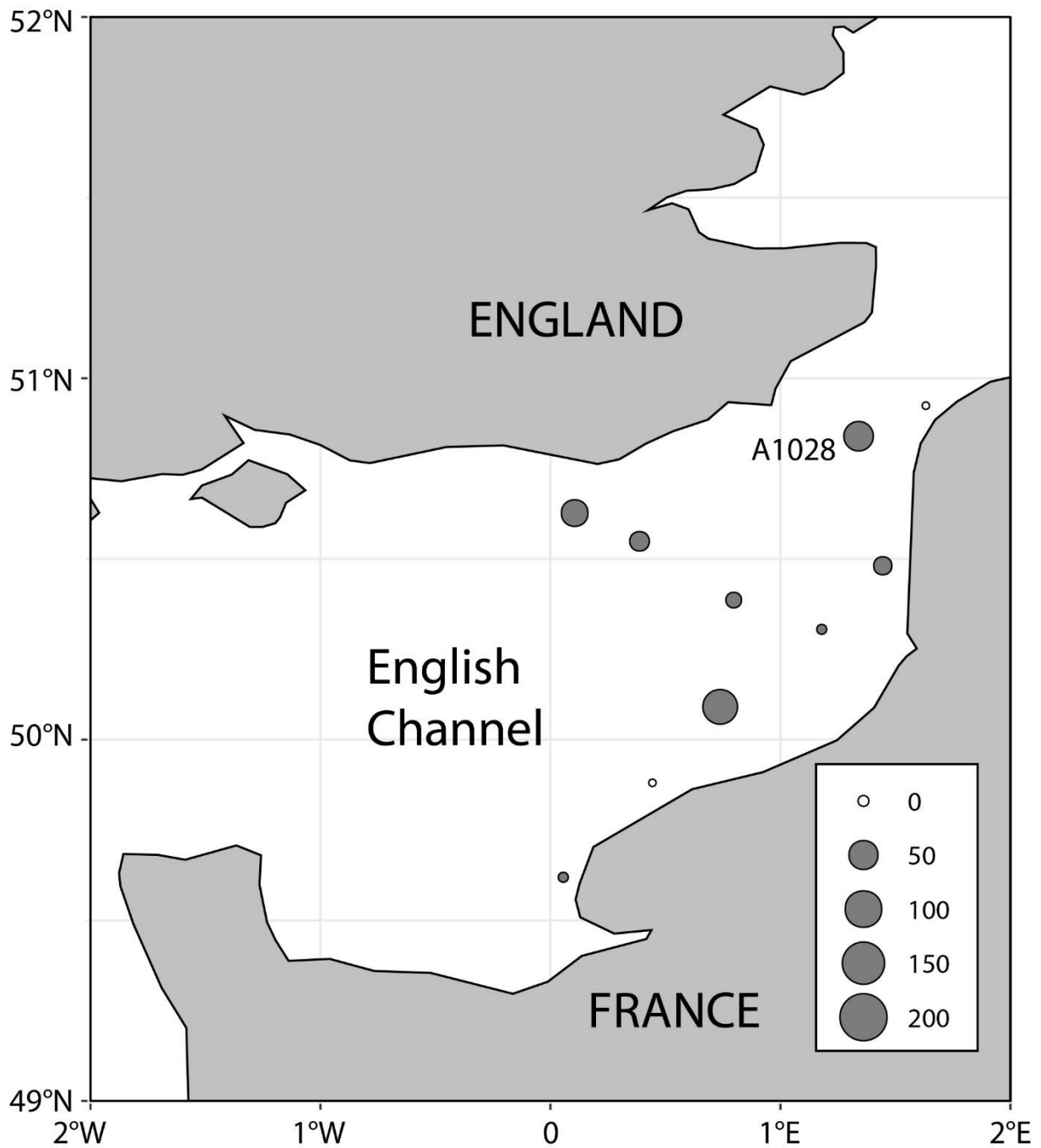
Figure 1: IBTS 2021 trawling stations in the Eastern English Channel. Size of the dots represents the number of red mullets collected at each station, with white dots standing for stations where no red mullet was caught. Numbers were retrieved from ICES DATRAS database (https://datras.ices.dk/Data_products/Download/Download_Data_public.aspx). Individuals analysed in the present study were caught at Station A1028

C and N stable isotope ratios were measured with a Thermo Delta V isotope mass ratio spectrometer coupled with a Carlo Erba NC2500 elemental analyser, used to analyse C and N elemental content. Accuracy of the isotopic ratios measurements was checked by repeated

analyses of an in-house standard (one analysis of the standard after every 10 samples). For this analytical sample run, the overall standard deviation for the internal animal standard was 0.08‰ for δ^{15}N and 0.17‰ for δ^{13}C. Stable isotopes ratios were expressed following the classical δ notation:

$$\delta X = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \times 10^3, \tag{1}$$

with X ^{13}C or ^{15}N, respectively, and R the isotopic ratios (^{13}C/^{12}C or ^{15}N/^{14}N, respectively) measured in fish muscle (sample) or in standard (Vienna Pee Dee Belemnite for C and atmospheric nitrogen for N). C:N ratios were measured and used a proxy of lipid content, notably to correct an effect of high lipid content on δ^{13}C (Post *et al.*, 2007). δ^{13}C ratios measured in muscle with C:N ratios higher than 3.5 were corrected using the Post *et al.* (2007) formula: δ^{13}C$_{lipid-corrected}$ = δ^{13}C$_{raw}$ -3.32 +0.99 C:N.

All biometric, isotopic and elemental individual data are presented in Table I. Isotopic and C:N ratios were compared with values previously measured for *M. surmuletus* in the English Channel in autumn 2009 (63 individuals), autumn 2015 (3 individuals) and winter 2016 (10 individuals) (Kopp *et al.*, 2015; Cresson *et al.*, 2018). Difference were checked by ANOVAs, followed by Tukey posthoc tests (*multcomp* package; Hothorn *et al.*, 2008). Assumption of parametric tests were checked by visual examinations of diagnostic plots produced by the *check.model()* function of the *performance* package (Lüdecke *et al.*, 2020). Correlation between isotopic and C:N ratios and eggs number in stomach content was estimated by calculating Pearson correlation coefficient. All analyses and plots were done using R core software version 4.1.2 (R Core Team, 2020) and packages *ggplot2* and *ggcorrplot* (Wickham, 2016; Kassambara and Kassambara, 2019).

Table I. – Biometric and isotopic data recorded on red mullet individuals.

Individual number	Total length (cm)	Total mass (g)	Muscle			
			δ^{13}C raw (‰)	δ^{13}C corrected (‰)	δ^{15}N (‰)	C:N
H0220	22.0	124	-19.46	-17.43	15.19	5.40
H0221	21.9	121	-18.10	-17.29	16.22	4.17
H0222	22.7	132	-17.67	-17.22	16.05	3.80
H0223	22.7	145	-20.20	-18.91	15.08	4.66
H0224	22.0	124	-18.96	-18.26	14.75	4.05

In the laboratory, stomachs were thawed, opened and prepared before being digitized with a ZooScan. Individual items were placed upon the screen of the ZooScan to be digitized following the appropriate protocol (Gorsky et al., 2010; Jalabert et al., 2022). Dietary items were individualized as much as possible, by gently scraping it and cleaning it from the stomacal mucus. This was not always possible for herring eggs: as they are sticky, they remained in bunches, and scraping it may cause their destruction. A 16-bit grey-level high resolution image was obtained for each stomach sample (2400 dpi, pixel size: 10.6μm, Gorsky et al., 2010). This raw image is then processed using Zooprocess software (based on ImageJ macro language). It is submitted to a background subtraction, which allows a normalization and the comparability of ZooScan images. This image is then segmented to automatically separate the objects. Each object is then individually and automatically processed for the measurement of morphological features (size, area…, see details in Gorsky et al., 2010). At the end of the image processing, one image ("vignette") is obtained for each object and is associated to a set of ~35 morphological parameters in a metadata file). These data (vignettes + metadata file) are uploaded in the Ecotaxa web based application to identify each item (https://ecotaxa.obs-vlfr.fr/prj/6258; Picheral et al., 2017)

As no Ecotaxa dataset dedicated to stomach samples nor benthic organisms is available, a learning set was created by manually assigning preys of the first individual into 15 categories of prey items (Table II). Items of the four other individuals were then predicted using the resulting learning set. All vignettes assignment were then manually validated to ensure a correct identification of each item. Non-dietary items (gravel or detritus) and artefacts (bubbles) were identified and excluded from the analysis Diet was then expressed as a modified percentage of abundance, *i.e.* as the number of prey item of a category in a stomach divided by the total number of preys.

Accurately estimating the number of eggs was problematic. Due to the sticky nature of herring eggs, the number of eggs in a picture was variable. Some eggs occurred alone, whether because they actually occurred alone in the stomach or because they were manually scrapped before digitization (Fig. 2A). As separation of multiples (a zooprocess function)was not always possible whithout degradating the eggs shape, bunches of eggs remained and were counted as one, even if bunches of more than 10 eggs were digitized (Fig. 2B).
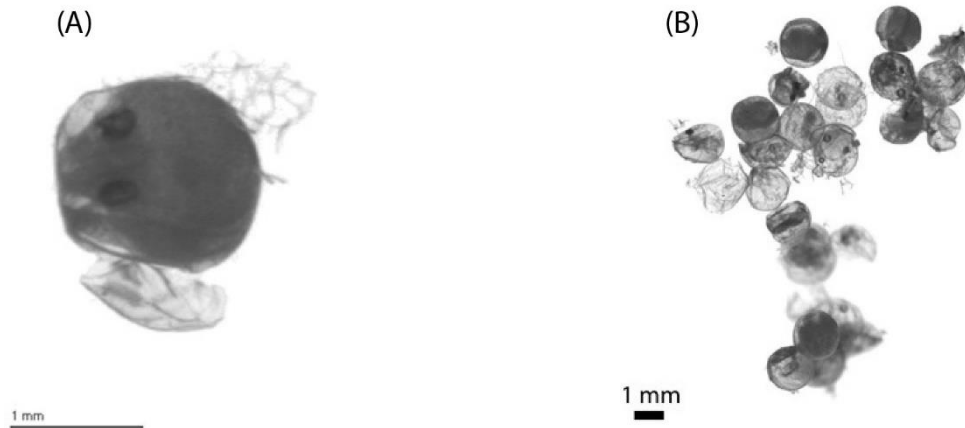
Figure 2. – Examples of vignettes picturing eggs, whether alone (A) or in bunches of numerous eggs (B)

The actual number of eggs consumed is thus underestimated by this method, but it remained consistent with the cautious approach of the routine protocol of the visual analysis of stomachs we have applied in previous works (Timmerman *et al*., 2020; Denamiel *et al*., 2021; Marchal *et al*., 2021). Namely, the protocol is designed to exclude overestimation of the preys, by considering the minimal number of individual preys only. Nevertheless, the effect of this potential bias was estimated by a manual counting of the number of eggs on each vignette.

An estimated Total Number of eggs was then calculated as follows

$$TN_{eggs} = \sum_{i=1}^{10} i \times n_{vignettes} \tag{3}$$

with i the number of eggs on the vignette and $n_{vignettes}$ the number of vignettes with this number of eggs. The maximum number of eggs was considered to be 10, due to the heterogeneity in the number of eggs when more that 10 occur in a vignette, and the difficultly to count the exact number of eggs in bunches of more than 10 eggs. This manual counting was also an opportunity to assess the level of degradation of the eggs. Each vignette was allocated to the category "intact" if all eggs on the vignette were intact, or to the category "degraded" if one egg at least was degraded.

Finally, two automated size measurement were recorded for each vignette, Feret diameter (*i.e.* the maximal distance between any two points along the object boundary) and Area (*i.e.* the surface area of the object). These two parameters were selected as representative of distance and area parameters, and were compared for classes of objects picturing the same number of eggs, to determine if these parameters could be used as proxies of the number of eggs, applying

linear or nonlinear (LOESS) regressions. Pictures displaying 10 or more eggs were excluded from this analysis due to their high variability.

RESULTS

All collected red mullets were females acquiring maturity (stage II) of similar length (~20 to 22 cm) and mass (120 to 140 g). Carbon isotopic ratios and C:N were largely variable. Difference between minimal and maximal values were of ~2.5‰ (–20.20‰ to –17.67‰) for raw δ^{13}C and of 1.6 for C:N (3.80 to 5.40). Variability was slightly reduced by lipid correction, as the range of corrected δ^{13}C values was of 1.69‰ (–17.22 to –18.91‰). Nitrogen isotopic ratios were less variable and ranged between 14.75‰ and 16.22‰.

Comparison between measured and literature values (Fig. 3) highlighted differences for δ^{13}C (ANOVA $F_{3,77}$ = 8.89, pvalue <10^{-3}) and C:N ratios (ANOVA $F_{3,77}$ = 8.89, pvalue <10^{-3}). Carbon isotopic ratios varied seasonally, with lower values in winter 2022 (–17.82 ± 0.74‰) and 2016 (–17.79 ± 0.59‰) than in autumn 2009 (–17.28 ± 0.54‰) and 2015 (–16.69 ± 0.20‰). C:N ratios were significantly higher in winter 2022 (4.42 ± 0.63) than in winter 2016 (3.76 ± 0.44) and autumn 2009 (3.47 ± 0.37). They were also higher than values measured in autumn 2015 (4.26 ± 0.68), but this difference was not significant. Differences between winter 2016, autumn 2009 and autumn 2015 were not significant either. Regarding δ^{15}N, the difference of ~1‰ between winter 2022 (δ^{15}N = 15.46 ± 0.64‰) and the other seasons (14.53 ± 0.88‰ in winter 2016, 14.77 ± 0.69‰ in autumn 2009 and 14.86 ± 0.14‰ in autumn 2015) was not significantly different (ANOVA $F_{3,77}$=1.96, pvalue = 0.13), potentially as a result of the low sample size.
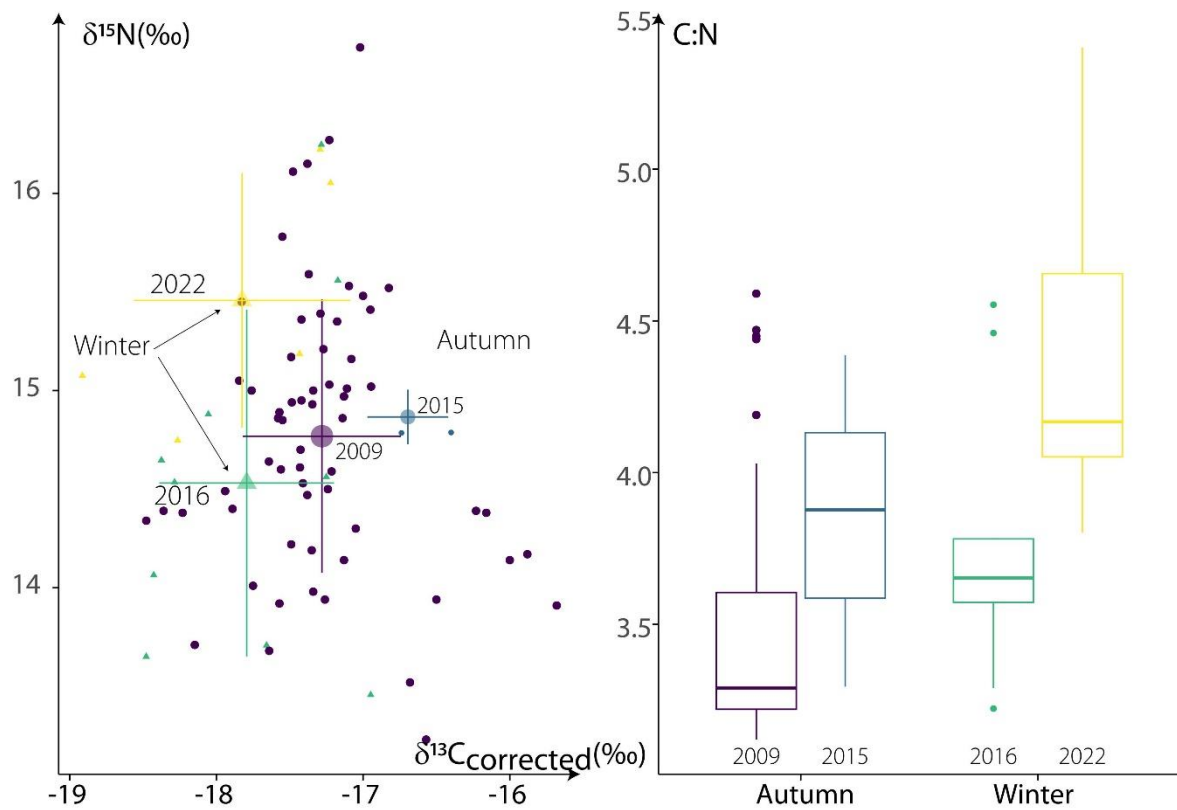
Figure 3. – Variation of isotopic (Lipid-corrected δ13C vs. δ15N, left plot) and C:N ratios (right plot). Individual isotopic ratios are represented by small symbols (triangles: winter, circles: autumn; purple: 2009, blue: 2015, green: 2016; yellow: 2022). Color-coding is consistent between plots.

Stomach contents revealed the predominance of eggs in the diet of the red mullet individuals sampled (Fig. 4). Herring eggs always represented more than 90% of the items (excluding objects with a non-living origin) recorded in the stomachs. Other preys were benthic invertebrates, mollusks and crustaceans, but with minor numerical importance when compared with eggs. A stomach without eggs was observed for one individual only, but the almost emptiness of this stomach (10 vignettes recorded) called for cautions regarding this stomach.
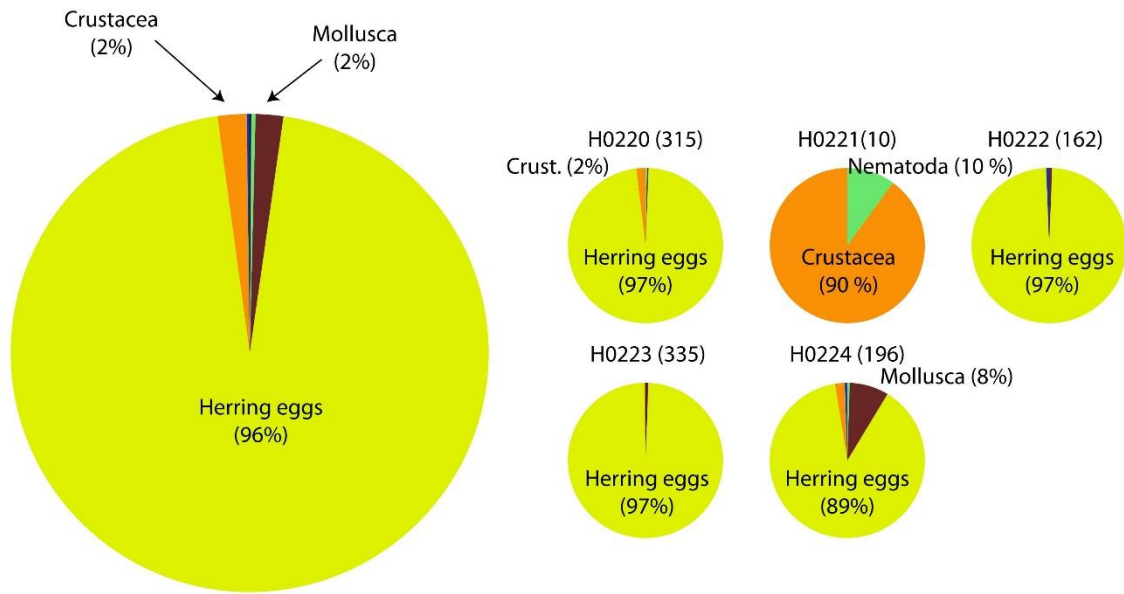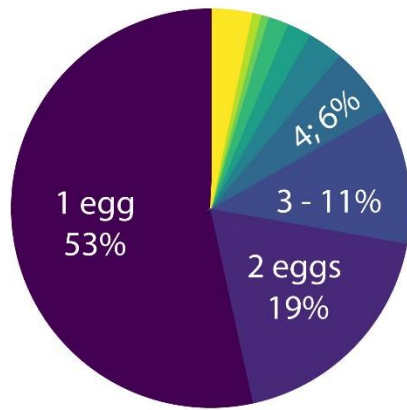
Figure 4. – Average (left plot) and individual (right plots) diets of the red mullets. Number of vignettes recorded for each individual is indicated between brackets after individual number

Most of the vignettes comprised one egg (Fig. 5A). Individual eggs, whether picturing intact or degraded, represented 531 out of the 971 vignettes corresponding to eggs (55%). Adding vignettes with two (190 vignettes, 20%) and three eggs (82; 8%) allowed reaching 83% of all vignettes. The calculation of the total number of eggs from visual observation of vignettes resulted in an estimated number of 2 204, *i.e.* more than twice the number of vignettes. The majority of vignettes (57%) displayed degraded eggs (Fig. 5B).
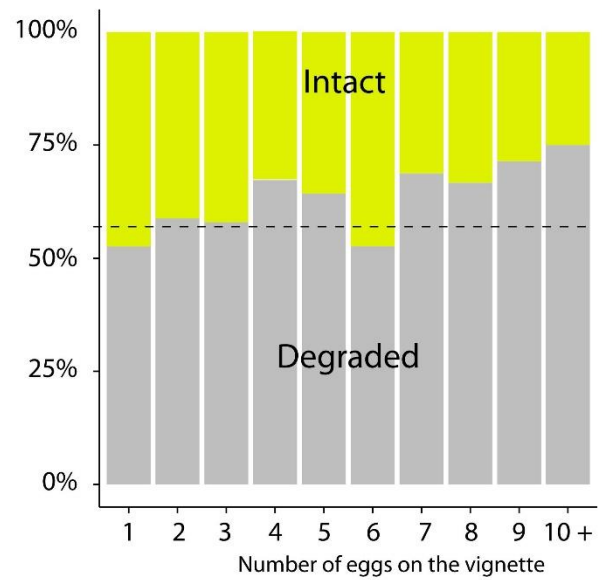
Figure 5. – (A) Number of eggs per vignette in proportion of the total number of vignettes corresponding to eggs and (B) proportion of vignettes picturing intact (in yellow) or degraded eggs (at least one in the vignette, in grey), depending on the number of eggs on the vignette. Horizontal dashed line represent the average percentage of vignettes classified as degraded (value).

Automated classification was powerful to detect and classify eggs: classification scores for eggs, whether intact or degraded, were higher (69.0 ± 14.0% and 61.0 ± 18.0 % respectively; Table II) than for all other dietary items (33 to 58%). Automated image analysis was also powerful to identify (and potentially exclude) non-dietary items (all classification scores higher than 77%).

Table II. – Prey categories, sorted by decreasing order of importance, and total number of vignettes attributed to each category after visual examination and manual classification. Classification score is the probability of an object to be part of a manually pre-established group in the learning set) attributed to the initial automated classification of each vignette from the four predicted samples, whether the classification was manually validated or attributed to another category afterwards. Items in the five final lines (artefacts, bubbles, detritus, gravel and fibres) have no trophic importance. They were consequently not considered in the dietary analyses and are presented here for informative purposes only.

	Category	Number of vignettes	Classification score
Dietary items	Herring eggs (intact)	421	69.0 ± 14.0 %
	Herring eggs (degraded)	550	61.0 ± 18.0 %
	Crustaceans (remains)	509	48.6 ± 17.1 %
	Mollusks (degraded)	95	58.2 ± 18.7 %
	Annelida (remains)	11	41.7 ± 17.2 %
	Galatheidea	7	33.7 ± 12.6 %
	Mysida	7	42.9 ± 19.0 %
	Crustaceans not identified	4	43.6 ± 14.2 %
	Nematoda	4	46.4 ± 17.1 %
	Amphipoda	2	43.4 ± 28.1 %
	Ophiuridea	1	33.1 %
Artefacts and detritus	Detritus not identified	4647	79.1 ± 16.7%
	Gravel	2541	86.0 ± 17.3 %
	Artefacts	1536	83.7 ± 18.2 %
	Fibers	623	81.4 ± 16.7 %
	Bubbles	17	77.6 ± 10.6 %

Objects Feret diameter and area were related with the number of eggs (n_{eggs}) but varied differently (Fig. 6). Area varied linearly with the number of eggs (Area (in $10^5 px^2$) = 0.05 + 0.19 n_{eggs}; adjusted R^2 = 0.67, pvalue $<10^{-3}$) throughout the range of value (1 to 9 eggs) tested. Feret diameter also varied linearly (Feret = 163.64 +78.52 n_{eggs}; adjusted R^2=0.63, pvalue $<10^{-3}$) but pattern was better described by a LOESS regression, as a result of the inflexion of the

Feret diameter for vignette picturing 6 eggs and more. As expected, values for vignettes picturing 10 eggs or more were heterogeneous and largely lied outside off the general pattern.
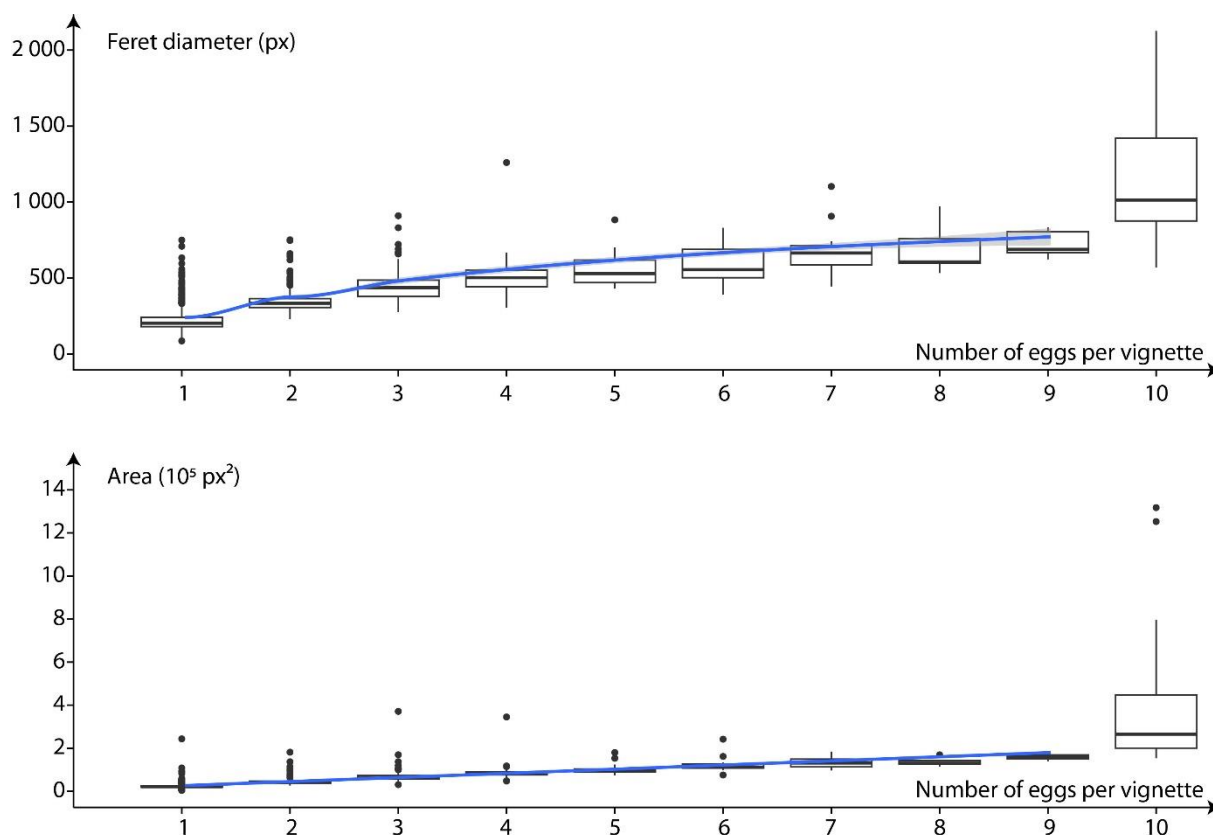

Figure 6: Automated size (Feret diameter, above panel) and area (below panel) parameters automatically measured by Ecotaxa web-based application on all vignettes picturing eggs. Blue lines represent nonlinear (LOESS, above panel) and linear (below panel) regressions

Strong correlations were observed between isotopic and C:N ratios and the numbers of eggs (Table III). Higher number of eggs was associated with lower $\delta^{13}C$ and $\delta^{15}N$ ratios (Spearman's $\rho = -0.69$), and higher C:N ratios ($\rho = 0.66$). Interestingly, correlation was stronger for raw $\delta^{13}C$ ($\rho = -0.81$) than lipid-corrected $\delta^{13}C$ ($\rho = -0.56$). Similarly, C:N ratios was more strongly correlated with raw $\delta^{13}C$ ($\rho = -0.70$) than with lipid-corrected $\delta^{13}C$ ($\rho = -0.12$), as a result of the lipid correction step. Finally, $\delta^{15}N$ was slightly correlated with fish length ($\rho = -0.49$) while all other descriptors were not correlated (ρ ranging between -0.01 and 0.26).

Table III: Spearman's ρ correlation coefficients between egg numbers, and isotopic and C:N ratios.

	Number of eggs	Raw $\delta^{13}C$	Lipid-corrected $\delta^{13}C$	$\delta^{15}N$	C:N	Length
Number of eggs	-					
Raw $\delta^{13}C$	-0.81	-				
Lipid-corrected $\delta^{13}C$	-0.56	0.79	-			
$\delta^{15}N$	-0.69	0.77	0.73	-		
C:N	0.66	-0.70	-0.12		-	
Length	0.26	-0.01	0.10	0.49	0.14	-

DISCUSSION

Despite the importance of herring spawning migration in the Eastern English Channel and the numerous lines of evidence supporting the trophic importance of fish eggs in marine ecosystems worldwide, the consumption of herring eggs was never observed nor demonstrated in the English Channel. This preliminary study was an opportunity to demonstrate for the first time that herring eggs could represent an important dietary item for a benthic fish locally. Combination of stomach content and stable isotopes demonstrate that eggs were consumed all along the herring spawning period. Automated image analysis could also provide a convenient tool to facilitate stomach content analysis but that further developments would be necessary, notably when accuracy is required.

On a technical point of view, there is a long history of papers discussing pros and cons of stomach content analysis. The question of the most appropriate and robust quantitative indicator of the diet is classical, along with solutions to limit the impacts of the time-consuming nature of this method (*e.g.* Hyslop, 1980; Baker *et al.*, 2014; Saikia, 2016; Amundsen and Sánchez-Hernández, 2019). In this context, present application of a method initially developed to analyse zooplankton samples to the diet of a benthic feeding fish was a powerful opportunity to add a new tool to the toolbox of trophic ecology, to test its limits and explore future developments requested to gain accuracy.

The major conclusion is that the automated analysis as it stands now provides a semi-quantitative vision of the diet of red mullets, as the exact number of eggs cannot be ascertained. This issue was similarly raised for DNA-based analysis of stomach contents, as the number of sequences cannot be related to the number of preys (Amundsen and Sánchez-Hernández, 2019). In the present case study, the predominance of eggs in the diet of red mullets diminished the importance of an accurate estimation of the exact number of eggs consumed. The result based on the number of vignettes or on the exact number of eggs would be similar and confirm the

trophic importance of eggs. In addition, occurrence was advocated as the most robust indicator of dietary patterns, as long as the sample size is sufficient (Hyslop, 1980). In these two cases, where the exact number of eggs, and more largely the exact number of all prey items, is not requested, automated analysis of stomach content could be viewed as a useful method to diminish the time requested to analyse stomachs. In addition, classification scores were good for eggs, even if the learning set is somehow limited. This may largely result from the fact that the shape of eggs is simple and very different from invertebrates. It also demonstrates that Zooscan and Ecotaxa routine can be powerful to identify eggs in the stomachs.

Nevertheless, manual *a posteriori* analysis of the vignettes revealed that the number of recorded vignettes was more than half the number of eggs. This underestimation could be problematic, depending on the research question. For example, estimating the carbon fluxes or energy inputs provided by eggs consumption would require further analysis of the images. Here, as the sample size was low, and as this work was aimed at testing the method, all vignettes were post-processed manually to count the number of eggs. This would no longer be possible in the future, notably if this process have to be applied to limit the treatment time of large samples, consistently with the initial purpose of Zooscan. Performing such a task would lower the interest of the automated method. Another option could be to use the tool implemented in ZooProcess to manually separate bunches of eggs (Grandremy *et al.*, 2023). Even if this would also be time-consuming and contradictory with the idea of automatizing analyses, this could be helpful for small bunches of eggs, but not for larger ones. However, the issue related with eggs sticking together was already observed and dedicated algorithms were developed to separate eggs in pictures (Duan *et al.*, 2019). Future implementation of such methods in Ecotaxa could be powerful to tackle this issue. More largely, the issue related with sticking object, and their impact on the results of the study is a well-known limitation of this approach. Vandromme *et al.* (2012) notably estimated that manually eliminating touching objects decreased the total biovolume of zooplankton of ~2 times, *i.e.* something similar to the number observed here for eggs. Another way to tackle this issue could be to estimate the average size of a vignette displaying one egg, automatically measured by EcoTaxa for each vignette (Grandremy *et al.*, 2023). This number could be then be used as a ratio between the size of vignette displaying multiple eggs and the number of eggs (Friedland *et al.*, 2005). Nevertheless, results of the preliminary analyses performed here demonstrated that the relationship is not straightforward and that further developments are also requested before being able to link directly size or area parameters measured on each vignette to the number of eggs.

This study is largely preliminary, due to the limited sample size and the peculiar trophic profile of red mullets, with stomach full of eggs. It is thus complex to generalize the methodological conclusion of this work to all case studies, but it opens new perspective. Similarly, it was not pertinent to deeply investigate the strengths and drawbacks of the method, *i.e.* by comparing outputs of the visual examination and of Ecotaxa, notably as detection efficiency may be taxon-specific. Future studies should thus be considered, with more individuals, and with different species belonging to different feeding guilds. Zooplankton-feeding species appear as the perfect next case study: zooplankton will be predominant in the stomach of these species, and this would allow using the existing learning sets of zooplankton. Further steps could also include other benthic invertebrate feeders, to continue exploring the trophic importance of herring eggs and to continue building a learning set dedicated to this habitat. Nevertheless, and consistently with the conclusion of Vogelmann *et al.* (2022), some technical limitations may be anticipated for both case studies. The effect of digestion and of the different digestibility of the preys could be viewed as the first caveat. In both ours and Vogelmann *et al.* (2022) studies, manual processing of the vignettes was required to correct some misclassifications. Thus, even if learning sets include many zooplankton vignettes, digested items may not be accurately identified. The application of Zooscan/Ecotaxa to benthic feeders will request new learning sets, and new preys categories, as benthic invertebrates are not the focus group of this tool. For both approaches, at this initial stage, experienced eye would be thus more performant that the automated analysis. However, the interest of a learning set lies in the number and the diversity of vignettes. The development of a large learning set through repeated analyses and confirmation would thus reveal powerful, not at the beginning but after some time.

More generally, in the current context of the implementation of Ecosystemic Approach for Fisheries globally, there is a demand for a better integration of environmental drivers and of biotic interactions, like predator-prey interactions, in processes of fisheries management. For example, analysis of stomach contents for exploited fish species has been integrated in the European Data Collection Framework up to 2022 (EC, 2021). However, this analysis will not be exhaustive, because of the time requested to do it. Combining several methods to analyse stomach contents, as the one presented here, along with other trophic descriptors like stable isotopes could be viewed as a solution to compensate pros and cons of each method and to increase the robustness of models used for management (Hernvann et al., 2022). The ability to estimate automatically the size of eggs is also supports the application this method, after further

methodological developments, as this parameter is crucial for ecosystem functioning and to parametrize models (Travers-Trolet et al., 2019), but complex to acquire.

On an ecological point of view, eggs were almost the unique dietary item observed in all non-empty stomachs analysed. As this sampling was largely based on an opportunistic sampling, and not on a planned sampling, the number of individuals observed is rather low and preclude from a formal conclusion. It is for example difficult to determine if the almost empty stomach found for one of the five individuals represents the natural variability of the feeding behaviour, or is an outlier of a pattern of general egg consumption.

Nevertheless, the timing of this sampling, in the late morning (10:40 GMT) was consistent with feeding rhythm of red mullet, as it is a diurnal feeder. Feeding activity of red mullet begins with the sunrise and is maximal in the morning (Mazzola et al., 1999; Onay and Dalgic, 2019). Sampling was thus coincidental with the feeding activity, and allowed the observation of eggs. The importance of degraded eggs is consistent with their fast digestion, and illustrate the importance of the coincidence between feeding and sampling. Such a coincidence was potentially missed in previous samplings (Timmerman et al., 2020).

The overwhelming presence of eggs in stomachs, along with isotopic and elemental ratios in muscle could testify of the trophic importance of herring eggs in red mullets in winter. It is interesting to note that results from instantaneous (stomach content) and integrated (stable isotope and C:N ratios) trophic tracers both testified the consumption of eggs. Isotopic framework consider that isotopic ratios of the diet request some months to be integrated in muscle, even if metabolic activity may alter this pattern (Guelinckx et al., 2007; Ballutaud et al., 2022). Mullets in the present study were caught in January. As herring begins spawning in November in the English Channel, mullets sampled in the present study may have been repeatedly feeding on eggs for the last 2 months before.

Nevertheless, this pattern is contradictory to all literature data, whether in the English Channel or other European seas, notably in the Mediterranean Sea and the Bay of Biscay. Comparison with literature should nevertheless be taken with caution, as no study occurred during period of massive spawning. Red mullets are considered to eat benthic invertebrates, mostly crustaceans or annelids (N'Da, 1992; Mazzola et al., 1999; Bautista-Vega et al., 2008; Derbal et al., 2010; Pinnegar, 2014; Giraldo et al., 2017). These preys were also observed here, but in very low numbers as eggs overwhelmed them. Trophic theory classically consider that feeding choices are driven by an optimization of the ratio between trophic inputs and handling costs. Consuming invertebrate preys require energy to catch, while eggs are highly energetic,

and easy to catch. They would have thus a major trophic interest for benthic feeders like red mullets, notably as they are laid in large and dense patches. By deploying artificial spawning units, Kotterba *et al.* (2014, 2017) estimated that the average density of herring eggs is of ~50,000 eggs m^{-2} but can reach more than 594,000 eggs m^{-2}. These authors also demonstrated that the higher the eggs density, the higher the feeding intensity of predators (threespine stickleback *Gasterosteus aculeatus* in their study). Similar patches in the English Channel could thus represent a massive trophic resource that allow avoiding usual preys, notably in a resource-poor season like the winter in the English Channel (Timmerman *et al.*, 2020).

Isotopic and elemental ratios also testify the consumption of eggs, even if this conclusion could be strengthen by analyses performed on a larger sample size. Lower $\delta^{13}C$ values measured in red mullets collected in winter 2016 and 2022 could be interpreted whether as the integration of more pelagic derived organic matter, and/or as the consumption of a diet richer in lipids. These two hypotheses are consistent with the consumption of herring eggs. Application of the lower $\delta^{13}C$ values of pelagic production to estimate relative importance of benthic and pelagic pathways is potentially one of the most usual applications of stable isotopes in marine ecology (Pethybridge *et al.*, 2018), and was powerful to demonstrate the connection between benthic and pelagic productions (Cresson *et al.*, 2020). Predation on herring, or on their eggs, would thus lead to very negative values, even for a benthic fish. The absence of major seasonal variation in $\delta^{13}C$ ratios measured in benthic invertebrates (Timmerman *et al.*, 2021; P. Cresson unpubl. results) also confirms that the variation observed here does not result from a difference in isotopic ratio of the baseline, but from an actual trophic difference. In addition, eggs exhibit lower $\delta^{13}C$ values than spawning females, because of their high lipid content. Lower $\delta^{13}C$ ratios in lipid rich tissues is usually interpreted as a result of the isotopic discrimination against ^{13}C during lipogenesis (Jardine *et al.*, 2005). Finally, even if C:N is less accurate than an actual measurement of lipid content, it is usually considered as an efficient proxy for lipid content, notably when coupled with isotopic analysis (Post *et al.*, 2007). Here, the markedly higher C:N ratios in winter 2022 and the correlations between C:N and raw and corrected $\delta^{13}C$ are also consistent with the consumption of lipid-rich eggs. C:N values observed for red mullet in the present study (4.42 ± 0.63) are markedly higher than values usually observed for the majority of fish species in the English Channel (3.1 to 3.2). They are more similar with values recorded for Atlantic horse mackerel *Trachurus trachurus* (3.92 ± 0.61), or jack mackerel *Scomber scombrus* (4.91 ± 1.79), species largely recognized as a being lipid-rich species (Cresson *et al.*, 2017, 2018, 2023). The consumption of eggs is thus largely consistent with lower $\delta^{13}C$ ratios, whether because of the use of a pelagic carbon, or the consumption of

lipid-richer preys. Similarly, the markedly higher $\delta^{15}N$ ratios for red mullets sampled in 2022 could also testify the integration of egg-derived organic matter. As reviewed by Jardine *et al.* (2005), oogenesis generated an increase of $\delta^{15}N$, with higher values in eggs measured just before spawning. It is thus reasonable to assume that laid eggs are N-enriched compared to adults. This may result from the final transfer of specific amino acids at the final stage of oogenesis and the associated isotopic effect (Jardine *et al.*, 2005; Fuiman and Faulk, 2013; Tanaka *et al.*, 2016). Thus, high $\delta^{15}N$ ratios in consumers was previously used as a proxy of eggs consumption (Vander Zanden *et al.*, 1998).

More largely, even if the number of red mullet sampled here is not sufficient to provide a robust conclusion about the trophic importance of herring eggs for the benthic community, such a conclusion appear plausible, and would be efficiently confirmed by further analyses on more red mullets, and on other benthic fish species. Nevertheless, as number of red mullets at all stations are important (Figure 1), one can expect that predation on herring eggs may affect survival of herring eggs, just as in other ecosystems (Kotterba *et al.*, 2014, 2017). The spatio-temporal variations of red mullet occurrence in the English Channel were previously demonstrated to be related to environmental parameters (Pinto *et al.*, 2019), but the ability to access a trophic resource like eggs could also be an important factor. In this sense, mapping the distribution of herring eggs, by example by underwater towed videos (*e.g.* Jac *et al.*, 2021) would be a crucial step towards a better understanding of the role of herring eggs.

As a conclusion, this opportunistic sampling of five red mullet provides some evidences that herring eggs are a major dietary item for a benthic fish species in the English Channel, even if the small sample size precludes from drawing general conclusions. It is nevertheless consistent with the general framework of an important predation on eggs. It is also a preliminary confirmation that herring has a forage role all through its life cycle. From a methodological point of view, this preliminary study demonstrated that automated image analysis allow the production of semi-quantitative indicators of fish diet, that can be sufficient by itself for some questions, but that some further development is requested when accuracy is needed. It also initiated the development of a learning set adapted to benthic fish species, which can be used in similar studies in the future. It finally demonstrated its efficiency to estimate size for small preys like eggs, one of the blind spots of stomach content analyses and despite the importance of size in predator-preys interactions. This study also open new questions regarding population dynamics and management of exploited fish species, whether being preys or predators, and confirmed the need for holistic approaches in fisheries ecology.

Acknowledgement

Author contribution

P. Cresson: Fieldwork, data treatment and conceptualization, writing original and final drafts of manuscript, data curation and visualization,

R. Cordier: Lab work, data treatment, draft review

A. Delegrange: Data treatment and conceptualization, writing final draft, draft review

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