Effect of diet composition and temperature on the gastric evacuation rate of European sardine: implication for egg predation estimates

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ABSTRACT: Cannibalism and intraguild predation of fish eggs are frequently observed for small and medium pelagic fish and can be an important cause of natural mortality. The European sardine *Sardina pilchardus* is the major predator of pelagic fish eggs in Atlantic Iberian waters. Estimates of egg mortality due to predation rely on a number of parameters that are frequently unknown for most species, such as gastric evacuation rate (GER) and feeding periodicity, and rates obtained for other fish species are generally used. These assumptions can severely affect egg mortality estimates. We conducted laboratory experiments to estimate the GER of European sardine that were fed diets composed of different proportions of fish eggs and other food types, such as *Artemia franciscana* nauplii, *Brachionus plicatilis* and microalgae, at different temperatures experienced by sardine in the wild (15, 17 and 21°C). Temperature, predator size and sex did not influence the gastric rates. Prey type significantly affected the GER of sardines, with slower evacuation when offered diets containing a higher concentration of fish eggs rather than microalgae, rotifers and *Artemia* nauplii. Fish eggs are more nutritionally rich compared to microalgae, rotifers and *Artemia* nauplii, and therefore more difficult to digest. GERs obtained here for *S. pilchardus* were similar to those obtained for *Sardinops sagax* in South Africa. This study provides important information that allows for improving estimates of daily feeding rates and egg mortality by predation, thus increasing our understanding of small pelagic fish population dynamics.

KEY WORDS: Gastric evacuation · Prey type · Fish eggs · Intraguild predation · *Sardina pilchardus*

1. INTRODUCTION

Predation on fish eggs by small pelagic fish is widely described for a variety of species, such as sardine, anchovy, chub mackerel, Atlantic chub mackerel, horse mackerel, Mediterranean horse mackerel, bogue and European sprat (Smith & Reay 1991, Irigoien & de Roos 2011, Bachiller et al. 2015, Garrido et al. 2015, reviewed by Garrido & van der Lingen 2014). Some of these species are known to prey on their own eggs, as described for anchovies (Hunter & Kimbrell 1980, Alheit 1987, Valdés et al. 1987, Gennotte et al. 2007, Pájaro et al. 2007) and sardines (Santander et al. 1983, Valdés-Szeinfeld 1991, Garrido et al. 2008, 2015, Fonseca et al. 2022). Intraguild predation can have a direct impact on several pelagic species. In sardine populations, cannibalism on eggs may be sufficient to cause fluctuations in annual class strength, affecting recruitment variability (Garrido & van der Lingen 2014). Indeed, a recent decline

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in the Iberian sardine population has been associated with trophic interactions and predation on their eggs, not only by sardines, but also by other competing species such as chub mackerel, horse mackerel and bogue (Szalaj et al. 2021).

The European sardine *Sardina pilchardus* is widely distributed throughout Northeast Atlantic waters, extending from Iceland and the North Sea to Senegal, and it is also common in the Mediterranean and Black Sea (Whitehead 1985). The main recruitment 'hotspots' for sardines are located along the northwestern coast of Morocco (with 2 main spawning areas: North of Dakhla and near Cintra Bay) (Ettahiri et al. 2003), followed by the Iberian Atlantic coast (Gulf of Cadiz and the North Portuguese coast) and the Bay of Biscay (Massé et al. 2018).

In laboratory experiments, European sardines were shown to preferentially select fish eggs over other zooplankton prey types, with high ingestion rates (Garrido et al. 2007). European sardines are one of the major consumers of fish eggs amongst small pelagic fish species, particularly in Ibero-Atlantic waters, where fish eggs are one of the most important prey items in terms of dietary carbon (Garrido et al. 2008, 2015, Fonseca et al. 2022). Sardine shoals off the Iberian coast ascend towards the surface at dawn and descend at night (Zwolinski et al. 2007), coinciding with the upward movement of positively buoyant eggs that were spawned at depth (Garrido & van der Lingen 2014). Sardine eggs are frequently found in sardine stomachs, particularly in winter months, at the peak of the spawning season. Cannibalistic behavior may be related to a density-dependent control mechanism within sardine populations (Valdés-Szeinfeld 1991) as a self-regulatory mechanism, used as a way to compensate for reduced food availability. The high frequency and abundance of fish eggs in sardine stomachs, including those of sardine and anchovy, means that egg mortality due to predation might be an important factor in egg loss, given the high number of juvenile and adult sardine individuals in the area compared to other fish species. Daily egg mortality due to predation is generally estimated by counting the number of eggs consumed in one day (daily ration) in relation to the total number of eggs produced by the population in one day. To be able to estimate the daily ration of a given predator based on stomach content composition, it is necessary to have estimates of the number of hours spent feeding per day and of the gastric evacuation rate (GER).

There is species-specific information that allows us to infer the number of hours per day that sardines are able to feed on fish eggs. Sardines are planktivorous and can use 2 feeding modes: filter feeding, which can be used all day for small prey, and particulate feeding (which is a visual feeding mode and is used for prey larger than 780 µm, such as fish eggs; Garrido et al. 2007), which is likely restricted to daylight hours. However, there are no species-specific estimates of GERs for the European sardine. For this reason, previous estimates on the potential impact of sardine predation on fish eggs have used GERs obtained for other species. For example, previous studies of the potential impact of sardine predation on anchovy and sardine eggs estimated that sardines may be responsible for approximately 71% of anchovy egg mortality in the northwestern Portuguese shelf during spring months and approximately 6% of sardine egg mortality in the southwestern Portuguese shelf during winter months (Fonseca et al. 2022). However, these estimates assumed a theoretical GER defined by Hunter & Kimbrell (1980) as 0.701 h^{-1} for the northern anchovy *Engraulis mordax*. In order to obtain accurate estimates, it is important to apply species-specific GERs in calculations of egg mortality.

Various studies have been conducted using different prey types, with factors such as prey type and size, meal size, predator size and temperature known to influence the GER for fish (Bromley 1994). Such studies provide important information on GER, daily rations and feeding rates, enabling the development of multispecies assessment models for the management of fish stocks. To determine the food intake and GERs of fish, various techniques have been employed (Bromley 1994). The 24 h fishery method can produce misleading results if fish with different feeding histories are sampled at different times of the day, while ship-board tank experiments have drawbacks related to stress associated with capture and transfer methods (Bernreuther et al. 2009). Controlled laboratory experiments are preferred, but only a few taxonomic groups have been well studied.

For robust fish species that can be maintained individually, gastric evacuation experiments under controlled laboratory conditions are standard practice, with a large number of studies carried out on gadids (e.g. Bromley 1988, 1991, dos Santos & Jobling 1988, 1995, Temming & Andersen 1992, 1994, Andersen 1998, 1999, Anderson & Beyer 2005a,b, 2008a,b, Couturier et al. 2013, Anderson et al. 2016). In other studies, with piscivorous species such as mackerel, several fish per tank are used in order to allow them to form shoals (e.g. Temming & Herrmann 2001a,b, Temming et al. 2002). Experiments on clupeoids are rare, not only because they need to be conducted

using groups of fish but also due to their sensitivity to handling.

For small pelagic fish, several laboratory experiments were conducted to study GERs in juvenile herring *Clupea harengus*, juvenile sprat *Sprattus sprattus* and adult Benguela sardine (Bernreuther et al. 2008, 2009, van der Lingen 1998). For juvenile herring fed with copepods (frozen, natural and stained) in short- and long-term feeding experiments, a positive effect of temperature (13 and 16°C) on the evacuation rate, described by an exponential evacuation model, was found (Bernreuther et al. 2008). For sprat fed with brine shrimp (*Artemia* sp. nauplii), gastric evacuation was described by a general gastric evacuation model, determining an evacuation constant (*R*) which increased exponentially with temperature from 7.5 to 19°C, above which there was a slight decrease (up to 21.5°C). However, no significant effect of temperature on evacuation rates of sardine was found (van der Lingen 1998). For the southern Benguela sardine, experiments assessed feeding periodicity and estimated the daily ration of sardines fed with natural plankton (van der Lingen 1998), revealing a marked effect of food type on evacuation rates, where microalgae were evacuated 2–5 times faster than zooplankton (van der Lingen 1998).

In this study, we aimed to determine the GERs of the European sardine for different meal sizes with different proportions of prey items. The species-specific GERs obtained in this work can be used to improve estimates of egg mortality by predation for sardine throughout the Atlanto-Iberian waters.

2. MATERIALS AND METHODS

2.1. Gastric evacuation experiments

European sardine *Sardina pilchardus* adults were captured off southern Portugal and kept in captivity in a 9000 l tank at an average (±SD) temperature of 16.9 ± 2.26°C for approximately 16 mo prior to the experiments. A total of 18 sardines were introduced into each of the 9 experimental tanks (1500 l fiberglass tanks) with a continuous flow of seawater and controlled temperature, where they underwent a 2 wk acclimation period to the tanks before the start of the experiments. Three different temperatures were tested (15, 17 and 21°C), and for each temperature, 3 different diets were used. Diets included fish eggs (*Sparus aurata* and *Argyrosomus regius* eggs), brine shrimp (*Artemia franciscana* nauplii), rotifers (*Brachionus plicatilis*) and microalgae (*Nannochloropsis*

oculata and *Tisochrysis lutea*) in different proportions (see Table 1). Diets had approximately the same total weight of food organisms but with different proportions of eggs to other prey. Diet 1 had a proportion of 25% of fish eggs, Diet 2 had 70% of fish eggs, and Diet 3 had 5% of fish eggs. The biomass of the different prey in each of the 9 tanks was calculated based on the carbon content of each prey, using estimates from the equations of Garrido & van der Lingen (2014) based on prey size. The experimental design focused on varying natural conditions that included temperature, the amount of eggs in the stomachs and the availability of other prey in the environment, to obtain the GER of the sardines. Previous studies have shown that fish eggs are a main prey item for sardines but were never consumed as the sole prey. There is high variability in egg consumption and it seems to depend on food availability (Garrido et al. 2008).

Fish were not fed prior to the experiments for a period of 48 h to allow a complete emptying of the stomachs. At the beginning of the experiments, the water inflow of the tanks was turned off and adjusted to a volume of 1000 l. Then, food was introduced into the 9 tanks according to the concentrations of the 3 diets (see Table 1); the introduction of food corresponded to time *t* = 0 h of the experiment. A porous air tube was used for aeration and to ensure homogeneous distribution of the food in the tank. Water renewal was restored after 30 min to eliminate any remaining food in the tanks, and 30 min after, samples were taken to confirm that the tanks no longer contained food.

Water samples were taken at the beginning of the experiment to quantify the decrease in the percentage of food in the tanks after prey introduction and then to verify that the tanks had no prey remaining at *t* = 1 h. Three water samples were collected with 200 ml flasks for counting microalgae after the introduction of the food (*t* = 0 h). Three water samples were collected with a PVC tube that sampled the entire water column with a 45 μm net and a volume of 1.3 l to count fish eggs and zooplankton at times *t* = 0, 1, and 2 h.

At times 1, 2, 3, 4, 5 and 7 h after the feeding, 3 individuals were removed from each tank, anaesthetized (with a solution of MS-222) and sacrificed with a cut to the anterior spine. For each fish sacrificed, total length (cm), total and gutted weight (g), gonad weight (g), sex, and maturity stage were determined. In total, 162 sardines were sacrificed at the end of the experiment.

In the laboratory, the stomachs were defrosted and weighed, and then the stomach contents were re-

moved and weighed. Only the contents inside the eso-phagus and the stomach until the junction of the pyloric stomach were analyzed. All weight measurements were made to the nearest 0.01 mg. Stomach contents were filtered through a 45 µm sieve, resulting in 2 samples. Those that were <45 µm, equivalent to phytoplankton, were identified using an inverted microscope Zeiss Axioplan 2 (magnification 400×) with AxioVision Software and counted with a Neubauer chamber. The >45 µm fraction, corresponding to zooplankton and fish eggs, were identified using a stereomicroscope Leica S APO (magnification 80×) with Leica Application Suite (LAS X 3.0) software and were counted using a Bogorov chamber.

2.2. Data analysis

Estimates of the degree of curvilinearity of the gastric evacuation curve from experimental results are preferably performed using a mathematical model, not defining *a priori* the parameter that describes the degree of curvilinearity (Temming & Anderson 1994). The gastric evacuation model applied in this study had previously been given by Temming & Andersen (1994) and is flexible regarding the shape of the evacuation curve. The model is defined as:

$$S_t = \left[S_0^{(1-B)} - R \times (1-B) \times t \right]^{\left(\frac{1}{1-B} \right)} \tag{1}$$

where S_t is residual stomach contents at time t, t is time after ingestion, S_0 is initial meal size, B is a shape parameter and R is a gastric evacuation constant (g^{1-B} h^{-1}) that is dependent on temperature, food type, predator weight and other factors. The model may include linear, convex, exponential and intermediate curve types, with the degree of curvilinearity being defined by the value of B and with the degree of concave curvilinearity increasing with B (Temming & Andersen 1994). If $B < 0$, then it is a curvilinear model with convex curve with increasing negative slope; $B = 0$ is a linear model with a negative slope; $0 < B < 1$ is a curvilinear model with concave curve with decreasing negative slope; $B = 1$ is a curvilinear model with an exponential decay curve; $B > 1$ is a curvilinear model with a concave curve, and the dependence of evacuation rate on the stomach content is stronger than in the exponential case.

With $B = 1$, the integrated form of the general equation (Eq. 1) results in an exponential function to determine the constant R (h^{-1}):

$$S_t = S_0 \, e^{-Rt} \tag{2}$$

In order to estimate evacuation curves for a specific prey species inside the stomach content of sardines, we adapted the general equation:

$$P_t = \left[P_0^{(1-B)} - R \times (1-B) \times t \right]^{\left(\frac{1}{1-B} \right)} \tag{3}$$

Eq. (3) is an adaptation of Eq. (1), in which the values are expressed by number of prey instead of by weight (g), and where meal sizes are expressed by number of prey items instead of dry weight of the prey. P_t is the residual number of prey inside the stomach at time t and P_0 is the initial number of prey. The gastric evacuation constant R is described in units of $prey^{(1-B)}$ h^{-1}. If $B = 1$, an exponential function similar to Eq. (2) is applied to determine R (h^{-1}):

$$P_t = P_0 \, e^{-Rt} \tag{4}$$

The general and exponential models, defined by Eqs. (1) & (2), were fitted to untransformed data by means of non-linear regression with a Levenberg-Marquardt algorithm, and the models defined by Eqs. (3) & (4) were fitted by a non-linear regression with a sequential quadratic programming algorithm. All non-linear regressions were performed in SPSS version 25 statistical software.

The homogeneity of variances of the data was tested using Levene's test with multiple independent variables, and the normality of the data was checked using the Shapiro-Wilk test. Non-parametric Kruskal-Wallis test was applied to determine the statistical significance of the different independent variables in the model. Subsequently, to perform multiple pairwise comparisons, Dunn's test with Bonferroni adjustment was done. Significance tests were done using the open-source software R version 4.1.2 (R Core Team 2021). The package 'car' (Fox & Weisberg 2019) was used for homogeneity tests and the package 'rstatix' (Kassambara 2022) was used for normality tests, non-parametric tests and respective post hoc tests. A significance level of 0.05 was considered for all statistical analyses.

For correcting sardine egg predation estimates using the new species-specific GER data, the average number of eggs eaten per kilogram of sardine per day was estimated using the equation proposed by Hunter & Kimbrell (1980):

$$C = EE \times R \times t \tag{5}$$

where C is the mean number of eggs eaten per kilogram of fish during time t, EE is the mean number of eggs observed per kilogram of fish mass, R is the

GER (h^{-1}) for eggs and t is the duration of feeding (h). Estimates of daily egg production are described in Fonseca et al. (2022).

3. RESULTS

3.1. Variable selection

Shapiro-Wilk tests of the variation in stomach content weight over time revealed that the data was not normally distributed (p < 0.05). For this reason, non-parametric tests (Kruskal-Wallis) were conducted to determine the significance of temperature, diet type, sardine length class and sex in the GER model.

A total of 162 adult sardines were used in the experiments, of which 129 were females and 33 were males, resulting in a sex ratio of 4:1 (F:M). The Kruskal-Wallis test revealed no significant differences in the variation of the weight of sardine stomach contents over time between sexes (p = 0.615).

The total length of the sardines used in the experiments ranged from 18.40 to 22.90 cm (mean ± SD: 20.61 ± 0.95 cm; Table 1). Kruskal-Wallis test revealed no significant differences in the variation of the weight of sardine stomach contents over time between individuals of different length classes (p = 0.508).

Three different temperatures (15, 17, 21°C) were tested during the experiment. When analyzing the influence of temperature over time, no significant differences were observed in the variation of the weight of sardine stomach contents between the different temperatures tested (Kruskal-Wallis test, p = 0.692).

Three different diets with different proportions of fish eggs, *Artemia* nauplii, rotifers and microalgae were tested (Table 1). Statistically significant differences

were found in the variation of the weight of sardine stomach contents between the 3 diets (p < 0.001). Pairwise comparisons, adjusted by Bonferroni correction, revealed significant differences between Diets 1 and 3 (p < 0.001) and between Diets 2 and 3 (p < 0.001).

3.2. Univariable gastric evacuation model

The variables sex, length and temperature were not included in the gastric evacuation model due to the lack of statistical significance (see previous subsection).

Results of the parameter estimates for the general model (Eq. 1) of the 3 distinct diets (Table 2) show that the curvilinearity of the model is more intense for Diet 1 ($B = 0.523$), while Diets 2 and 3 approach linear models. Diet 2, despite presenting a model with slight concavity, approaches a linear model ($B = -0.168$), and Diet 3 presents a slightly more curvilinear model with a small increase in negative slope ($B = -0.210$). However, the 95% confidence limits of parameter B are quite large, considering the estimates of B for each of the diets. For Diet 1, the minimum limit is −1.565, and the maximum limit is 2.611. For Diet 2, it ranges from −4.278 to 3.941, and for Diet 3, it ranges from −1.971 to 1.550 (Table 2).

The parameter R, which corresponds to the gastric evacuation constant according to Eq. (1), depends exclusively on the type of diet used in each model. The model for Diet 1 had a higher value ($R = 0.239 \ g^{1-B} \ h^{-1}$), while Diet 2, which had the higher proportion of fish eggs, had $R = 0.168 \ g^{1-B} \ h^{-1}$ and Diet 3 (with the lower proportion of fish eggs) had $R = 0.071 \ g^{1-B} \ h^{-1}$ (Table 2, Fig. 1). The exponential model (Eq. 2), which considers $B = 1$, shows less difference in R be-

Table 1. Experimental tanks used with European sardine, showing respective diet, temperature, average (±SD) fish total length (TL) and average (±SD) fish body weight. Meal composition is given by dry weight of the 4 prey types used (microalgae, rotifers, *Artemia* sp. nauplii and fish eggs). Meal size is given by wet weight (WW) and percentage of body weight (BW)

Tank	Diet	Temperature (°C)	TL (cm)	Weight (g)	Algae	Rotifers	*Artemia*	Eggs	WW (g)	% BW
						Dry weight of prey (g)			Meal size	
1	1	15	21.0 ± 0.7	79.9 ± 12.6	0.07	0.27	0.37	1.68	5.96	7.46
2	2	15	20.5 ± 1.1	77.3 ± 10.5	0.08	0.34	0.46	2.10	12.31	15.92
3	3	15	21.4 ± 1.0	83.2 ± 16.0	0.08	0.34	0.46	2.10	9.47	11.38
4	1	17	20.2 ± 1.1	72.7 ± 14.0	0.01	0.09	0.12	4.70	7.44	10.23
5	2	17	20.7 ± 0.9	76.6 ± 17.0	0.01	0.11	0.15	5.88	15.38	20.08
6	3	17	20.7 ± 1.0	79.4 ± 16.2	0.001	0.11	0.15	5.88	11.83	14.90
7	1	21	20.3 ± 0.8	69.5 ± 11.8	0.05	1.43	1.97	0.34	7.45	10.73
8	2	21	20.4 ± 0.5	73.7 ± 10.1	0.06	1.79	2.46	0.42	15.37	20.85
9	3	21	20.4 ± 0.7	73.0 ± 10.0	0.07	1.79	2.46	0.42	11.84	16.21

Table 2. Results of gastric evacuation rate for European sardine, using the non-linear regressions of stomach content weight (g) with the general equation (Eq. 1, constant R units: g^{1-B} h^{-1}) and the exponential equation (Eq. 2, constant R units: h^{-1}). See Section 2.2 for explanation of variables. The models were applied to the 3 different diets (Diet 2: +eggs; Diet 3: −eggs; Diet 1: ±eggs) as well as a single model (with all 9 tanks)

Model	Tanks	Diet	SS regress.	SS residual	r^2	B estim.	B 95% conf. limit Lower	Upper	R Estim.	R 95% conf. limit Lower	Upper	S_0 Estim.	S_0 95% conf. limit Lower	Upper
General	1, 4, 7	1	75.188	29.056	0.285	0.523	−1.565	2.611	0.239	0.119	0.359	2.121	1.202	3.039
	2, 5, 8	2	97.253	19.429	0.222	−0.168	−4.278	3.941	0.168	0.010	0.326	1.880	1.306	2.453
	3, 6, 9	3	10.611	4.427	0.275	−0.210	−1.971	1.550	0.071	−0.072	0.214	0.711	0.463	0.959
	−	−	157.109	78.855	0.173	0.169	−1.714	2.052	0.166	0.090	0.243	1.553	1.150	1.955
Exponential	1, 4, 7	1	75.074	29.170	0.282	−	−	−	0.228	0.108	0.348	2.288	1.547	3.029
	2, 5, 8	2	97.084	19.598	0.215	−	−	−	0.125	0.052	0.198	2.004	1.525	2.484
	3, 6, 9	3	10.468	4.570	0.251	−	−	−	0.210	0.087	0.333	0.819	0.538	1.101
	−	−	156.710	79.255	0.168	−	−	−	0.177	0.107	0.247	1.688	1.335	2.040

tween diets. The values of R significantly increase for Diet 3 and slightly decrease for Diet 2 (Table 2).

The sample size for each of the 3 diets was 54 observations, with the general models explaining 29% of the variance for Diet 1, 22% for Diet 2, and 28% for Diet 3 (Table 2). The exponential models explained similar percentages of the variance, with 28% for Diet 1, 22% for Diet 2 and 25% for Diet 3 (Table 2).

3.3. Adapted gastric evacuation model: prey count as a proxy for meal size

Gastric evacuation of fish eggs was expressed as the number of eggs instead of egg weight, using Eq. (3). The constant R (gastric evacuation constant) registered high values for diets with very low numbers of eggs in the initial meal size; with an increase in fish eggs in the diet, the R values decrease. The diet with the low proportion of eggs (Diet 3) had $R = 0.570$ prey$^{(1-B)}$ h^{-1}; Diet 1, with an intermediate proportion of eggs, had $R = 0.257$ prey$^{(1-B)}$ h^{-1}; and Diet 2, in which the proportion of eggs was higher, had $R = 0.295$ prey$^{(1-B)}$ h^{-1}.

The curvilinearity of the model is determined by parameter B. According to the model, an exponential curve was observed in the gastric evacuation of fish eggs in Diet 1 ($B = 1$), while curvilinear models tended towards exponential curves with a decreasing negative slope for Diet 2 ($B = 0.805$) and Diet 3 ($B = $

0.789) (Table 3). However, the 95% confidence limits for the models with Diet 2 and Diet 3 were considerably large, with minimum and maximum limits deviating significantly from the estimated values for parameters B and R. Based on the low r^2 values obtained, the models explained 39% of the variance in data for Diet 1, 16% for Diet 2 and 31% for Diet 3 (Table 3, Fig. 2).

The exponential model (Eq. 4) that considers $B = 1$ shows significantly lower values of R for Diets 2 and 3 (Table 3). However, with the exponential model, the
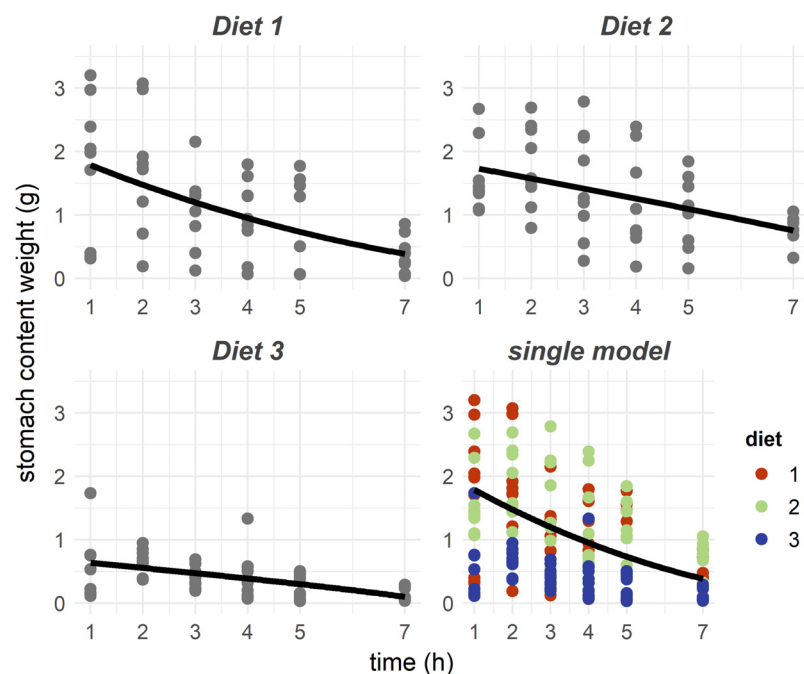

Fig. 1. Non-linear regression of the European sardine gastric evacuation models. Curves were calculated using the general equation (Eq. 1). Three of the plots illustrate the individual diets (Diet 2: +eggs; Diet 3: −eggs; Diet 1: ±eggs) and one plot shows the single model (with all 9 tanks)

Table 3. Results of the non-linear regressions using the general equation adapted to number of prey inside European sardine stomachs (Eq. 3, constant R units: prey$^{(1-B)}$ h^{-1}) and the exponential equation adapted to the number of prey items inside stomachs (Eq. 4, constant R units: h^{-1}). See Section 2.2 for explanation of variables. The models were only applied to fish eggs as prey inside stomachs. The models were applied to the 3 different diets (Diet 2: +eggs; Diet 3: −eggs; Diet 1: ±eggs) as well as a single model (with all 9 tanks)

Model	Tanks	Diet	r^2	B Estim.	B 95% conf. limit Lower	Upper	R Estim.	R 95% conf. limit Lower	Upper	P_0 Estim.	P_0 95% conf. limit Lower	Upper
General	1, 4, 7	1	0.393	1.000	0.848	1.152	0.257	−0.041	0.556	10436	6858	14014
	2, 5, 8	2	0.159	0.805	−18.906	20.516	0.295	−48.509	49.099	5487	3268	7705
	3, 6, 9	3	0.309	0.789	−5.348	6.927	0.570	−25.297	26.437	2479	1309	3650
	−	−	0.211	0.825	−1.049	2.700	0.767	−11.024	12.559	6623	4697	8548
Exponential	1, 4, 7	1	0.393	−	−	−	0.257	0.153	0.362	10437	7637	13236
	2, 5, 8	2	0.224	−	−	−	0.111	0.051	0.171	6881	5485	8277
	3, 6, 9	3	0.372	−	−	−	0.189	0.106	0.272	3328	2523	4133
	−	−	0.210	−	−	−	0.183	0.121	0.245	6766	5532	7999

confidence limits of the constant R have narrowed significantly for the 3 diets. The r^2 value also increased and the exponential models explained 22% of the variance of the data in Diet 2 and 37% in Diet 3. The GER adjusted to estimates of egg mortality by predation was defined as 0.183 h^{-1}. This value of GER is equivalent to the R constant of the exponential model applied to all 9 tanks together (Table 3).

Similar to the exponential model for fish eggs, an exponential model was adjusted separately to the gastric evacuation of the 4 prey types used (fish eggs, *Artemia* nauplii, rotifers and microalgae) in order to describe the evacuation for each prey (Table 4). Instead of number of prey items, the data was analyzed by carbon content (μg) of each prey type, given the large size range of prey types used. Generally, gastric evacuation was higher for *Artemia* spp., followed by fish eggs and rotifers, and lower for microalgae (Fig. 3). Sardines that were fed with Diet 3 (lowest egg concentration) had the highest intake of *Artemia* nauplii, rotifers and microalgae. Sardines that were fed with Diet 2 (highest egg concentration) had the lowest intake of *Artemia* nauplii, rotifers and microalgae (Fig. 3).

3.4. Egg mortality by sardine predation

Previous estimates of egg mortality due to sardine predation off the Iberian coast during different seasons and areas

(Garrido et al. 2008, Fonseca et al. 2022) have used the GER estimated for the northern anchovy *Engraulis mordax* (−0.701 h^{-1}; Hunter & Kimbrell 1980). With the new species-specific GER value obtained in this work, estimates of egg mortality by sardine predation in Garrido et al. (2008) and Fonseca et al. (2022) were recalculated; egg mortality using the diet composition of sardine eggs described in Garrido et al. (2015) was estimated and results were compiled in Table 5.

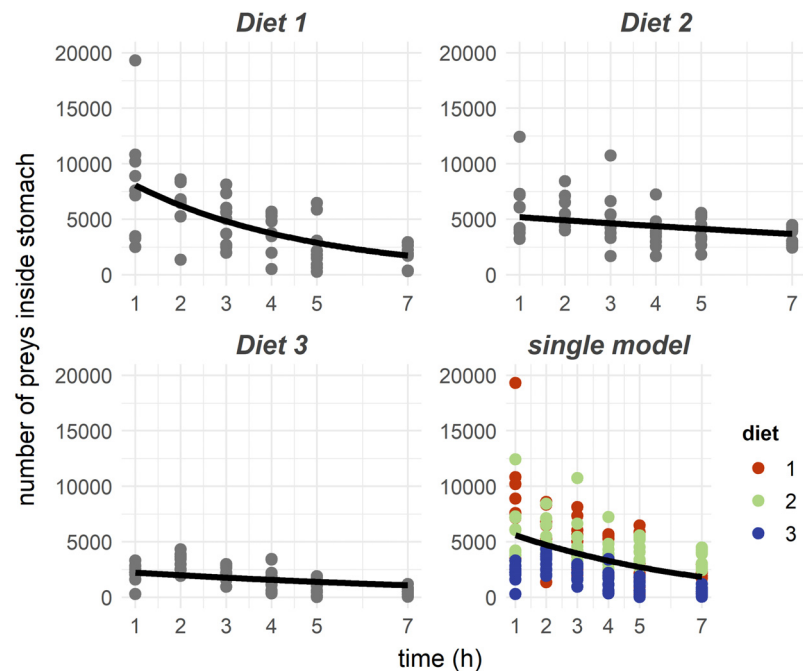Using the species-specific GER for fish eggs (0.183 h^{-1}) obtained in the current work, estimates

Fig. 2. Same as Fig. 1, but with curves calculated using the general equation adapted to number of prey items inside the stomachs (Eq. 3). The models were only applied to fish eggs as prey inside stomachs

Table 4. Results of the non-linear regressions of gastric evacuation rate (prey carbon content per hour) of European sardines using the exponential equation. Data of the models is divided by the 4 prey types fed to sardines. The models were applied to the 3 different diets (Diet 2: +eggs; Diet 3: −eggs; Diet 1: ±eggs)

Prey type	Diet	r^2	R Estim.	R 95% conf. limit		P_0 Estim.	P_0 95% conf. limit	
				Lower	Upper		Lower	Upper
Fish eggs	1	0.393	0.257	0.153	0.362	125238	91642	158835
	2	0.224	0.111	0.051	0.171	82568	65816	99320
	3	0.372	0.189	0.106	0.272	39935	30277	49594
Artemia nauplii	1	0.358	1.000	0.259	1.741	1682	147	3217
	2	0.224	0.621	0.092	1.150	81	13	148
	3	0.261	1.000	0.075	1.925	15059	2107	32225
Rotifers	1	0.271	0.500	0.188	0.811	74	33	116
	2	0.337	0.293	0.150	0.436	6	4	8
	3	0.109	0.135	0.013	0.256	1004	611	1397
Microalgae	1	0.633	0.367	0.267	0.468	575	451	698
	2	0.455	0.216	0.137	0.295	229	179	278
	3	0.227	0.201	0.078	0.323	680	442	917

of egg mortality per day by sardine predation determined by Garrido et al. (2008) for sardines collected throughout 1.5 yr in Western and Southern Iberia decrease from 30% to 8%. According to estimates of egg consumption by sardines collected off the western and southern Iberian coast in 2014 (Garrido et al. 2015), egg mortality would be calculated as 6% on Western Iberia coast and 16% on the Southern Iberia coast.

Recalculating the estimations of Fonseca et al. (2022), sardine egg mortality by cannibalism per day decreases from 6% to 2%. When considering the mortality of anchovy eggs per day by sardine predation during spring, estimates decreased from 71% to 19% in Northwest Iberia and from 13% to 3% in South Iberia (Table 5).

4. DISCUSSION

4.1. Limitations of the experimental design

It is important to address certain aspects of this study that may have influenced the outcomes and interpretation of the results. Firstly, it would have been desirable to have replicate tanks to draw more robust conclusions. However, due to logistical constraints, it was not feasible to have 9 tanks housing adult fish simultaneously, lim-

iting the replication of the experimental setup. Logistic constraints also limited the duration of the experiments to 7 h; however, it would have been desirable to extend the period to more accurately describe the digestion curve. Fish in captivity may not exhibit normal feeding behavior when subjected to stress, and force-feeding practices can further disrupt their feeding patterns, potentially leading to an underestimation of GERs (Bromley 1994). For this reason, sar-
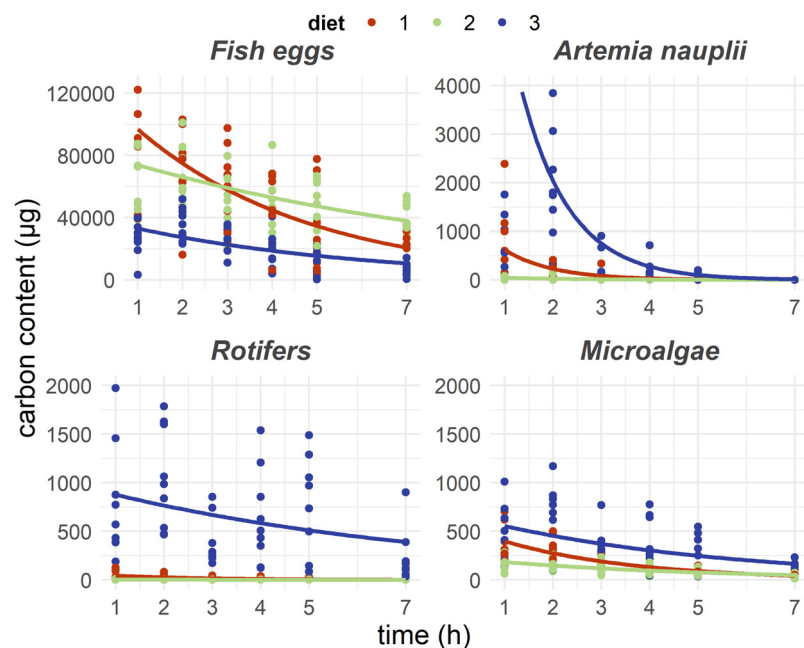

Fig. 3. Non-linear regression of the European sardine gastric evacuation models for the 4 prey types. Curves were calculated using the exponential equation with the prey carbon content instead of number of prey. Diet 2: +eggs; Diet 3: −eggs; Diet 1: ±eggs. Note that the *y*-axes have different scales

Table 5. Compilation of the results from different studies on egg mortality by predation of *Sardina pilchardus*. The last column shows the results of recalculating egg mortality by predation of sardines using the gastric evacuation rate (GER) determined in this study. EE: mean number of eggs observed per kilogram of fish mass; *C*: mean number of eggs per kilogram of fish during the time of feeding; BF: batch fecundity; SF: spawning fraction; SO: spatial overlap

Source	Location	Egg species	GER (h^{-1})	Feeding duration (h)	EE	*C*	BF (no. of eggs)	SF (%)	Sex ratio	SO (%)	Eggs consumed per day (%)	Eggs consumed per day (with new GER) (%)
Garrido et al. (2008)	Portuguese continental shelf	*S. pilchardus*	0.701	9	688	4344	14255	10	0.61	–	30	8
Garrido et al. (2015)	Western Iberia	*S. pilchardus*	0.211	13	493	1754	21322	7.5	0.505	–	–	6
Garrido et al. (2015)	Southern Iberia	*S. pilchardus*	0.211	13	1510	5368	22673	8.0	0.602	–	–	16
Fonseca et al. (2022)	Northwest Portuguese shelf	*E. encrasicolus*	0.701	13	279	2546	7502	23.4	0.53	21	71	19
Fonseca et al. (2022)	Algarve and Cadiz region	*E. encrasicolus*	0.701	13	3	29	7502	23.4	0.53	44	13	3
Fonseca et al. (2022)	Southwest Portuguese shelf	*S. pilchardus*	0.701	10	6	43	20698	11.5	0.609	78	6	2

dines underwent an acclimation period of 2 wk in the experimental tanks, with constant temperature, salinity and photoperiod. Careful handling and transfer techniques were applied, and feeding and removal of food were conducted without major disturbance. Overall, no abnormal behaviors were observed for sardines either during the feeding phase or food removal, indicating that a minimal level of stress was experienced by the fish throughout the experiment. Similar techniques were employed by Garrido et al. (2007) in their study on the feeding behavior of sardines, yielding promising results in terms of minimizing stress in the observed fish.

One of the main objectives of the present work was to obtain gastric evacuation data for sardine eggs, to assess the impact of egg cannibalism on egg mortality of the European sardine. Although sardine eggs were unavailable during the experimental period, we used live eggs of 2 species reared in the aquaculture facilities (*Sparus aurata* and *Argyrosomus regius*) that are similar to sardine eggs, being pelagic, floating near the surface of the water and having similar although slightly smaller size and weight.

4.2. Temperature, predator size and sex effect on gastric evacuation

Our results show that only the type of diet, characterized by different meal sizes, had a significant influence on the GER. Temperature did not significantly influence GER. In the experiments, we tested for 3 temperatures (15, 17 and 21°C) that replicate the natural conditions experienced by sardines in Atlantic Iberian waters during fall, winter and spring months, when most coastal pelagic fish in the area spawn. By testing only temperatures within the species' optimum range for survival, the sardines may not have experienced any stress or pressure that could have affected their normal digestive functioning. Several studies have explored the relationship between temperature and gastric evacuation in similar species. Similar to our results, van der Lingen (1998) found no significant effect of temperature (ranging from 14.6 to 17.8°C) on the rate of gastric evacuation for *Sardinops sagax*. Bernreuther et al. (2009) observed that temperature increased the gastric evacuation constant in *Sprattus sprattus*, with maximum rates observed around 19°C. Bernreuther et al. (2008) noted a high temperature dependency compared to other studies with herring and sprat, while Temming et al. (2002) reported a strong temperature effect on gastric evacuation in *Scomber scombrus* feeding on sprat, sandeel and krill.

Predator size and sex did not significantly affect the GERs, contrasting with other studies such as Temming & Herrmann (2001a) for *Trachurus trachurus* and Bernreuther et al. (2009) for *S. sprattus*. This can be explained by the small range of fish sizes used in the present work (18–23 cm).

4.3. Diet type effect on gastric evacuation

In this study, a general univariable gastric evacuation model was employed to analyze the gastric emptying curve of sardines fed different diets, and an exponential model was adjusted to allow comparisons of the constant R with previous studies. Contrary to previous studies, which stated that exponential evacuation ($B = 1$) was prevalent in microphagus fish species whereas piscivorous fishes exhibited values closer to $B = 0.5$ (Temming et al. 2002), our results showed that both a high proportion of eggs (Diet 2) and a reduced proportion of eggs (Diet 3) resulted in an approximately linear gastric evacuation curve. However, Diet 1, representing an intermediate egg proportion, exhibited a more pronounced curve ($B = 0.5$), similar to piscivorous fishes. These results contradict the expectations based on previous studies conducted on related species such as *S. sagax* and juvenile *Clupea harengus*, where exponential evacuation was observed. On the other hand, Bernreuther et al. (2009) excluded both linear and exponential evacuation models for small *S. sprattus* partly as a consequence of the meal size variation.

While the exponential model has been commonly applied to describe the evacuation of small and easily digested prey such as zooplankton, our results and those of other studies challenge this assumption. The general model, which allows for greater flexibility, provided a better prediction of evacuation for large meals compared to strict linear or exponential models. The estimates of parameter B varied across studies, indicating that the shape of the evacuation curves depends on various factors, including prey size and species-specific feeding behavior. While previous studies on clupeid species have predominantly applied the exponential model, our findings with *Sardina pilchardus* suggest a more complex pattern, varying from approximately linear to curvilinearity. However, results should be analyzed carefully, taking into consideration all the variables, especially the high variability in meal sizes between diets, since not only the shape parameter (B) but also the respective confident limits (not excluding different models from linear to exponential) varied significantly.

The GER denoted as the R parameter was lower for Diet 2 (higher proportion of eggs) and higher for Diets 1 and 3, which had lower proportion of eggs and a higher proportion of microalgae, rotifers and *Artemia* nauplii (ranging from 0.125 to 0.228 h^{-1}, exponential model). Fish eggs are more nutritionally rich and larger than microalgae, rotifers and *Artemia* nauplii, and therefore more difficult to digest. Results of

GER were similar to previous studies conducted on clupeid species. For *S. sagax*, R values (mean ± SD) ranged from 0.009 ± 0.03 h^{-1} for zooplankton to 0.27 ± 0.03 h^{-1} for phytoplankton, using plankton of natural origin (van der Lingen 1998). For *S. sprattus* fed with similar prey (*Artemia* spp. nauplii), R values ranged from 0.07 to 0.19 h^{-1}, considering temperatures between 7.5 and 21.5°C, applying an exponential model and using mostly juvenile sprat (Bernreuther et al. 2009). For juvenile *C. harengus*, R values range from 0.082 at 13°C to 0.843 at 16°C, applying the general gastric evacuation model; and between 0.098 h^{-1} at 13°C and 0.202 h^{-1} at 16°C by assuming exponential gastric evacuation (Bernreuther et al. 2008).

The results of the exponential models of fish egg evacuation agree with the exponential models for the stomach content weight of sardines, previously used to compared diets, where the GER of fish eggs was lower in Diet 2 (with a high concentration of fish eggs) and slightly higher for Diet 3 (with a low concentration of fish eggs). On the other hand, The GER of fish eggs in Diet 1 (similar proportions of prey) was unusual in that it displayed a higher value than Diet 3 despite the high ingestion of fish eggs by sardines in tanks 1, 4 and 7 in the beginning of the experiments ($t = 1$ h; Fig. 2).

Unexpectedly, Diet 2, with larger proportion of eggs than Diet 1, had lower egg intake. This is probably related to the fact that sardines on Diet 2 and some sardines on Diet 1 had completely full stomachs (bursting with eggs) at the start of the experiment $t = 1$ h. This means that despite the higher concentration of eggs in Diet 2, some sardines may have reached the maximum limit of egg intake in both diets (1 and 2). Therefore, these values could possibly be explained by the greater ability of some of the fish on Diet 1 to ingest eggs (slightly larger stomachs).

4.4. Egg mortality by predation

Cannibalism and intraguild predation of fish eggs are frequently observed for small pelagic fish and can be an important source of mortality. In Atlanto-Iberian waters, sardines have been identified as the major predator of pelagic fish eggs (Garrido et al. 2015) and may have a direct impact on the mortality of sardines and competing species such as the anchovy (Fonseca et al. 2022). Several authors have estimated the percentage of mortality caused by egg predation; for example, Garrido et al. (2008) and Fonseca et al. (2022) for Atlanto-Iberian waters, using GERs found in the literature for other species as there

was no data for the European sardine. Re-calculating egg mortality by predation using sardine GER obtained in the current work showed that the values obtained by Garrido et al. (2008) and Fonseca et al. (2022) were overestimated. Estimates of sardine cannibalism by Garrido et al. (2015) off the Iberian coast are in line with the estimates of Garrido et al. (2008) and Fonseca et al. (2022). Despite corrections, results show that a significant percentage of spawned sardine eggs may be consumed by sardines (ranging from 2 to 16%). Estimates of anchovy egg mortality consumed by sardine (ranging from 3 to 19%) can significantly impact population dynamics off Western Iberia and justify why anchovy populations have only increased the abundance in the area during the lowest period of sardine abundance (ICES 2022).

It is important to have an accurate assessment of the impact of cannibalism and intraguild predation by sardines because it might be a significant source of mortality and, consequently, may have a negative impact on recruitment (Irigoien and de Roos 2011, Garrido et al. 2015). In small pelagic fish, the survival rates in the early life stages are highly volatile (Garrido & van der Lingen 2014). Higher mortality in developing eggs and early life stages can drastically affect recruitment in small pelagic fish like sardines. Atlantic chub mackerel was identified as a major predator of sardine eggs off the Iberian coast (Fonseca et al. 2022). For this reason, it is important to obtain digestion data for Atlantic chub mackerel, allowing future estimates of its impact on sardine population dynamics.

This study provides important information on GERs of sardines that will allow an improvement of previous models that estimate egg mortality by predation. These refined estimates of egg mortality by predation provide a way to improve our understanding of small pelagic fish population dynamics and inform models for an ecosystem approach to fisheries management.

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