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A real-time PCR assay to estimate invertebrate and fish predation on anchovy eggs in the Bay of Biscay.

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Highlights:

-An assay capable of detecting traces of *Engraulis encrasicolus* DNA was designed

-Fifty four taxa of potential predators were assayed in May 2010

-A contrasting predation pressure corresponded to the 2 main spawning centers of anchovy

-Mortality due to macrozooplankton ranged from 1-4 % (shelf-break) to 14-89 % (shelf)

-Predation by sardine accounted for a 7 % of the daily anchovy egg mortality

Keywords:

Predator prey interactions; *Engraulis encrasicolus*; Clupeoid fisheries; Zooplankton; Molecular assay; DNA; Bay of Biscay.

Abstract

In order to investigate the role of predation on eggs and larvae in the recruitment of anchovy (*Engraulis encrasicolus*), sardine (*Sardina pilchardus*), sprat (*Sprattus sprattus*) and 52 macrozooplankton taxa were assayed for anchovy remains in the gut during the 2010 spawning season using a molecular method. This real-time PCR based assay was capable of detecting 0.005 ng of anchovy DNA (roughly 1/100 of a single egg assay) in a reliable way and allowed detecting predation events up to 6h after ingestion by small zooplankton taxa. A total of 1069 macrozooplankton individuals, 237 sardines and 213 sprats were tested. Both fish species and 32 macrozooplankton taxa showed remains of anchovy DNA within their stomach contents. The two main findings are (1) that the previously neglected macrozooplankton impact in anchovy eggs/larvae mortality is in the same order of magnitude of that due to planktivorous fishes and that, (2) the predation pressure was notably different in the two main spawning centers of Bay of Biscay anchovy. While relatively low mortality rates were recorded at the shelf-break spawning center, a higher predation pressure from both fish and macrozooplankton was exerted at the shelf one.

1 Introduction

It is generally accepted that much of the marine fish year-class strength variation is determined by early life stages (ELS) mortality (e.g. Houde, 1987; Bailey and Houde, 1989; Bailey, 1994; Bax, 1998; Bunn et al., 2000; North et al., 2009). Between others (starvation, transport away from nursery grounds, disease, parasitism and pollutants) predation is considered the main cause of natural mortality of marine fish ELS. Mortality rates for temperate species ELS are between 5-20 % per day (Bunn et al., 2000), leading to cumulative mortalities of 98-99 %. Consequently, small shifts in the mortality rate lead to large changes in survivorship and associated recruitment. There is a large range of potential predators responsible for egg and larval mortality including gelatinous organisms, amphipods, mysids and euphausiids, carnivorous copepods, chaetognaths and fish (e.g. Bailey and Houde, 1989; Bunn et al., 2000). However the knowledge on which are the actual predators *in situ* is limited, in particular with respect to invertebrates. In this sense, changes in the predator community and/or in prey abundance have the potential to impact the recruitment success (e.g. Koster and Mollmann, 2000; Lynam et al., 2005; Richardson et al., 2011; Irigoien and de Roos, 2011).

There are a number of competitors for food of the adult anchovy such as sardine, sprat, mackerel and horse mackerel that also predate on their ELS (Szeinfeld, 1991) and the consequences of intraguild predation have been discussed (Irigoien and de Roos, 2011). However, predation by marine invertebrate has been largely absent from fish recruitment models although it is known that euphausiacea (Krautz et al., 2007) and chaetognatha (Terazaki, 2005) may be important causes of mortality of anchovy eggs. For example, predation by euphausiacea accounted for 24 to 27 % of natural mortality in the Chilean anchoveta *Engraulis*

ringens (Krautz et al., 2007) and 47-78 % of the natural mortality on northern anchovy (*Engraulis mordax*) eggs and yolk-sac larvae (Theilacker et al., 1993).

The main reason underlying this lack of knowledge on the role of ELS predation in fish recruitment success is that studying predation in the field has proven challenging because accurate identification of partially ELS remains in predator stomachs is difficult (Bailey and Houde, 1989; Heath, 1992). Fish eggs and larvae can be counted in the guts of other fish, but only for a few minutes/hours after ingestion as they are digested quickly (e.g. Hunter and Kimbrell, 1980; Folkvord, 1993; Schooley et al., 2008). In the case of invertebrates, accurate identification of fish eggs, larvae and juveniles in the stomach contents is usually much more difficult because invertebrates, particularly the crustacean, macerate their prey (e.g. Theilacker et al., 1993; Vestheim et al., 2005; Coston-Clements et al., 2009). Accurate quantification is an additional problem and Baker et al. (2014) have recently proposed to limit analyses to presence/absence data. Beside this, identifying remains visually (if possible) is a labour-intensive task, limiting the number of samples that can be analyzed. However, a growing number of studies have employed molecular methods to identify target species in the guts of predators (e.g. reviews in Symondson, 2002 and King et al., 2008). Briefly, two molecular approaches have been applied intensively to detect predation in the field: 1) immunoassays and 2) DNA based assays. Although immunoassays have been used to measure predation on other anchovy species ELS (Theilacker et al., 1986, 1993; Krautz et al., 2003, 2007), DNA-based methods present several advantages making them the method of choice when facing the molecular determination of diet. Briefly DNA-based methods are not only easier and cheaper to develop than immunoassays, but also allow rapid screening against a multitude of different prey likely to be encountered in the field (e.g. Symondson, 2002). To date all the methods targeting prey DNA in predators have relied upon a polymerase chain reaction (PCR) step. Among them, those involving monitoring via real-time PCR detection of an species-specific DNA sequence are faster, more sensitive (capable of detecting DNA traces) and offer improved specificity over conventional PCR approaches generally involving visualization of gel bands (e.g. McBeath et al., 2006). This allows the reliable species assignment of a single fish egg DNA overcoming traditional methods´ limitation (e.g. Fox et al., 2005; Fox et al., 2008; Minegishi et al., 2009). Examples of real-time PCR application to characterize marine animals diet include copepods (Nejstgaard et al., 2008; Troedsson et al., 2009; Durbin et al., 2008, 2011), decapods (Tobe et al., 2010; Albaina et al., 2010, 2012; Cleary et al., 2012; Redd et al., 2014), fishes (Hunter et al., 2012; Fox et al., 2012; Baerwald et al., 2012) and mammals (Bowles et al., 2011).

The Bay of Biscay anchovy (*Engraulis encrasicolus*), a species with large socio-economic impact in the Bay of Biscay area (e.g. Uriarte et al., 1996), experienced, since 2002, a succession of low recruitments, resulting in the collapse of the stock in 2005 that led to successive closures of the fishery until reopening in 2010 (Andrés and Prellezo, 2012; ICES, 2013). It has been suggested that anchovy recruitment in the Bay of Biscay could be partially controlled by ELS predation (Irigoien et al., 2007) and in particular by intraguild

predation (Irigoien et al., 2007; Irigoien and de Roos, 2011). However, predation ecology on ELS has hardly been studied and only limited to fish predators (Goñi et al., 2011; Bachiller, 2013). In this study, our objectives were to determinate the range of predators consuming anchovy ELS and to provide an estimation on the contribution of mortality by predation in Bay of Biscay anchovy eggs survival. To accomplish this, we designed, validated and applied a molecular method capable of detecting traces of *E. encrasicolus* DNA.

2 Material and methods

2.1 European anchovy DNA detection assay

2.1.1 Assay design

Based on the high sensibility and specificity of the real-time PCR based TaqMan assays and on the suitable characteristics of mtDNA for trace DNA detection (see reviews in Symondson, 2002 and King et al., 2008), we aimed to design and validate a TaqMan assay targeting a mtDNA specific sequence for European anchovy (*Engraulis encrasicolus*) detection in predators' stomach contents.

We screened 5 potential mtDNA target genes [12S rRNA, 16S rRNA, cytochrome oxidase subunit I (COI), cytochrome-b (cytB) and D-loop] using a set of *E. encrasicolus* mtDNA sequences [see http://tomato.bio.trinity.edu/manuscripts/12-2/mer-11-0256.pdf, accompanying paper of Molecular Ecology Resources Primer Development Consortium et al. (2012)] along with other clupeids´ sequences retrieved from NCBI´s GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). We selected cytB as target due to the absence of insertions and deletions, the combination of conserved and variable regions along it and, to the relatively larger availability of clupeid specific sequences in public databases. In order to include the highest *E. encrasicolus* genetic variability in our assay, further sequencing of the cytB gene was performed following Jerome et al. (2008) primers (CytBI-7F and TruccytB-R) and PCR conditions. Combining these new sequences (GenBank accession numbers KF972469-KF972488 and KF972503-KF972539) with all the GenBank available matching sequences (9th May 2012 search) a total of 81 *E. encrasicolus* individuals sequences, distributed along the species distributional area, were then aligned using the Clustal W algorithm (Thompson et al., 1997) implemented in Bioedit (Hall, 1999) (Table 1; Supplementary Material Table 1). A total of 482 bp were considered for the assay design including the *in silico* assessment of both intra- and inter-species specificity. To accomplish this, sequences from other 12 clupeid species, aiming to cover the highest possible spatial coverage within them, were either produced within this study (sequenced with the previous primers and conditions; accession numbers KF972489-KF972502 and KF972540-KF972543) or retrieved from GenBank (9th May 2012 search) adding a total of 105 sequences that were then aligned

against the target species (Table 1, Supp. Mat. Table 1). Providing that the assay differentiate *E. encrasicolus* from the closest genetically relatives, the assay will then discriminate from the less related ones. From these 186 cytB aligned sequences, a real-time PCR based TaqMan assay for *E. encrasicolus* DNA discrimination was designed using Primer Express software (Applied Biosystem) and manufacturer recommendations. Both primers and probe were located on conserved areas for the target species but showing variation against the rest of species (Figure 1). The assay amplified a total of 87 bp. BLASTn algorithm (https://blast.ncbi.nlm.nih.gov) was used to discard the alignment of other taxa apart from clupeids. The 5′ end of the MGB (minor groove binding) TaqMan probe was labelled with fluorescent dye FAM (Applied Biosystems) and the 3′ end labelled with a non-fluorescent quencher. The 15 bp *E. encrasicolus* probe showed at least 1 SNP against the rest of clupeid species except for *E. japonicus* where only a SNP in the last position of the reverse primer was noted. This does not compromise the objectives of the research as these species never co-occur in the wild.

2.1.2 Assay validation

The assay intra- and inter-species specificities were tested against DNA extracted from wild specimens distinct from the ones used in the assay development. Assay sensibility was also tested against a series of *E. encrasicolus* DNA dilutions including both muscle tissue and single eggs. DNA extractions and real-time PCR settings for assay validation were based in those established by Albaina et al. (2010, 2012). DNA was extracted using a modified salt extraction protocol (Aljanabi and Martinez, 1997). Following dissection, tissues were partially homogenized in 1.5 ml autoclaved Eppendorf tubes containing 675 ml of extraction buffer (30 mM Tris, 10 mM EDTA and 1 % SDS) and left overnight at 55 °C to digest after addition of 10 µl Proteinase K (20 mg/ml; Roche). Dissection tools were flamed with ethanol after each sample and an extraction blank (EB) control (negative control, where no tissue is added to the extraction buffer prior to DNA extraction protocol) was included every 11 samples to prevent cross-contamination. Next, 225 µl 5 M NaCl was added to the homogenate and briefly vortexed. After centrifuging for 5 min at 13,000 rpm, 450 µl of the supernatant was collected in new 1.5 ml Eppendorf tube, 900 µl of 100 % ice cold ethanol added before leaving for 1 h at −80 °C. After 30 min centrifugation at 13,000 rpm, the liquid phase was removed and 1 ml of 70 % ethanol was added to wash the remaining pellet. Finally, after 10 min centrifugation at 13,000 rpm the ethanol was poured off, the DNA pellet was then dried at 37 °C, then resuspended in 100 µl ultrapure H_2O and stored at−20 °C. TaqMan assays were run on an Applied Biosystems 7900 real-time sequence detection system. 10 µl volume reactions were run in 384-well reaction plates using Optical Adhesive Covers (Applied Biosystems). Each reaction contained 0.083 µl of 60X assay (corresponding to 125 nM of anchovy probe and 450 nM of both the F and R primers), 5 µl of Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies), 0.15 µl of ROX reference dye (1 mM; Agilent Technologies), 1.25µl

BSA (#B9001S New England Biolabs; 10 mg/ml), 2.517 µl of ultrapure H_2O and 1 µl extracted DNA. DNA yield (ng μl^{-1}) and purity indexes [determined from the absorbance (A) at different wave lengths (nm); A260/A280 and A260/ A230 ratios] for the extractions were determined using a ND-1000 Spectrophotometer (NanoDrop). Plates were run under FAST cycling conditions on a single dye layer with 20 no template controls (NTCs) and 12 positive controls (DNA extracted from anchovy muscle tissue) per 384-well plate. The assay was run using 40 PCR cycles following Master Mix manufacturer´s conditions: after a first stage of 3 min at 95 °C, the run comprised 40 cycles of 5 s at 95 °C followed by 15 s at 60 °C. After PCR, the results were analysed using the Sequence Detection Software version 2.3 (Applied Biosystems) and threshold cycle (Ct) values were computed. Ct values are directly correlated with the number of copies of target DNA present in the reaction. A lower Ct value means higher amount of target DNA in the reaction and, vice versa. All amplification curves were checked visually to remove false positive/negative signals.

The designed real-time PCR based TaqMan assay amplified 100 % of *E. encrasicolus* samples from different locations (including > 10 individuals sampled at Bay of Biscay and Celtic Sea waters). Ten-fold dilutions of an *E. encrasicolus* DNA extract (standard curve) were used to estimate the detection limit of the assay (sensibility). Figure 2a shows the magnitude of the detection signal (expressed as Ct value) along the decreasing values of template DNA (ng). The TaqMan assay was able to detect up to 0.0005 ng of anchovy DNA. However, full (100%) reproducibility among replicas was only attained at amounts of target DNA ≥ 0.005 ng. To assure reproducibility, the assay detection limit for a reliable detection of anchovy DNA was set at 35.4 Ct value (taking into account both average Ct and SD values of the last dilution yielding a Ct value for all the replicas). The efficiency of the reaction was > 90 % (following the equation in Galluzzi et al., 2004) for the different dilution series. As expected a ~3 Ct units increase corresponded to each 10 fold dilution (e.g. Albaina et al., 2010; Jungbluth et al., 2013). The high sensibility of the assay allowed detecting DNA extracted from a single anchovy egg. When testing 20 *E. encrasicolus* eggs, including all development stages (Moser and Alshtrom, 1985), every individual egg gave a positive signal well within the detection limits of the assay (average Ct of 26.6, SD = 2.3). No relationship with stage development was reported (Supp. Mat. Figure 1). Roughly 0.5 ng of DNA was assayed per anchovy egg. In this sense, the amount of DNA assayed from a single egg corresponded to~ 100 times the assay detection limit (Figure 2a).

Regarding inter-species specificity, the assay was tested against DNA from clupeid species potentially cohabiting with anchovy in the Bay of Biscay or surrounding regions. This included testing specimens of *Sardina pilchardus* (n = 15), *Sprattus sprattus* (n = 10) and *Clupea harengus* (n = 20) from the Bay of Biscay, Celtic sea and Irish Sea. The assay tested negative for 100 % of the cases for sardines and herrings including a wide range of assayed DNA amounts (up to > 200 ng; Figure 2b). However, non-complete specificity appeared with sprat DNA extracts. Six out of ten sprats gave signals below the 35.4 threshold. For these six cases an average Ct of 34.2 corresponded to an average assayed DNA amount of 150 ng.

Conversely, 150 ng of anchovy DNA would correspond to Cts of ~ 17.3. Furthermore, none of the 10-fold dilutions of sprat DNA yielded a positive signal (Figure 2b). If we assume the same DNA amount per egg as in anchovy, ~300 sprat eggs would be needed to yield a 34.2 signal that is around the Ct value corresponding to 1/100 dilution of a single anchovy egg (Figure 2a).

This contrasting sensibility of the assay granted the applicability of the assay to measure anchovy predation in the field in a reliable way. In this sense, new Ct thresholds were defined assuming that the entire DNA extracted from the stomach contents corresponded to raw (undigested) sprat DNA (worst case scenario). While the 35.4 threshold was sufficient when assaying < 50 ng of DNA extracted from stomach contents, a threshold of 32.4 Ct units corresponded to assays with between 50 and 500 ng DNA and, finally, a 29.4 Ct units threshold did to 500 - 5000 ng DNA ones (Figure 2b). This prevented false positive signals arising from hypothetical sprat DNA presence. Finally, a further distinction had to be done as, while in fish only stomach contents were extracted, the whole animal was extracted in the relatively small size invertebrates comprising the zooplankton predators (see section 2.3). For fish stomachs and large zooplankton, where a partial dissection was performed, the entire amount of extracted DNA was considered as potentially raw sprat tissue DNA. However, for the remaining zooplankton individuals extracted as a whole (1096 out of 1174), we assumed a 25 % of the whole individual extracted DNA corresponding to stomach contents DNA although this is also an overestimation reinforcing the conservative nature of the assay thresholds.

2.2 May 2010 sampling

The BIOMAN surveys are carried out annually by AZTI-Tecnalia since 1987 in the Bay of Biscay during the *E. encrasicolus* spawning peak (May-June; e.g. Motos et al., 1996, Santos et al., 2011) and are designed to cover the whole spawning area of this species. These surveys´ main objective is to determinate the Bay of Biscay anchovy spawning stock biomass (SSB) following the DEPM [Daily Egg Production Method; see Lasker (1985) and Somarakis et al. (2004) for further information] as to provide fisheries-independent information for stock assessment. The 2010 BIOMAN survey was carried out from the 5th to the 20th of May and involved two research vessels (RV), namely `RV Investigador´ and `RV Emma Bardán´ for, respectively, plankton and fish sampling. For a more detailed description of 2010 BIOMAN survey, see Santos et al. (2011).

2.2.1 Anchovy eggs sampling

A grid of 484 stations was sampled using vertical hauls of a 150 µm PairoVET net fitted with a flowmeter and lowered to 100 m or 5 m above the bottom at shallower stations. After allowing 10 seconds at the maximum depth for stabilisation, the net was retrieved to the surface at a speed of 1 m s^{-1}. Consecutive stations were 3 nautical miles apart located in transects spaced 15 nautical miles apart covering the Bay of Biscay from 43.3ºN to 46.9ºN and from 1.2ºW to 4.5ºW (Supp. Mat. Figure 2). PairoVET samples were preserved immediately after collection in formaldehyde 4 % buffered with sodium tetraborate in sea water. After six hours of fixing, anchovy, sardine and other eggs species ("other fish eggs" category) were identified, sorted out and counted. Apart from PairoVET samples, the Continuous Underway Fish Egg Sampler (CUFES, Checkley et al., 1997) was used to record the eggs found at 3m depth with a net mesh size of 350µm.

2.2.2 Adult fish sampling

Sardine (*S. pilchardus*) and sprat (*S. sprattus*) samples were obtained by pelagic trawling on board R/V Emma Bardán coinciding in space and time with the plankton sampling (see Santos et al., 2011 for more information). Although the fishing objectives are stock evaluation of anchovies, other fish species are captured. Immediately after fishing, hauls where sardine and/or sprat were present in sufficient number (aiming to sample 30 individuals per species and haul but with a minimum of 10 to proceed) were selected for fish sorting (Figure 3). In this sense, it has been reported that 20 stomachs per fish species ensured the detection of 75 % of the ingested prey groups in the Bay of Biscay (Bachiller, 2012). Less than one hour, after the haul was retrieved, was needed to complete sorting. Sardine and sprat whole individuals were preserved at -20ºC. Back in the laboratory, with the fish still frozen, individual sardines and sprats were weighted, measured, sexed, gonad stage determined following ICES methodology (ICES, 2008) and the stomach (cardiac stomach and pyloric caeca) was dissected and preserved in 50 ml Falcon tubes filled with fresh 100 % ethanol for further treatment. Dissecting tools were flame sterilized after each dissection. An insufficient number of individuals in certain hauls prevented statistical comparison among hauls; nevertheless, statistical tests were applied to both species considering all the tested animals together and also dividing them among three defined temporal ranges [midday (11:30 to 14:30 h local time), late afternoon (18:30 to 20:00 h) and midnight (22:30 to 00:30 h); respectively, MIDD, LAFT and MIDN].

2.2.3 Macrozooplankton sampling

A total of 5 MIK (Methot Isaac Kidd) net samples, with a mesh size of 1 mm and a mouth area of 1 m^2, were collected in order to characterize the macrozooplankton community and to sort potential predators of anchovy eggs for assay testing (Figure 3). All but one haul were towed at areas of high anchovy eggs abundance and during the night as to maximize predation events detection (e.g. Kaartvedt et al., 2002; Mauchline, 1998; Jumars et al., 2007). With the ship at 2 knots, oblique MIK net tows were carried out from 50-60 meters (below the incipient thermocline) to the surface at a speed of ~3 meters min^{-1}. Samples were preserved immediately after collection in 100 % ethanol. The ethanol was changed after 24 h (onboard) and at least another time back in laboratory as to assure optimum preservation of both morphology and DNA quality. The qualitative and quantitative analysis of MIK net samples was carried out under a stereoscopic microscope and identification was made to genus or species level when possible (Table 2). The assayed taxa were selected based on their reported carnivorous or, at least omnivorous, potential feeding character. In each sample, all the large animals were sorted, (mostly the myctophidae *Benthosema glaciale*, the pteropod *Cymbulia peroni*, amphipods over 9 mm total length (TL), malacostracans over 7 mm cephalothorax length (CL), and salps over 20 mm). Then, the remaining sample was aliquoted using a Motoda plankton splitter (Motoda, 1959; van Guelpen, 1982) and aliquots were sorted until a minimum of 300 individuals (including all categories except gelatinous organisms) were counted and measured. Further aliquots were analysed, if necessary, until at least 50 individuals from any of the taxa selected for assay testing were sorted (Table 2). Potential predators did not include gelatinous zooplankton and fish larvae (except for Myctophid larvae than showed a better condition related to their larger size) due to a relatively damaged condition caused by an inappropriate sampling device. Individuals to be assayed were transferred to 2 ml microtubes (Sarstedt) with fresh ethanol until DNA extraction. Multivariate analyses of the sampled stations and the relevant zooplankton taxa were carried out using via DCA (detrended correspondence analysis) using version 4.5 of CANOCO (ter Braak and Smilauer, 2002) applied to $\log_{10}$ transformed (x+1) abundance values using only those taxa that contributed more than 5 % of the zooplankton community abundance at any of the stations when gelatinous organisms were not considered (Supp. Mat. Table 2).

Both PairoVET and MIK nets were fitted with a RBR XR-420 CTD (Conductivity, Temperature, and Depth profiler; Sidmar) with a fluorescence sensor (Seapoint Chlorophyll Fluorometer; Seapoint Sensors, Inc.). Water density (expressed as sigma-t) profiles were calculated. In addition, surface temperature and salinity were recorded in each station with a manual thermosalinometer WTW LF197.

2.2.4 Digestion experiment

The capability of detecting the DNA extracted from one anchovy egg does not assure the assay capacity to detect predation events. Firstly, how digestion time affects the ability of the probe to detect the target DNA has to be estimated (e.g. Greenstone et al., 2013). To estimate the digestion time effects we considered previously published results (Albaina et al., 2010; Hunter et al., 2012), combined with an experiment for macrozooplankton performed onboard during the campaign. An extra MIK haul was carried out (46ºN, 2.42ºW; Figure 3) to collect a sufficient number of live macrozooplankton organisms for digestion experiments purposes. Although a lower retrieving speed was used (1-1.5 m min^{-1}) combined with a mere 0 to 16 m sampling depth, only Brachyuran larvae seemed active and in good condition (based on swimming behaviour and/or response to stimulus) and thus were selected for the experiment. A total of 16 *Liocarcinus depurator* and 12 *L. holsatus* megalopae (identified following Ingle 1991), were gently transferred in similar numbers to four 2 L borosilicate glass bottles (Schott), containing 20 µm filtered sea water, mounted on a plankton wheel that maintained sea surface temperature. After transferring animals, these were starved for 48 h before feeding trials commenced to assure predator gut clearance and animals feeding shortly after food is provided. As when the acclimation phase ended no anchovy eggs were present in the area, raw anchovy tissue was used to feed animals. Experimental animals were fed *ad libitum* for 2 h. Then, six actively swimming individuals were preserved (end of the feeding period; t = 0 h) and the remaining animals were transferred into new bottles with clean water and no anchovy tissue. Around five to nine actively swimming individuals were sampled at 3 different time points, 3h, 6h and 12h after the end of feeding period. Animals were preserved in 100 % ethanol in 2 ml microtubes until further DNA extraction. Half- life detectability rates of the target DNA (T_{50} values; the time after which only half of the individuals test positive for prey DNA) and maximum detection times were estimated.

2.3 Detection of anchovy DNA within BIOMAN 2010 stomach contents

Back in the laboratory sardine and sprat stomach contents were dissected for DNA extraction. At the same time, a visual fullness index was recorded for both cardiac and pyloric caeca: an index of 1 indicated stomachs with either not visible contents or with less than 1/10 of the volume filled; 2 indicated between 1/10 and 2/3 of the volume filled and 3 indicated more than 2/3 of the stomach volume filled but not completely full; for the latter a category of 4 was assigned. Prior to extraction, individual organisms were placed over a highly absorbent wiper and washed with distilled water using a Pasteur pipette. Forceps and dissecting tools were flame sterilized after each animal. DNA was extracted in 1.5 ml Eppendorf tubes

following the salt extraction based method detailed in section 2.1.2 and including an extraction blank control every 11 samples to detect cross-contamination. For krill and other large malacostracans, a plastic pestle, treated with bleach and UV radiation after each use, was used to aid homogenization. After extraction, DNA was resuspended in 100 µl ultrapure H2O and stored at −20°C. DNA yield (ng μl^{-1}) and DNA purity was assessed with the spectrophotometer. TaqMan assays were run under FAST cycling conditions as in section 2.1.2 with 20 NTCs and 12 positive controls per 384-well plate. In order to overcome PCR inhibition, apart from the addition of 1.25 µg μl^{-1} BSA to the reaction, two 10-fold dilutions were tested for each sample with at least one of those corresponding to a quantity tested of < 50 ng DNA (stomach contents extract) as this has been shown to reduce inhibition incidence to a minimum (Albaina et al., 2010). The defined thresholds (Ct versus ng of DNA tested; Figure 2b) were applied to call a positive signal. Finally, the percentage of positive signals per haul/station was computed.

2.3.1 Mortality estimation due to macrozooplankton predation.

In situ data for both predator and prey abundances were available at MIK nets location (Table 3) allowing the mortality estimation at haul point. We made the conservative assumption that each assay positive signal corresponded to one anchovy egg/larvae eaten in the last 24h. Our anchovy DNA detectability experiment, limited to one developmental stage of 2 crab species, showed that predation events were detectable during ~3h (Figure 4); therefore, an individual continuously feeding along the 24h cycle could consume up to 8 times the amount detected in the last 3h. However, the variety of taxa involved and the lack of information about macrozooplankton feeding behaviour and digestion times (e.g. Durbin et al., 2011) made us consider the "1 positive assay = 1 egg/larvae killed in the last 24h" as a reasonable conservative assumption representing minimum estimation of the predation impact of macrozooplankton on anchovy. As an example, between 1.6 and 1.9 fish larvae (respectively, *Brevoortia tyrannus* and *Anchoa mitchilli*) were ingested daily by the chaetognath *Sagitta hispida* in controlled laboratory experiments where high encounter rates were favoured (Coston-Clements et al., 2009). Due to the absence of anchovy larvae counts, daily mortality due to macrozooplankton predation was then computed as the fraction of anchovy eggs eaten in the last 24h (equation 1 and 2; adapted from Albaina et al., 2012). Both prey and predator abundances were transformed to abundances per unit area. Although the sampling depth were not the same (Table 3) the vast majority of anchovy eggs are distributed within the MIK haul sampling depth (Boyra et al., 2003; Coombs et al., 2004). For each assayed taxon:

$$N_P = p * D_C \quad (1)$$

where Np is the number of anchovy eggs consumed over the previous 24 h per unit area, p is the proportion of positive TaqMan assay for a certain taxon, and D_C is the estimated density of the predators per unit area. Then, for each sampled location taking into account every assayed taxon:

$$M_P = \frac{\sum N_P}{(D_P + \sum N_P)} * 100 \quad (2)$$

where M_P is the daily mortality at the sampling location exerted by macrozooplankton predation and D_P is the estimated abundance of anchovy eggs per unit area.

2.3.2 Mortality estimation due to fish predation.

The mortality exerted by sardine and sprat could be estimated following the classical approach (equations 3 and 4) due to the existence of feeding duration and gut evacuation rates from the literature. In this sense, given the quantitative nature of the real-time PCR technique (Ct ↔ ng of anchovy DNA) and the amount of DNA corresponding to one undigested/raw assayed egg (Figure 2a) we could estimate the number of anchovy eggs consumed by predator from the obtained Ct value. This estimation represents the minimum number of eggs predated by each fish as the same Ct value would correspond to a progressively higher number of eggs as digestion time progresses. Apart from this, the availability of biomass estimations for *Sardina pilchardus* and *Sprattus sprattus* from the parallel PELGAS 2010 acoustic survey (ICES, 2010b) along with the *E. encrasicolus* daily egg production and mortality rates from BIOMAN 2010 campaign (ICES, 2010a), allowed us to calculate the (minimum) proportion of anchovy egg mortality that can be assigned to sardine and sprat predation. Regarding sardine, the PELGAS 2010 biomass estimations for two discrete areas, the so-called Gironde and South-offshore ones, approximately corresponded to the two main anchovy spawning centers in 2010 (from here onwards Gironde shelf and shelf-break ones; Figure 3). For sprat, however, the provided biomass included that of the sprats inhabiting Gironde shelf waters along with the more abundant ones at northern locations thus preventing more accurate mortality estimation in the anchovy spawning areas (ICES 2010b).

We calculated the proportion of anchovy egg mortality explained by sardine and sprat predation (P_C) following the method given by Hunter & Kimbrell (1980) including the modification introduced by MacCall (1981) as in Szeinfeld (1991). We estimate the number of eggs consumed daily per predator:

$$C = EE * g * t'$$

(3)

where C = average numbers of eggs eaten per kg of fish during time t´, EE average number of eggs per predator kg as estimated by the real-time PCR assay, t´ = duration of feeding (h) and g = gastric evacuation rate (h^{-1}). While whole day long feeding behaviour of sardine and one restricted to daylight hours in sprat (07:00 to 21:00 for May in the Bay of Biscay) have been reported (respectively, Garrido et al., 2007, 2008 and Peck et al., 2012), evacuation rates of 0.2105 h^{-1} (Nikolioudakis et al., 2011) corresponded to sardine and were also applied to sprat due to the lack of comparable values in the literature. Then, the proportion of anchovy egg mortality caused by fish predation (P_C):

$$P_C = \frac{Z_C}{Z_t} = \frac{C_t / G_t}{(1 - e^{-tZ})}$$

(4)

where Zc = proportion of egg production consumed by fish predation (C_t/G_t), C_t = daily total anchovy egg consumption by sardine or sprat (C * predator biomass) and G_t = total daily anchovy egg production; Z_t = proportion of egg production lost due to all causes of mortality ($1 - e^{-tz}$); Z = hourly instantaneous rate of egg mortality and t = 72 h (anchovy egg development time, following Motos, 1994).

3 Results

A total of 190 extraction blanks and 260 no template controls (NTCs) were included in order to check cross-contamination. Only 2 extractions blanks and 2 NTCs gave a positive signal, which after repetition in a different real-time PCR run showed negative signal thus being more than likely associated to contamination due to incorrect pipetting when setting the 384-well real-time PCR plates. The same could explain the 2 out of 260 positive signals in the NTCs included in the plates. Based on these results a marginal 0.9 % of positive signals (4 out of 450) could be assigned to false positives.

3.1 Detectability of anchovy DNA over predator´s digestion

The *E. encrasicolus* DNA detectability along digestion time was tested with an experiment during the plankton sampling cruise (section 2.2.4). Figure 4 shows the decay of *E. encrasicolus* DNA detection rates in two Brachyuran species along the digestion process (up to 12 hours after ingestion) at temperatures

ranging from 13.2 to 14.4 ºC. For both species (namely *Liocarcinus depurator* and *L. holsatus*) the same signal decay pattern was reported showing comparable detectability along digestive process. Half-life detection rates (T_{50}) of ~5 h were reported for *E. encrasicolus* DNA within stomach contents. Positive signals could be measured up to 6h after ingestion (2 out of 9 individuals) but not after 12h.

3.2 Plankton survey

3.2.1 Anchovy eggs distribution

Anchovy eggs were mostly distributed in two main areas that correspond well with the two main spawning centers for the species in the last decade (Bellier et al., 2007; ICES, 2010a). The first center was located in front of the Gironde river mouth in the inner shelf (< 100m) waters (from here onwards "Gironde shelf" one) and the other in the shelf-break region between Arcachon bay and Adour river locations (shelf-break one; Figure 3). Abundances up to 19.8 eggs m^{-3} were recorded. All but one (MIK-C) of the five MIK hauls collected with predation assessment purposes were carried out in *E. encrasicolus* eggs high density areas (Figure 3, Table 3) with a maximum of 14.5 eggs m^{-3} (MIK-E). Fish hauls were performed in areas with a moderate to high anchovy eggs abundance (up to ~5 eggs m^{-3} in two of the hauls and never below 0.1 eggs m^{-3}).

3.2.2 Macrozooplankton abundance and distribution

A total of 80 taxa were identified from MIK samples and 52 of them were sorted for assay testing (Table 2). The most abundant taxa apart from gelatinous organisms (mainly siphonophores and salps, 34 % of total abundance), were the euphausiid *Meganyctiphanes norvegica*, mysids (mainly *Schistomysis ornata* and *Haplostylus normani*), decapod larvae (Brachyuran and Solenocera larvae being the most abundant) and the copepod *Calanus helgolandicus*. The copepods were the most diverse group with 19 species identified. Interestingly, no anchovy egg or larvae was recorded. When plotting the most abundant taxa (21 taxa; Table 2) in a detrended correspondence analysis (DCA) with CANOCO software (see section 2.2.3), two assemblages were distinguished (Figure 5; codes as in Table 2). Stations collected at the shelf-break (MIK-A and B) occupied the left part of the biplot and were characterized by salps, euphausiids and large copepod species. Gironde shelf stations (MIK-C, D and E) occupied the opposite section of the DCA plot and were characterised by decapod larvae and mysidacea. Macrozooplankton abundances over 7 ind. m^{-3} were reported in the latter stations (with a maximum of 10.3 ind. m^{-3} in station E) whereas in oceanic samples values were, respectively, 5.5 and 6.3 ind.m^{-3} for stations A and B (Supp. Mat. Table 2).

3.2.3 Detection of anchovy DNA within macrozooplankton stomach contents

From 1069 organisms tested (ranging from 156 to 248 individuals assayed per MIK haul), 353 yielded a positive signal including 32 different taxa (Table 3). While shelf-break stations presented low frequencies of positive signals (4.8 and 3.6 % of the individuals testing positive for anchovy DNA for, respectively, MIK-A and B), the Gironde shelf ones presented higher frequencies ranging from 5.6 % in Station MIK-C to 68.6 % in MIK-D and to 99.5 % in station MIK-E. Interestingly, predation incidence within the Gironde shelf stations increased with prey abundance in a logarithmic way (Figure 6).

Sorted taxa in shelf-break stations were mainly represented by large calanoid copepods and euphausiacea species. Among those, the most abundant taxa, the euphausiid *M. norvegica*, hardly predated on anchovy eggs/larvae (only 2 individuals, out of 114, presented anchovy DNA remains within their contents). Regarding copepods, a total of 7 species (*Candacia armata*, *Euchirella curticauda*, *Euchaeta hebes*, *Paraeuchaeta gracilis*, *P. tonsa*, *Undeuchaeta major* and *U. plumosa*) presented positive assays (12 positive signals out of 215 copepod individuals for MIK-A and B). While one out of 13 myctophidae larvae showed a positive signal, larger *Benthosema glaciale* did not test positive. Apart from these species, positive assays were recorded also in the pelagic Polychaeta *Tomopteris* spp. and the decapods *Systellaspis debilis*, *Polybius henslowi* and *Pasiphaea sivado* in the shelf-break stations (one positive assay per taxa).

Regarding the Gironde shelf stations (MIK-C, D and E), mysid species along with a variety of decapods larvae taxa represented the bulk of organisms assayed. Only one targeted copepod species (*Candacia armata*) was present in these samples. Mysids and the cumacean *Diastylidae* spp. comprised the bulk of positive signals in MIK-C. Regarding MIK-D and E, the bulk of positive signals corresponded to *Solenocera* larvae (especially in MIK-D where this taxon comprised 85 % of the assayed taxa) and Brachyuran megalopae, followed by the mysids assemblage (S*chistomysis ornata*, *Haplostylus normani*, *Haplostylus lobatus*, *Leptomysis gracilis* and unidentified or immature individuals) and other decapods larvae. Apart from those taxa, positive signals in MIK-D and E hauls were also recorded for *Tomopteris* spp., *Candacia armata*, the amphipods *Hyperia galba* and Gammarids spp., *Diastylidae* spp., Chaetognatha spp. and one juvenile *Merlangus merlangus*.

3.3 Adult fish sampling

3.3.1 Sardine and sprat hauls characterization

A total of 14 pelagic trawling hauls were collected in the anchovy spawning area (Figure 3). A total of 237 sardines and 213 sprat individuals were sorted and tested for anchovy DNA presence in their guts (Table 4). While sardine was distributed along the whole fished area including both anchovy spawning centers, sprat was only caught in the Gironde shelf area. Sorted animals´ length ranged from 138 to 227 mm for sardines and from 79 to 134 mm in sprats with average weight of, respectively, 58 and 10 g (Supp. Mat. Tables 3 and 4). Pyloric fullness was constantly high, showing average values close to 3 in both species, and no significant difference between the three different times of the day (Kruskal Wallis $p > 0.001$). Cardiac fullness index was more variable and there were significant differences between sampling times for both species (Kruskal Wallis $p < 0.001$). Gut fullness data suggests feeding intensity peaking at dusk in both species but a higher preference for dark hours in sardine than in sprat. Finally, no differences in both fullness indexes were recorded regarding size and gonad stage.

3.3.2 Detection of anchovy DNA within fish stomach contents

3.3.2.1 Sardine samples

A total of 82 % of the sardine´s stomachs showed a positive signal (Supp. Mat. Table 3). Interestingly we found sardines with anchovy DNA remains in their stomachs in every sorted haul (nine hauls with values ranging from 48 to 100 % of positive signals; Table 4). No significant differences (Kruskal-Wallis tests) were found when comparing predation incidence against defined categories for cardiac and pyloric fullness (respectively, $p = 0.06$ and $p = 0.21$), size ($p = 0.58$), weight ($p = 0.49$) and female gonad stages ($p = 0.36$). The same lack of significance was reported when comparing the three distinct times of day sampled ($p = 0.14$). Estimated anchovy eggs abundance at haul location and percentage of positive signals are plotted in Figure 7a. Although positive signals incidence is higher at higher prey values, the correlation is not significant (Pearson´s rho 0.44, $p = 0.24$) and high percentages of fish testing positive for anchovy DNA are found even at areas with relatively low abundances of anchovy eggs. The same lack of correlation corresponded to the number of anchovy eggs consumed per predator, as estimated by Ct values, showing a slight increase associated with higher prey abundances (Pearson´s rho 0.351, $p = 0.355$; Figure 7b). The maximum number of eggs estimated for a sardine stomach was 252.

3.3.2.2 Sprat samples

A total of 42 % of the assayed stomach contents for sprat showed a positive signal for *E. encrasicolus* DNA presence (Supp. Mat. Table 4). This value ranged between 10 % and 63 % depending on the haul (Table 4). No significant differences (Kruskal-Wallis tests) were found when comparing predation incidence against cardiac and pyloric fullness indexes ´categories (respectively, p = 0.21 and p = 0.74) and the three distinct times of day sampled (p = 0.06). Although not significant the latter low p-values corresponded to a higher predation incidence in daylight hours (Supp. Mat. Table 4). Regarding size and weight categories, there was a significant difference with larger fish presenting higher percentage of positives (p = 0.004 for size and p = 0.03 for weight). However the average number of anchovy eggs consumed per individual, as estimated by assay Ct values, was similar in both size categories (5.3 and 5 for, respectively, fish below or over 100 mm total length). A maximum of 71 eggs was estimated for one sprat individual. Finally, when plotting predation incidence and anchovy egg abundance, the obtained pattern resembled the sardine one, but with considerable lower incidence of positive signals given a particular prey abundance and a higher dispersion of values (Pearson´s rho 0.27, p = 0.56) (Figure 7a). However, for sprat, the average number of anchovy eggs consumed per individual decreased with prey abundance (Pearson´s rho -0.362, p = 0.425; Figure 7b).

3.4 Predation mortality of anchovy eggs

3.4.1 Mortality due to macrozooplankton (M_P)

Mortality due to macrozooplankton predation (M_P, see section 2.3.1) ranged from 1 to 89 % of the egg abundance (Figure 6). Lowest mortalities were found at the shelf-break area (3.6 and 1.3 % for, respectively, MIK-A and MIK-B), whereas the Gironde shelf area presented higher mortalities (from 14.3 and 14.6 % at, respectively, MIK-D and E, to 89 % at MIK-C).

3.4.2 Mortality due to fish (P_C)

A minimum of 7.1 % of the whole Bay of Biscay anchovy egg daily mortality was explained by sardine predation when considering both anchovy main spawning centers (Table 5; see section 2.3.2 for further explanation). While a P_C of 1.8 % corresponded to sardine predation at the shelf-break spawning center, the remaining 5.3 % did to those at Gironde shelf one. Regarding sprat, it has to be noted that this species´ impact on anchovy egg survival is limited to the Gironde shelf spawning center due to its distribution (ICES

2010b). However, the lack of an acoustic biomass value related to the Gironde shelf area from the PELGAS campaign prevented estimating mortality in an accurate way. If we consider the whole species biomass for the Bay of Biscay when computing P_C [an obvious overestimation due to the larger part of the 2010 sprat biomass being located in coastal areas north of 47ºN (ICES 2010b) well out of anchovy spawning area], this would lead to a 7.2 % of the anchovy egg daily mortality.

4. Discussion

The method developed was able to detect up to 0.005 ng of anchovy DNA (corresponding to ~1/100 fraction of one egg signal) in a reliable way (Figure 2). Furthermore, the assay was able to detect anchovy DNA up to 6 h after ingestion by Brachyuran larvae (Figure 4). This indicates that the assay is suitable to detect fish eggs ingestion by macrozooplankton where target DNA concentrations are expected to be low and the possibilities of microscopic analysis limited. As the DNA based assay cannot distinguish between the egg and larval stages we would refer to the predation on European anchovy eggs/larvae throughout. However, when relating these predation rates to prey fields and, when computing natural mortality by predation, we will restrict to anchovy egg distribution data as these were the only available prey abundances. As anchovy eggs would represent the bulk of anchovy early life stages (ELS) at the sampling moment due to the species spawning dynamics (e.g. Irigoien et al., 2007; Cotano et al. 2008) a significant bias due to the previous simplification is not to be expected.

4.1 Putative biases of the molecular method based diet assessment

First of all, we consider the impact of the assay´s lack of total discrimination against sprat DNA (see section 2.1.2). However, due to the reported different sensibility of the assay for sprat and the target species´ DNA, this issue was overcome by setting new thresholds (Ct to extracted DNA ng threshold; Figure 2b) to call a positive signal in field samples where, we assumed that the entire DNA extracted from the guts was potentially undigested sprat DNA. This prevented false positive signals coming from the putative presence of sprat DNA within the contents. Apart from this particular factor, two other factors, inherent to the application of molecular methods to diet assessment, need to be evaluated: cross-contamination and PCR inhibition. Given the sensitivity of these methods, cross-contamination has to be assessed (e.g. King et al., 2008). Special care has to be taken to prevent contamination of samples from collection to the real-time PCR plate setting and, with special care, in the dissection procedure. Sterility has to be kept during all the process and both on-board and lab protocols have to be adapted to these new issues including controls at different process points and dedicated laboratories for predator sorting, DNA extraction and real-time PCR steps. In this sense, present findings are not to be affected by this issue in a significant way because less than 1 % of

the controls (EBs and NTCs) presented a positive signal pointing to pipetting malfunction. Beside this, PCR inhibitors presence, potentially causing false negatives, has been reported within DNA extracts from a wide range of sources including human blood and soil (e.g. Rådström et al., 2004; Hedman and Rådström, 2013). Furthermore, PCR inhibition is a common issue when dealing with DNA extracts from stomach contents (e.g. Symondson, 2002; King et al., 2008) and has been reported when analyzing stomach contents from a variety of crustaceans, including *M. norvegica*, and fish species (Albaina et al., 2010, 2012; Cleary et al., 2012; Fox et al., 2012). Dilution of template DNA combined with the addition of 1.25 µg/µl bovine serum albumin (BSA) to the PCR reaction mix has been shown as effective in overcoming PCR inhibition both in fish and marine invertebrates (Albaina et al., 2010, 2012; Fox et al., 2012) and this was replicated here. Apart from this, other biases associated with the assessment of predation, independently of the method applied, include regurgitation, the risk of feeding within the sampling gear, scavenging and secondary predation. Regurgitation, a common issue in fish diet assessment (e.g. Bowman, 1986), had a low if any incidence in the present results as reflected in the high stomach fullness indexes reported for the assayed fish (Supp. Mat. Table 3 and 4). Moreover, the high sensibility and detectability of the real-time PCR based method allows detecting feeding events even with stomachs visually empty and thus reduces the potential affectation by this issue (Albaina et al., 2010; Hunter et al., 2012). Feeding within the sampling gear has been cited as a potential bias affecting zooplankton´s predation rate estimations (Nicol, 1984); however, as expected due to the MIK net mesh size (1mm Ø), no *E. encrasicolus* egg (neither larva) was identified in the taxonomy analysis thus avoiding this issue. Other factor to take into account are alternative routes by which prey DNA might get into the guts of predators which include secondary predation and scavenging (King et al., 2008). While the secondary predation of anchovy eggs/larvae by zooplankters would be highly unlikely and potentially undetectable by the assay, this probably does occur in fish predators due to the combination of a higher predatory capacity and higher detectability of DNA (e.g. Hunter et al., 2012). Apart from this, scavenging would also imply an incorrect assignation of the egg/larvae death cause but present method is not able to distinguish this from predation and thus this issue cannot be dismissed entirely.

Finally, for mortality estimation due to fish predation, we use the Ct values of the real-time PCR assay as a proxy of the amounts of eggs consumed per predator. As stated in section 2.3.2, to avoid the bias of the unknown digestion time, we followed the most conservative approach calculating the minimum number of predated eggs that would yield the observed Ct signal. This allowed us to compute the minimum percentage of anchovy eggs mortality that could be assigned to predation by sardines and sprats. The validity of our approach is supported by the fact that the average number of anchovy eggs estimated for sardine and sprat stomach contents (respectively, 15 and 5) fell within the range obtained by Bachiller (2013). It has to be noted that traditional visual identification of egg remains in the stomach contents is subjected to the same bias as the effect of digestion time in the capacity to identify prey remains is also ignored and cannot be compensated. Further developments combining quantitative PCR with fragment-length analysis may have the potential to overcome the latter limitation (Deagle et al., 2006; Troedsson et al., 2009). Apart from this, the real-time PCR approach allows to screen hundreds-thousands of fish in a comparable and cost effective

way (< 1$ sample) without requiring any taxonomical expertise. Regarding small crustacean, comprising the bulk of zooplankton, molecular approaches stand as the only option when studying predation on fish eggs where no hard remains could resist the involved maceration.

4.2 Predation on anchovy ELS on May 2010

4.2.1 Predation incidence

A total of 32 taxa (out of 52 assayed) from the macrozooplankton community tested positive for the European anchovy DNA assay indicating that the majority of the macrozooplankton predator community was a potential predator on anchovy eggs at the Gironde shelf spawning centre. This is probably due to the high concentration of eggs in the area and the resulting encounter rate. However, relatively high prey abundances were also found in the shelf-break stations (Figure 6) where predation was low. In this sense, further studies should investigate the putative role of alternative prey availability and/or species-specific feeding behaviour.

Regarding fish predators, although our survey covered a broad range of prey abundances, high percentages of positive values for anchovy DNA were found in every sardine haul with a minimum predation incidence of 48 % (haul 5) and the remaining 8 hauls well over 70 % incidence values (Table 4). Regarding sprat, although positive signals were recorded in the seven hauls, predation incidence was lower than in sardine, ranging from 10 to 60 % (Table 4). Apart from this, both visual fullness and the molecular assay results suggest that sprat have a diurnal feeding behaviour while sardines feed also during dark hours. This corresponds well with the current knowledge on the physiology of the two species (Garrido et al., 2007, 2008; Peck et al., 2012). This contrasting feeding behaviour would increase the chances to eat anchovy eggs by sardine as maximum anchovy spawning occurs at night (e.g. Uriarte et al., 1996). The herein observed predation incidence (82 % for sardines and 42 % for sprat) corresponds well with microscopic gut analysis where 55 and 30 % of sardines and sprats presented anchovy eggs/larvae (Bachiller, 2013).

4.2.2 Estimations of egg mortality

In spatial terms our results suggest a high impact of the predation by macrozooplankton taxa on the spawning center at Gironde shelf waters while this mortality at the shelf-break was considerably lower. The results indicate that a large fraction of anchovy eggs mortality at the Gironde shelf spawning center is due to predation by macrozooplankton. This observation raises two points: 1) Egg mortality is probably higher in

shelf waters than in the shelf-break. This has implications on the study of the effects of transport in recruitment that has been generally directed to larvae starvation but not to egg mortality (Allain et al., 2007a). And: 2) Mortality due to macrozooplankton predation can be of the same magnitude than that due to planktivorous fish predation. This has been previously ignored when discussing potential factors affecting Bay of Biscay anchovy recruitment (Irigoien et al., 2007). It has also to be considered that gelatinous plankton has not been included in this analysis. However, gelatinous organisms are important predators of fish eggs and, for example, average daily mortalities of > 20 % of eggs and larvae daily production has been reported for gelatinous predators feeding on *Anchoa mitchilli* in Chesapeake Bay (Purcell, 1985; Purcell et al., 1994; Purcell and Arai, 2001).

Comparing to sardine, sprat showed lower predatory capacity both in terms of anchovy eggs consumed per individual and of frequency of sprat individuals testing positive for anchovy DNA. This could be counterbalance somehow by this species´ higher spatial overlap with anchovy eggs at the Gironde shelf spawning center. However, even considering the whole Bay of Biscay sprat biomass, including the larger part of the stock located north of the anchovy spawning centers (ICES 2010b), the mortality does not exceed that of sardine (~7 % for both species) that was computed with a more precise predator biomass data (see section 2.3.2). Regarding sardine, the herein reported P_C value (7 %) is lower than the ones obtained by visual identification of contents (14-48 %; Bachiller, 2013). However, this is probably due to interannual differences in sardine biomass and overlap with anchovy distribution. Finally, an important impact of cannibalism has been reported for anchovy species (Hunter and Kimbrell, 1980; Alheit, 1987; Szeinfeld, 1991). Unfortunately, cannibalism cannot be addressed with the herein developed method.

4. 3 Concluding remarks

The large range of macrozooplankton taxa assayed (52 taxa) and the fact that, apart from anchovy, sardine and sprat comprised the bulk of planktivorous fish in the area (78 %, ICES 2010b), allows us to consider our results as a holistic view of anchovy eggs predation mortality. It is noticeable that, at least for the Gironde shelf spawning center, the observed mortality due to macrozooplankton predation is in the same order of magnitude than the one computed for fish. Considered together, macrozooplankton and fish predation accounted for a large percentage of the anchovy egg mortality. Apart from the previously neglected impact of macrozooplankton, the main finding is the contrasting predation pressure that corresponded to the two main spawning centers of Bay of Biscay anchovy. While relatively low eggs mortality rates, mainly due to sardine, were recorded at the shelf-break spawning center, a higher predation pressure was exerted by sardine, sprat and macrozooplankton community at the Gironde shelf one.

Interestingly, reduced predation mortality at offshore environment has been proposed for anchovy juveniles. Irigoien et al (2007) suggested that anchovy juveniles could be recruiting through a spatial *loophole* (*sensu*

Bakun and Broad, 2003) for predation in these waters. Furthermore, a higher mortality on the Gironde shelf, compared with the offshore domain, was reported by Cotano et al. (2008) when studying anchovy larval growth. Beside this, present results suggest that the shelf-break spawning center could be more favourable for anchovy eggs survival due to a lower predation pressure. However, other factors such as differential spawning intensity or advection patterns could be playing a role (e.g. Allain et al., 2007a). In this sense, future studies could give insights on the role of predation in the higher survival rate reported for anchovy eggs produced after the peak spawning moment (Allain et al., 2007b; Aldanondo et al., 2010). Related to this, it has to be noted that the bulk of the taxa consuming anchovy eggs/larvae in the Gironde shelf area belongs to the meroplankton (Table 3; 67 % of the positive signals). Because of this, the impact exerted by this fraction of the macrozooplankton community, is limited by the duration of both the predator planktonic stage and the species-specific reproductive season. Wider temporal sampling could assess the impact of macrozooplankton phenology on the Bay of Biscay anchovy eggs/larvae survival.

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TABLES

Table 1. Species and geographical origin of the 186 cytochrome-b (cytB) sequences used in the study. NEA stands for North-Eastern Atlantic samples (Celtic Sea, North Sea and Baltic Sea); Bay of Biscay includes Cantabrian Sea samples.

Species	Code	n	NEA	Bay of Biscay	W. Medit.	E. Medit.	Canary I.	South Africa	Unknown loc.	Others
Engraulis encrasicolus	EENC	81	21	27	9	9	6	7	2	
Sprattus sprattus	SSPR	23	4	17					2	
Clupea harengus	CHAR	14	6	2					1	5
Sardina pilchardus	SPIL	10	2	3		2	1		2	
Sardinella aurita	SAUR	9			2	2	3			2
Sardinops sagax	SSAG	3						2		1
Alosa fallax	AFAL	9	7	2						
Alosa alosa	AALO	3		2					1	

Other anchovy species		n	Origin
Engraulis japonicus	EJAP	19	Japon
Engraulis anchoita	EANC	5	Brasil
Engraulis ringens	ERIN	5	Peru
Engraulis mordax	EMOR	3	USA (Pacific coast)
Anchoa mitchilli	AMIT	2	USA (Gulf of Mexico)

Total		186

Table 2. Macrozooplankton species list, showing presence/absence (1/0) at each MIK net haul (A, B, C, D and E) along with average and maximum abundance (individuals 1000 m^{-3}). While Assay column signals the taxa selected for the application of the real-time PCR assay for *E. encrasicolus* DNA detection, the Code column identifies the taxa selected for DCA analysis (see section 2.2.3)

	A	B	C	D	E	Code	Assay	Average	Maximum
Gelatinous									
Jellyfishes	1	0	1	1	1	JELLY		283.7	1317.1
Siphonophora	1	1	1	1	1	SIPHO		1280.6	3855.9
Ctenophora	1	1	0	0	0			1.6	5.4
Salps	1	1	0	0	0	SALPS		401.2	1623.5
Gelatinous remains	1	1	0	1	0	GELAT		494.4	1864.3
Non-Gelatinous									
Tomopteris spp.	1	1	0	1	0	TOMOP	+	49.0	229.0
Cymbulia peroni	1	1	0	0	0		+	2.1	5.4
Diacria trispinosa	0	1	0	0	0		+	1.2	6.2
Pteropod spp.	0	1	0	0	0			0.3	1.6
Podon spp.	0	0	0	0	1			2.9	14.3
Calanus helgolandicus	1	1	1	1	1	CHELG		439.3	1299.3
Calanus robustior	0	1	0	0	0			2.5	12.4
Eucalanus crassus	0	0	0	1	0			2.0	9.8
Temora longicornis	0	0	0	0	1			11.4	57.1
Centropages typicus	0	1	0	0	0			5.0	24.9
Candacia armata	1	0	0	1	1		+	30.6	114.5
Euchirella rostrata	1	1	0	0	0	EUCRO	+	100.0	354.2
Euchirella curticauda	1	1	0	0	0		+	8.7	31.2
Metridia lucens	1	1	0	0	0		+	5.8	18.6
Pleuromamma robusta	1	1	0	0	0	PLERO	+	45.2	174.0
Pleuromamma spp.	1	1	0	0	0		+	5.4	20.8
Euchaeta acuta	1	1	0	0	0		+	12.1	41.6
Euchaeta hebes	1	1	0	0	0		+	32.0	104.1
Euchaeta spp.	1	1	0	0	0		+	24.1	68.4
Paraeuchaeta gracilis	1	1	0	0	0	PARGR	+	56.5	208.1
Paraeuchaeta norvegica	1	1	0	0	0		+	9.6	41.6
Paraeuchaeta tonsa	1	1	0	0	0	PARTO	+	63.2	260.2
Paraeuchaeta spp.	1	0	0	0	0		+	2.1	10.4
Undeuchaeta major	1	1	0	0	0		+	34.9	93.7
Undeuchaeta plumosa	1	1	0	0	0	UNDPL	+	66.4	248.6
Undeuchaeta spp.	0	1	0	0	0		+	1.2	6.2
Acartia clausi	0	0	1	0	0			4.7	23.5
Oithona plumifera	0	0	0	0	1			2.9	14.3
Other/damaged Copepods	1	1	1	1	0			14.6	23.5
Conchoecissa imbricata	1	0	0	0	0		+	2.1	10.4
Conchoecilla daphnoides	1	0	0	0	0		+	2.1	10.4
Schistomysis ornata	0	0	1	0	1	SCHOR	+	351.9	1716.8
Schistomysis/Paramysis spp.	0	0	1	0	0		+	37.6	188.1
Haplostylus normani	0	0	1	0	1	HAPNO	+	390.1	1693.3
Haplostylus lobatus	0	0	1	1	1		+	34.3	71.4
Leptomysis gracilis	0	0	0	1	1		+	4.8	14.3
Mysid inmature/juvenile	0	0	1	1	1	MYSID	+	460.6	1716.8
Mysid damaged	0	0	1	0	1			26.4	117.6
Parathemisto abyssorum	1	0	0	0	0		+	0.1	0.7
Hyperia galba	0	0	0	0	1		+	0.2	0.9
Amphipod Gammaridae	0	0	1	0	1		+	21.8	85.7
Unknown amphipods	1	0	0	0	1			3.1	14.3
Diastylidae	0	0	1	0	1		+	51.1	141.1
Meganyctiphanes norvegica	1	1	0	0	0	MEGNO	+	351.2	1156.5
Nematoscelis megalops	1	1	0	0	0		+	16.0	42.9
Thysanoessa longicauda	1	0	0	0	0		+	2.1	10.4
Nematobrachion boopis	0	1	0	0	0		+	1.2	6.2
Euphausiacea spp.	1	1	0	0	0	EUPHA		181.9	468.3
Pasiphaea sivado	1	1	0	0	0		+	3.2	13.2
Pasiphaea spp.	1	0	0	0	0		+	4.2	20.8
Systellaspis debilis	1	0	0	0	0		+	0.1	0.7
Solenocera larvae	0	1	1	1	1	SOLEN	+	335.5	1367.3
Galatheidean megalopae	0	0	1	0	1		+	27.5	114.2
Paguridean megalopae	0	0	0	0	1		+	11.4	57.1
Zoea Porcellana	1	0	1	0	1			72.3	258.7
Zoea Galatheoidea ≠ Porcell.	0	0	0	0	1			2.9	14.3
Liocarcinus holsatus	0	0	1	0	1		+	26.3	128.5
Liocarcinus pusillus	0	0	0	0	1		+	2.9	14.3
Liocarcinus spp. damaged	0	0	0	0	1			2.9	14.3
Polybius henslowi	0	1	0	0	0		+	0.2	0.8
Discarded Swimming Crabs	0	1	1	0	0			1.3	5.9
Corystes cassivelaunus megalopa	0	0	0	0	1		+	51.4	257.0
Brachyuran zoeae	1	1	1	1	1	BRAZO		472.8	1442.1
Other brachyuran megalopae	1	0	1	1	1	BRAME	+	200.8	842.4
Other decapod larvae	1	1	1	1	1		+	94.2	299.8
Chaetognatha	1	1	0	1	1		+	20.1	31.2
Echinodermata larvae	0	0	1	1	1	ECHIN		134.8	428.3
Oikopleura spp.	0	0	1	0	1			7.6	23.5
Ammodytidae	0	0	1	0	0		+	1.2	5.9
Merlangius merlangus	0	0	0	0	1		+	0.2	0.9
Benthosema glaciale	1	1	0	0	0		+	3.7	16.3
Myctophidae larvae	1	1	0	0	0		+	21.6	83.3
Clupeid larvae ≠ anchovy	1	1	1	1	1	OCLUP		216.8	656.8
Other fish larvae	1	1	1	1	1			128.5	385.5
Others (non-gelatinous)	1	1	1	1	1			141.7	285.6
Gelatinous								2461.5	4405.5
Non-Gelatinous								4960.2	7897.4
Total								7421.6	10299.6

Table 3. Results of the application of the assay targeting *Engraulis encrasicolus* DNA to the selected macrozooplankton predators. MIK hauls data including *E. encrasicolus* eggs abundance (ind. 1000 m^{-3}) are shown along with the percentage of the assays testing positive for *E. encrasicolus* DNA. The total number of predators assayed per species and station is also shown as well as the abundance of these putative predators at the sampled point (ind. 1000 m^{-3}).

	MIK-A			MIK-B			MIK-C			MIK-D			MIK-E		
Date	5/8/2010			5/11/2010			5/13/2010			5/14/2010			5/15/2010		
Time of haul (local time)	3:28			4:46			1:12			5:33			1:13		
Haul depth (m)	65.6			63.6			45.8			54.8			53.5		
Bottom depth (m)	1600			1153			79			94			73		
Anchovy eggs (PairoVET)	2291.9			2568.4			0.0			4075.2			14482.1		
Anchovy eggs at 3m depth (CUFES)	22404.8			28790.6			166.2			25926.8			60850.6		
	% + assays	n assayed	Abundance	% + assays	n assayed	Abundance	% + assays	n assayed	Abundance	% + assays	n assayed	Abundance	% + assays	n assayed	Abundance
Tomopteris spp.	4.5	22	229.0	0.0	1	6.2		0	0.0	100.0	1	9.8		0	0.0
Cymbulia peroni	0.0	8	5.2	0.0	7	5.4		0	0.0		0	0.0		0	0.0
Diacria trispinosa		0	0.0	0.0	1	6.2		0	0.0		0	0.0		0	0.0
Candacia armata	12.5	8	114.5		0	0.0		0	0.0	0.0	1	9.8	100.0	2	28.6
Euchirella rostrata	0.0	13	145.7	0.0	14	354.2		0	0.0		0	0.0		0	0.0
Euchirella curticauda	0.0	3	31.2	50.0	2	12.4		0	0.0		0	0.0		0	0.0
Metridia lucens	0.0	1	10.4	0.0	1	18.6		0	0.0		0	0.0		0	0.0
Pleuromamma robusta	0.0	4	52.0	0.0	15	174.0		0	0.0		0	0.0		0	0.0
Pleuromamma spp.	0.0	1	20.8		0	6.2		0	0.0		0	0.0		0	0.0
Euchaeta acuta	0.0	4	41.6	0.0	1	18.6		0	0.0		0	0.0		0	0.0
Euchaeta hebes	10.0	10	104.1	0.0	6	55.9		0	0.0		0	0.0		0	0.0
Euchaeta spp.	0.0	4	52.0	0.0	10	68.4		0	0.0		0	0.0		0	0.0
Paraeuchaeta gracilis	15.0	20	208.1	12.5	8	74.6		0	0.0		0	0.0		0	0.0
Paraeuchaeta norvegica	0.0	4	41.6	0.0	1	6.2		0	0.0		0	0.0		0	0.0
Paraeuchaeta tonsa	4.2	24	260.2	0.0	8	55.9		0	0.0		0	0.0		0	0.0
Paraeuchaeta spp.	0.0	1	10.4		0	0.0		0	0.0		0	0.0		0	0.0
Undeuchaeta major	0.0	8	93.7	10.0	10	80.8		0	0.0		0	0.0		0	0.0
Undeuchaeta plumosa	11.1	9	83.3	8.3	24	248.6		0	0.0		0	0.0		0	0.0
Undeuchaeta spp.		0	0.0	0.0	1	6.2		0	0.0		0	0.0		0	0.0
Conchoecissa imbricata	0.0	1	10.4		0	0.0		0	0.0		0	0.0		0	0.0
Conchoecilla daphnoides	0.0	1	10.4		0	0.0		0	0.0		0	0.0		0	0.0
Schistomysis ornata		0	0.0		0	0.0	0.0	73	1716.8		0	0.0	100.0	3	42.8
Schistomysis/Paramysis spp.		0	0.0		0	0.0	25.0	8	188.1		0	0.0		0	0.0
Haplostylus normani		0	0.0		0	0.0	5.6	72	1693.3		0	0.0	100.0	18	257.0
Haplostylus lobatus		0	0.0		0	0.0	0.0	3	70.6	0.0	3	29.5	100.0	5	71.4
Leptomysis gracilis		0	0.0		0	0.0		0	0.0	100.0	1	9.8	100.0	1	14.3
Mysid inmature/juvenile		0	0.0		0	0.0	2.7	73	1716.8	0.0	3	29.5	100.0	40	556.8
Parathemisto abyssorum	0.0	1	0.7		0	0.0		0	0.0		0	0.0		0	0.0
Hyperia galba		0	0.0		0	0.0		0	0.0		0	0.0	100.0	1	0.9
Amphipod Gammaridae		0	0.0		0	0.0	0.0	1	23.5		0	0.0	83.3	6	85.7
Diastylidae		0	0.0		0	0.0	66.7	6	141.1		0	0.0	100.0	8	114.2
Meganyctiphanes norvegica	3.8	52	1156.5	0.0	62	599.7		0	0.0		0	0.0		0	0.0
Nematoscelis megalops	0.0	6	42.9	0.0	6	37.3		0	0.0		0	0.0		0	0.0
Thysanoessa longicauda	0.0	1	10.4		0	0.0		0	0.0		0	0.0		0	0.0
Nematobrachion boopis		0	0.0	0.0	1	6.2		0	0.0		0	0.0		0	0.0
Pasiphaea sivado	0.0	3	2.6	10.0	10	13.2		0	0.0		0	0.0		0	0.0
Pasiphaea spp.	0.0	2	20.8		0	0.0		0	0.0		0	0.0		0	0.0
Systellaspis debilis	100.0	1	0.7		0	0.0		0	0.0		0	0.0		0	0.0
Solenocera larvae		0	0.0	0.0	1	6.2	50.0	2	47.0	72.7	132	1367.3	100.0	14	257.0
Galatheidean megalopae		0	0.0		0	0.0	100.0	1	23.5		0	0.0	100.0	9	114.2
Paguridean megalopae		0	0.0		0	0.0		0	0.0		0	0.0	100.0	4	57.1
Liocarcinus holsatus		0	0.0		0	0.0	0.0	2	2.9		0	0.0	100.0	9	128.5
Liocarcinus pusillus		0	0.0		0	0.0		0	0.0		0	0.0	100.0	1	14.3
Polybius henslowi		0	0.0	100.0	1	0.8		0	0.0		0	0.0		0	0.0
Corystes cassivelaunus megalopa		0	0.0		0	0.0		0	0.0		0	0.0	100.0	18	257.0
Other brachyuran megalopae	0.0	1	10.4		0	0.0	0.0	1	23.5	66.7	12	127.9	100.0	59	842.4
Other decapod larvae	0.0	3	62.4		0	18.6	0.0	2	70.6	50.0	2	19.7	100.0	13	299.8
Chaetognatha	0.0	3	31.2	0.0	4	31.1		0	0.0	0.0	1	9.8	100.0	2	28.6
Ammodytidae		0	0.0		0	0.0	0.0	4	5.9		0	0.0		0	0.0
Merlangius merlangus		0	0.0		0	0.0		0	0.0		0	0.0	100.0	1	0.9
Benthosema glaciale	0.0	3	2.0	0.0	21	16.3		0	0.0		0	0.0		0	0.0
Myctophidae larvae	0.0	9	83.3	25.0	4	24.9		0	0.0		0	0.0		0	0.0
Total	4.8	231	2948.4	3.6	220	1952.9	5.6	248	5723.6	68.6	156	1613.2	99.5	214	3171.5

Table 4. Fish hauls. Data on fish haul including local time, depth and captured fish. The information on assayed sardines and sprats is also shown (average values and range): cardiac and pyloric stomach visual fullness, female gonad stages, length, weight and the percentage of fish testing positive for *E. encrasicolus* DNA presence along with the average number of consumed anchovy eggs (minimum value as estimated by Ct values, see section 2.1.2). For sprat no female gonad data is provided as every individual was immature/resting or spent. The PairoVET based *E. encrasicolus* eggs abundance is also shown (ind. 1000 m^{-3}), including the temporal lag with the fish haul (hours earlier or later for the fish haul with respect to the PairoVET net one). MIDD, LAFT and MIDN for, respectively, midday, late afternoon, and midnight.

Haul	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Date	5/6/2010	5/7/2010	5/9/2010	5/10/2010	5/13/2010	5/14/2010	5/15/2010	5/16/2010	5/17/2010	5/18/2010	5/18/2010	5/18/2010	5/18/2010	5/19/2010
Time of haul (local time)	18:56	12:40	19:12	19:58	12:19	19:00	14:29	23:50	11:30	0:29	12:10	19:50	22:41	11:55
MIDD-LAFT-MIDN	LAFT	MIDD	LAFT	LAFT	MIDD	LAFT	MIDD	MIDN	MIDD	MIDN	MIDD	LAFT	MIDN	MIDD
Haul depth (m)	17	14	70	95	88	30	35	15	113	5	40	53	5	66
Bottom depth (m)	>1000	31	100	110	105	42	48	40	125	36	60	67	38	75
Sardine Total (kg)	7.1	63.0	4.1	3.5	5.6	5.0	4.8	0.9	2.3	0.0	5.0	0.0	0.0	0.0
Sardine sorted for assay (n)	30	21	30	32	29		32	19	31		13			
Sprat total (kg)	0	0	0	0	0	46	0.5	0	0	0.85	110	550	0.66	5.2
Sprat sorted for assay (n)						32	31			30	30	30	30	30
Other fish (kg)	210.8	23.5	403.1	148.9	42.7	2000.0	26.5	0.0	144.5	31.0	386.5	48.0	143.1	119.6
Lag with DEPM sampling	<36h earlier	<36h earlier	<12h later	<36h later	<3 days later	<12h later	<24h earlier	<12h earlier	<5 days later	<4 days later	<3 days later	<3 days later	<3 days later	<3 days later
Anchovy eggs (PairoVET)	2694.6	222.7	3249.9	3466.5	1458.1	2108.0	4943.6	105.4	1761.3	323.7	1336.9	3492.1	1700.3	5677.5
Assayed sardine characteristics:														
Sardine gut fullness (cardiac)	2.5 (1-4)	3.1 (2-4)	3.3 (1-4)	2.3 (1-4)	1.1 (1-2)		2.0 (1-4)	2.5 (1-4)	1.1 (1-2)		1.1 (1-2)			
Sardine gut fullness (pyloric caeca)	3.0 (2-3)	3.2 (3-4)	2.8 (1-4)	2.7 (1-4)	3.0 (3-4)		2.9 (2-3)	2.7 (1-3)	1.9 (1-3)		1.0 (1-1)			
Sardine ♀ gonad stage	3.7 (3-5)	3.5 (2.5)	3.3 (3-5)	3.5 (3-4)	3.5 (2.5)		3.9 (3-5)	3.9 (3-5)	4.5 (4-5)		3.7 (3.5)			
Sardine total length (mm)	196 (182-225)	191 (167-227)	188 (141-208)	194 (176-217)	193 (178-217)		178 (138-211)	179 (160-213)	189 (164-209)		187 (178-198)			
Sardine total weight (g)	62 (45-94)	61 (39-90)	57 (20-83)	62 (46-94)	62 (45-81)		49 (21-80)	46 (32-76)	60 (38-86)		55 (46-71)			
Sardine % positive assays	83.3	76.2	93.3	87.5	48.3		100	84.2	74.2		100			
Average anchovy eggs per fish	2.7 (0-25)	3.6 (0-25.7)	19.8 (0-80.6)	18.8 (0-169)	2.5 (0-17.7)		41.8 (1-151.7)	18 (0-61.8)	17.2 (0-252.1)		2 (1-11.3)			
Assayed sprat characteristics:														
Sprat gut fullness (cardiac)						2.2 (1-3)	2.1 (1-3)			1.9 (1-3)	2.1 (1-3)	3.4 (2-4)	2.4 (1-3)	1.6 (1-3)
Sprat gut fullness (pyloric caeca)						3.0 (3-3)	3.0 (3-3)			3.0 (3-3)	3.0 (3-3)	3.0 (3-3)	2.9 (2-3)	3.0 (3-3)
Sprat total length (average-range)						99 (90-105)	122 (112-135)			88 (80-98)	102 (93-111)	106 (99-117)	100 (93-116)	121 (109-133)
Sprat total weight (average-range)						8 (6-11)	15 (12-18)			6 (4-8)	9 (7-13)	10 (7-13)	9 (6-13)	17 (13-22)
Sprat % positive assays						25	61.3			10	60	63.3	50	26.7
Average anchovy eggs per fish						8.3 (0-51.2)	7.2 (0-42)			1.9 (0-28.5)	12.4 (0-70.9)	0.8 (0-4.4)	3.9 (0-49.2)	1.1 (0-27.2)

Table 5. Proportion of anchovy eggs mortality explained by fish predation (P_C). Results are shown along with the data needed for computation. These includes data from the BIOMAN and PELGAS 2010 surveys (Z and B respectively; ICES 2010 a, b) along with *in situ* produced data and others from available literature (see section 2.3.2). Data are shown by geographical area for sardine but for the whole Bay of Biscay area for sprat due to the lack of regional acoustic biomass for the latter. For sardine, acoustic biomasses from PELGAS 2010 campaign´s "south-offshore" and "Gironde" areas were selected as they roughly corresponded to the two anchovy spawning centers in 2010.

	Sardina pilchardus		*Sprattus sprattus*
	Shelf-break	Gironde shelf	Bay of Biscay
t ´ = duration of feeding (h)	24	24	14
g = gastric evacuation rate (h^{-1})	0.2105	0.2105	0.2105
EE = Average number of eggs per predator kg during time t´	174.12	567.31	538.47
C = Average anchovy eggs eaten daily per kg of predator	879.63	2866.06	1586.87
B = Biomass from acoustics (kg)	3.02E+07	2.73E+07	6.70E+07
C_t = Total anchovy eggs eaten daily (C X B)	2.66E+10	7.83E+10	1.06E+11
G_t = Total daily anchovy egg production	2.32E+12	2.32E+12	2.32E+12
t = anchovy egg development time (h)	72	72	72
Anchovy eggs daily instantaneous mortality rate	0.34	0.34	0.34
Z = Anchovy eggs hourly instantaneous mortality rate	0.0142	0.0142	0.0142
P_C =	**1.79E-02**	**5.28E-02**	**7.17E-02**

FIGURES LEGENDS

-Figure 1. *Engraulis encrasicolus* DNA assay design. Alignment showing the location of the TaqMan assay primers and probe on the cytochrome-b gene. Forward primer (5´-3´): TTCTTACATGAATCGGAGGTATGC, Probe (5´-3´): CGAACACCCATTCAT, and Reverse primer (5´-3´) GGAARATAGAGAAGTAGAGTAGCGATGCT (reverse complement of the sequence shown in the alignment, grey highlighted). Species codes as in Table 1.

-Figure 2. *E. encrasicolus* DNA assay validation. a) Standard curve: rhombus representing threshold cycle (Ct, left axis) values plotted against serial dilutions of *E. encrasicolus* template DNA (ng, bottom axis); assay detection limit (0.005 ng DNA for a Ct of 35.4), average Ct values and standard deviations (in brackets) are superimposed along with the number of replicas tested and the number of assays giving a negative signal. Grey triangles signal one *E. encrasicolus* egg results (from right to left: undiluted, 1/10 and 1/100 dilutions from one egg extract). b) Superimposed lines and squares showing the thresholds defined to call a positive assay [Ct thresholds as a function of the amount (ng) of assayed DNA]; circles represent the results for all the tested *Sprattus sprattus* raw tissue´s DNA extractions. See section 2.1.2 for further information.

-Figure 3. Prey and predators´ spatial location. *E. encrasicolus* egg abundance (ind. 1000 m^{-3}, scale superimposed) during the BIOMAN 2010 campaign obtained with SURFER 10 (Golden Software) with PairoVET data. Apart from this, while the grid of black dots signals PairoVET stations (parallel cross-shelf transects perpendicular to the coast), locations of the macrozooplankton stations (MIK hauls, red stars) and fish hauls (pelagic trawling; blue crosses) are also shown. A pink circle locates the MIK haul performed to collect live animals for the digestion experiment (see section 2.2.4). Isobaths of 100, 200 and 1000 m are shown (dotted lines) along with the position of the Adour and Gironde river mouths and the Arcachon Bay. The two main spawning centers for anchovy, at the shelf in front of the Gironde river mouth ("Gironde shelf" one) and at the shelf-break between Arcachon Bay and Adour river mouth (shelf-break one), can be easily identified.

-Figure 4. Detectability curves for *Liocarcinus spp.* onboard digestion experiment. Respectively, white circles for *Liocarcinus depurator* specimens (n = 16; CW average of 1.53 mm, 0.10 SD) and, white squares for *Liocarcinus holsatus* (n = 12; average CW 1.58 mm, 0.14 SD). Detectability half-life (T_{50}) line is superimposed (see section 2.2.4 for further information).

-Figure 5. Macrozooplankton community. Detrended correspondence analysis (DCA; CANOCO software) of BIOMAN 2010 macrozooplankton taxa. Species codes as in Table 2, sampled MIK stations (MIK-A, B, C, D, and E) represented by black dots. The cumulative explained variance by the first axis was 81 %; when adding the second axis this shifted to 83 %.

-Figure 6. Macrozooplankton predation on anchovy eggs. Full symbols represent the relationship between the occurrence of *E. encrasicolus* DNA remains, predation incidence, in macrozooplankton taxa (rhombus and circles for the, respectively, Gironde shelf and shelf-break communities as clustered in CANOCO analysis) and the abundance of *E. encrasicolus* eggs at the MIK haul location. The relationship between egg abundance and the daily mortality (M_P; see section 2.3.1) due to macrozooplankton is shown by the empty symbols. For MIK-C station, due to the low resolution of PairoVET net counts at low egg abundances, anchovy egg abundance was obtained by transforming eggs counts at 3 m depth (CUFES sampling device) to those at the water column (0.15 * abundances at 3 m).

-Figure 7. Sardine and sprat predation on anchovy eggs. a) Relationship between the occurrence of *E. encrasicolus* DNA remains in stomachs of *S. pilchardus* (black rhombus and continuous line) and *S. sprattus* (white rhombus and broken line), the predation incidence, and the estimated abundance of *E. encrasicolus* eggs at the fish haul area (bottom axis). Superimposed lines represent optimal regression model based on Akaike Information Criterion (AIC) fitted to the data (respectively, P = 0.589 and P = 0.155 for sardine and sprat curves). b) Relationship between the average number of *E. encrasicolus* eggs predated by individual in each haul (minimum values based in assay´s Ct values; see section 2.3.2) and the estimated abundance of *E. encrasicolus* eggs (bottom axis). Optimal regression model superimposed (respectively, P = 0.316 and P = 0.311 for sardine and sprat).

SUPPLEMENTARY MATERIAL TABLES

Supp. Mat. Table 1. GenBank accession number and geographical location for the 186 clupeid sequences (cytochrome-b; cytB) used in the study. Locations for the AM911183-89 sequences obtained from personal communication with M. Jerome (GenBank submission responsible).

Species	Location	GenBank accession no.	Origin
Engraulis encrasicolus	Unknown	NC_009581	GenBank
	South Africa	EU552563-64	GenBank
	E. Mediterranean	JQ012359-60	GenBank
	Bay of Biscay	AF472579	GenBank
	Canary Islands	DQ197948	GenBank
	Cantabrian Sea	EF427558-59	GenBank
	W. Mediterranean	EF439526-27	GenBank
	Bay of Biscay	EU224051-52	GenBank
	North Sea	EU492081-82	GenBank
	Unknown	AM911183	GenBank
	Bay of Biscay	AM911184	GenBank
	Cantabrian Sea	AM911185	GenBank
	Celtic Sea	AM911186-89	GenBank
	E. Mediterranean	EU264006-07	GenBank
	Bay of Biscay	KF972469-88	This study
	Celtic Sea	KF972503-07	This study
	North Sea	KF972508-12	This study
	Baltic Sea	KF972513-17	This study
	W. Mediterranean	KF972518-24	This study
	E. Mediterranean	KF972525-29	This study
	Canary Islands	KF972530-34	This study
	South Africa	KF972535-39	This study
Engraulis japonicus	Unknown	NC_003097	GenBank
	Unknown	AB040676	GenBank
	Japan	AB374208-22	GenBank
	Unknown	AM911190	GenBank
	Unknown	KF972542	This study
Engraulis anchoita	Unknown	AM911194	GenBank
	Brazil	JQ012416-17	GenBank
	Unknown	KF972540-01	This study
Engraulis ringens	Peru	JQ012418	GenBank
	Peru	JQ012426	GenBank
	Unknown	AM911192-93	GenBank
	Unknown	KF972543	This study
Engraulis mordax	Pacific Ocean, USA	JQ012421	GenBank
	Pacific Ocean, USA	JQ012350	GenBank
	Unknown	AM911191	GenBank
Anchoa mitchilli	Gulf of Mexico, USA	JQ012357-58	GenBank
Sprattus sprattus	Unknown	NC_009593	GenBank
	Bay of Biscay	AF472581	GenBank
	North Sea	EU492085-86	GenBank
	Baltic Sea	EU492332-33	GenBank
	Bay of Biscay	EU224085-86	GenBank
	Unknown	AM911199	GenBank
	Bay of Biscay	KF972489-502	This study
Cuplea harengus	Unknown	NC_009577	GenBank
	Canada (Quebec)	EU552602-06	GenBank
	North Sea	AF472580	GenBank
	Norway	AB278564	GenBank
	Bay of Biscay	EU224003-04	GenBank
	North Sea	EU492087-88	GenBank
	Baltic Sea	EU492334-35	GenBank
Sardina pilchardus	Unknown	NC_009592	GenBank
	Bay of Biscay	AF472582	GenBank
	Canary Islands	DQ197989	GenBank
	North Sea	EU492102-03	GenBank
	Unknown	AM911198	GenBank
	E. Mediterranean	EU264017-18	GenBank
	Bay of Biscay	EU224030-31	GenBank
Sardinella aurita	W. Mediterranean	EF439569-70	GenBank
	Canary Islands	EF392611-12	GenBank
	E. Mediterranean	EV036480-81	GenBank
	Canary Islands	DQ197990	GenBank
	Ivory Coast	AF472584	GenBank
	Gulf of Mexico, USA	EU552619	GenBank
Sardinops sagax	South Africa	EU552565-66	GenBank
	Chile	AF472586	GenBank
Alosa fallax	Baltic Sea	EU492309-10	GenBank
	North Sea	EU492079-80	GenBank
	Bay of Biscay	EU223994-95	GenBank
	Unknown	EU552574-76	GenBank
Alosa alosa	Unknown	NC_009575	GenBank
	Bay of Biscay	EU224045-46	GenBank

Supp. Mat. Table 2. Macrozooplankton species list showing abundances (individuals 1000m^{-3}) at each MIK haul. Last four columns show the relative abundance (both average and maximum values) taking into account, respectively, all the counted taxa and, also without the gelatinous individuals.

	Abundance (ind. 1000m^{-3})					Relative abundance (%)			
	A	B	C	D	E	Average	Max.	Average	Max.
Gelatinous									
Jellyfishes	10.4	0.0	23.5	67.6	1317.1	2.8	12.8		
Siphonophora	1082.3	167.8	211.7	3855.9	1085.1	17.3	50.8		
Ctenophora	2.6	5.4	0.0	0.0	0.0	0.0	0.1		
Salps	382.5	1623.5	0.0	0.0	0.0	6.6	25.9		
Gelatinous remains	125.5	1864.3	0.0	482.0	0.0	7.7	29.7		
Non-Gelatinous									
Tomopteris spp.	229.0	6.2	0.0	9.8	0.0	0.9	4.1	1.3	5.8
Cymbulia peroni	5.2	5.4	0.0	0.0	0.0	0.0	0.1	0.1	0.2
Diacria trispinosa	0.0	6.2	0.0	0.0	0.0	0.0	0.1	0.0	0.2
Pteropod spp.	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Podon spp.	0.0	0.0	0.0	0.0	14.3	0.0	0.1	0.0	0.2
Calanus helgolandicus	124.9	68.4	94.1	609.9	1299.3	5.1	12.6	8.5	19.1
Calanus robustior	0.0	12.4	0.0	0.0	0.0	0.0	0.2	0.1	0.5
Eucalanus crassus	0.0	0.0	0.0	9.8	0.0	0.0	0.1	0.1	0.3
Temora longicornis	0.0	0.0	0.0	0.0	57.1	0.1	0.6	0.1	0.7
Centropages typicus	0.0	24.9	0.0	0.0	0.0	0.1	0.4	0.2	1.0
Candacia armata	114.5	0.0	0.0	9.8	28.6	0.5	2.1	0.7	2.9
Euchirella rostrata	145.7	354.2	0.0	0.0	0.0	1.7	5.6	3.5	13.6
Euchirella curticauda	31.2	12.4	0.0	0.0	0.0	0.2	0.6	0.3	0.8
Metridia lucens	10.4	18.6	0.0	0.0	0.0	0.1	0.3	0.2	0.7
Pleuromamma robusta	52.0	174.0	0.0	0.0	0.0	0.7	2.8	1.6	6.7
Pleuromamma spp.	20.8	6.2	0.0	0.0	0.0	0.1	0.4	0.2	0.5
Euchaeta acuta	41.6	18.6	0.0	0.0	0.0	0.2	0.8	0.4	1.1
Euchaeta hebes	104.1	55.9	0.0	0.0	0.0	0.6	1.9	1.0	2.7
Euchaeta spp.	52.0	68.4	0.0	0.0	0.0	0.4	1.1	0.8	2.6
Paraeuchaeta gracilis	208.1	74.6	0.0	0.0	0.0	1.0	3.8	1.6	5.3
Paraeuchaeta norvegica	41.6	6.2	0.0	0.0	0.0	0.2	0.8	0.3	1.1
Paraeuchaeta tonsa	260.2	55.9	0.0	0.0	0.0	1.1	4.7	1.8	6.6
Paraeuchaeta spp.	10.4	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.3
Undeuchaeta major	93.7	80.8	0.0	0.0	0.0	0.6	1.7	1.1	3.1
Undeuchaeta plumosa	83.3	248.6	0.0	0.0	0.0	1.1	4.0	2.3	9.5
Undeuchaeta spp.	0.0	6.2	0.0	0.0	0.0	0.0	0.1	0.0	0.2
Acartia clausi	0.0	0.0	23.5	0.0	0.0	0.1	0.3	0.1	0.3
Oithona plumifera	0.0	0.0	0.0	0.0	14.3	0.0	0.1	0.0	0.2
Other/damaged Copepods	20.8	18.6	23.5	9.8	0.0	0.2	0.4	0.4	0.7
Conchoecissa imbricata	10.4	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.3
Conchoecilla daphnoides	10.4	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.3
Schistomysis ornata	0.0	0.0	1716.8	0.0	42.8	4.7	23.1	4.9	23.9
Schistomysis/Paramysis spp.	0.0	0.0	188.1	0.0	0.0	0.5	2.5	0.5	2.6
Haplostylus normani	0.0	0.0	1693.3	0.0	257.0	5.1	22.8	5.4	23.6
Haplostylus lobatus	0.0	0.0	70.6	29.5	71.4	0.4	1.0	0.6	1.0
Leptomysis gracilis	0.0	0.0	0.0	9.8	14.3	0.1	0.1	0.1	0.3
Mysid inmature/juvenile	0.0	0.0	1716.8	29.5	556.8	5.8	23.1	6.4	23.9
Mysid damaged	0.0	0.0	117.6	0.0	14.3	0.3	1.6	0.4	1.6
Parathemisto abyssorum	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Hyperia galba	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0
Amphipod Gammaridae	0.0	0.0	23.5	0.0	85.7	0.2	0.8	0.3	1.1
Unknown amphipods	1.3	0.0	0.0	0.0	14.3	0.0	0.1	0.0	0.2
Diastylidae	0.0	0.0	141.1	0.0	114.2	0.6	1.9	0.7	2.0
Meganyctiphanes norvegica	1156.5	599.7	0.0	0.0	0.0	6.1	20.9	10.5	29.5
Nematoscelis megalops	42.9	37.3	0.0	0.0	0.0	0.3	0.8	0.5	1.4
Thysanoessa longicauda	10.4	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.3
Nematobrachion boopis	0.0	6.2	0.0	0.0	0.0	0.0	0.1	0.0	0.2
Euphausiacea spp.	468.3	441.2	0.0	0.0	0.0	3.1	8.5	5.8	16.9
Pasiphaea sivado	2.6	13.2	0.0	0.0	0.0	0.1	0.2	0.1	0.5
Pasiphaea spp.	20.8	0.0	0.0	0.0	0.0	0.1	0.4	0.1	0.5
Systellaspis debilis	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Solenocera larvae	0.0	6.2	47.0	1367.3	257.0	4.2	18.0	9.4	42.9
Galatheidean megalopae	0.0	0.0	23.5	0.0	114.2	0.3	1.1	0.4	1.4
Paguridean megalopae	0.0	0.0	0.0	0.0	57.1	0.1	0.6	0.1	0.7
Zoea Porcellana	31.2	0.0	258.7	0.0	71.4	0.9	3.5	1.1	3.6
Zoea Galatheoidea ≠ Porcell.	0.0	0.0	0.0	0.0	14.3	0.0	0.1	0.0	0.2
Liocarcinus holsatus	0.0	0.0	2.9	0.0	128.5	0.3	1.2	0.3	1.6
Liocarcinus pusillus	0.0	0.0	0.0	0.0	14.3	0.0	0.1	0.0	0.2
Liocarcinus spp. damaged	0.0	0.0	0.0	0.0	14.3	0.0	0.1	0.0	0.2
Polybius henslowi	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Discarded Swimming Crabs	0.0	0.8	5.9	0.0	0.0	0.0	0.1	0.0	0.1
Corystes cassivelaunus megalopa	0.0	0.0	0.0	0.0	257.0	0.5	2.5	0.7	3.3
Brachyuran zoeae	31.2	24.9	423.3	442.6	1442.1	5.3	14.0	8.0	18.3
Other brachyuran megalopae	10.4	0.0	23.5	127.9	842.4	2.1	8.2	3.1	10.7
Other decapod larvae	62.4	18.6	70.6	19.7	299.8	1.1	2.9	1.5	3.8
Chaetognatha	31.2	31.1	0.0	9.8	28.6	0.3	0.6	0.5	1.2
Echinodermata larvae	0.0	0.0	117.6	127.9	428.3	1.5	4.2	2.2	5.4
Oikopleura spp.	0.0	0.0	23.5	0.0	14.3	0.1	0.3	0.1	0.3
Ammodytidae	0.0	0.0	5.9	0.0	0.0	0.0	0.1	0.0	0.1
Merlangius merlangus	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0
Benthosema glaciale	2.0	16.3	0.0	0.0	0.0	0.1	0.3	0.1	0.6
Myctophidae larvae	83.3	24.9	0.0	0.0	0.0	0.4	1.5	0.6	2.1
Clupeid larvae ≠ anchovy	52.0	6.2	211.7	157.4	656.8	2.5	6.4	3.6	8.3
Other fish larvae	72.8	24.9	70.6	88.5	385.5	1.5	3.7	2.3	4.9
Others (non-gelatinous)	168.5	32.6	94.1	127.9	285.6	1.9	3.1	2.9	4.3
Gelatinous	1603.3	3661.0	235.2	4405.5	2402.2	34.4	58.4		
Non-Gelatinous	3919.5	2609.2	7187.6	3187.0	7897.4	65.6	96.8	100.0	100.0
Total	5522.9	6270.3	7422.8	7592.6	10299.6	100.0	100.0		

Supp. Mat. Table 3. *Sardina pilchardus*. Data sorted by haul time; MIDD, LAFT and MIDN for, respectively, midday, late afternoon, and midnight. The information on the assayed individuals is shown (average values and range). The percentage of fish testing positive for the *E. encrasicolus* DNA presence is shown for each defined category for length, weight, female gonad stages and cardiac stomach visual fullness (see section 2.2.2 and 2.3 for further information). The data for pyloric stomach fullness are not shown due to the reduced variability of this visual index. N/A stands for not available.

	All	MIDD	LAFT	MIDN
N hauls	9	5	3	1
Sardine sorted for assay (n)	237	126	92	19
Assayed sardine characteristics:				
Sardine gut fullness (cardiac)	2.1 (1-4)	1.6 (1-4)	2.7 (1-4)	2.5 (1-4)
Sardine gut fullness (pyloric caeca)	2.7 (1-4)	2.6 (1-4)	2.8 (1-4)	2.7 (1-3)
Sardine ♀ gonad stage	3.7 (2-5)	4 (2-5)	3.5 (3-5)	3.9 (3-5)
Sardine total length (mm)	189 (138-227)	187 (138-227)	193 (141-225)	179 (160-213)
Sardine total weight (g)	57.6 (20-94)	57.1 (21-90)	60.5 (20-94)	46.3 (32-76)
Sardine % positive assays (n)				
Total	82.3 (237)	77.8 (126)	88.0 (92)	84.2 (19)
size (≤ 190 mm TL)	82.8 (134)	81.3 (80)	86.8 (38)	81.25 (16)
size (> 190 mm TL)	81.6 (103)	71.7 (46)	88.9 (54)	100 (3)
weight (≤ 60 g)	83.7 (153)	83.5 (85)	84 (50)	83.3 (18)
weight (> 60 g)	79.8 (84)	65.9 (41)	92.9 (42)	100 (1)
female gonad stage 2	50 (2)	50 (2)	N/A (0)	N/A (0)
female gonad stage 3	90.3 (31)	100 (4)	88 (25)	100 (2)
female gonad stage 4	87.2 (47)	94.7 (19)	86.4 (22)	66.7 (6)
female gonad stage 5	90.9 (11)	87.5 (8)	100 (2)	100 (1)
cardiac fullness (1)	75 (96)	71.4 (77)	87.5 (16)	100 (3)
cardiac fullness (2)	83.3 (48)	82.8 (29)	85.7 (14)	80 (5)
cardiac fullness (3)	86.9 (61)	100 (7)	86.4 (44)	80 (10)
cardiac fullness (4)	93.8 (32)	92.3 (13)	94.4 (18)	100 (1)

Supp. Mat. Table 4. *Sprattus sprattus*. Data sorted by haul time; MIDD, LAFT and MIDN for, respectively, midday, late afternoon, and midnight. The information on the assayed individuals is shown (average values and range). The percentage of fish testing positive for the *E. encrasicolus* DNA presence is shown for each defined category for length, weight, and cardiac stomach visual fullness (see section 2.2.2 and 2.3 for further information). For sprat no female gonad data is provided as every individual was immature/resting or spent. The data for pyloric stomach fullness are not shown due to the reduced variability of this visual index. N/A stands for not available.

	All	MIDD	LAFT	MIDN
N hauls	7	3	2	2
Sprat sorted for assay (n)	213	91	62	60
Assayed sprat characteristics:				
Sprat gut fullness (cardiac)	2.2 (1-4)	2 (1-3)	2.8 (1-4)	2.2 (1-3)
Sprat gut fullness (pyloric caeca)	3.0 (2-3)	3.0 (3)	3 (3)	2.9 (2-3)
Sprat total length (average-range)	105 (80-135)	115 (93-135)	102 (90-117)	94 (80-116)
Sprat total weight (average-range)	10.5 (4-22)	13.9 (7-22)	9 (6-13)	7 (4-13)
Sprat % positive assays (n)				
Total	42.3 (213)	49.5 (91)	43.5 (62)	30 (60)
size (≤100 mm TL)	29.8 (84)	70 (10)	26.1 (23)	23.5 (51)
size (>100 mm TL)	50.4 (129)	46.9 (81)	53.8 (39)	66.7 (9)
weight (≤10 g)	36.1 (119)	55 (20)	38.6 (44)	27.3 (55)
weight (>10 g)	50 (94)	47.9 (71)	55.6 (18)	60 (5)
cardiac fullness (1)	40 (45)	45.8 (24)	40 (5)	31.2 (16)
cardiac fullness (2)	42.9 (84)	54.2 (48)	27.8 (18)	27.8 (18)
cardiac fullness (3)	38 (71)	42.1 (19)	42.3 (26)	30.8 (26)
cardiac fullness (4)	69.2 (13)	N/A(0)	69.2 (13)	N/A (0)

SUPPLEMENTARY MATERIAL FIGURE LEGENDS

-**Supp. Mat. Figure 1.** Assay signal for different anchovy eggs´ developmental stages. Squares representing threshold cycle (Ct, left axis) values for individual anchovy eggs´ assays including different egg developmental stages (from stage 2 to 11 following Moser and Alshtrom, 1985; bottom axis). Linear regression is superimposed.

-**Supp. Mat. Figure 2.** Plankton cruise track (RV 'Investigador'). The first plankton net was retrieved the 6th of May at the south-western edge of the domain and the last haul took place the 19th of May at the coastal station of the northernmost transect. Black and white arrows for respectively night and day time stations (night time from 07:00 to 21:00h for May in the Bay of Biscay). Superimposed black dots represent PairoVET stations location. Isobaths of 100, 200, 1000 and 2000 m are also shown.

Figure1

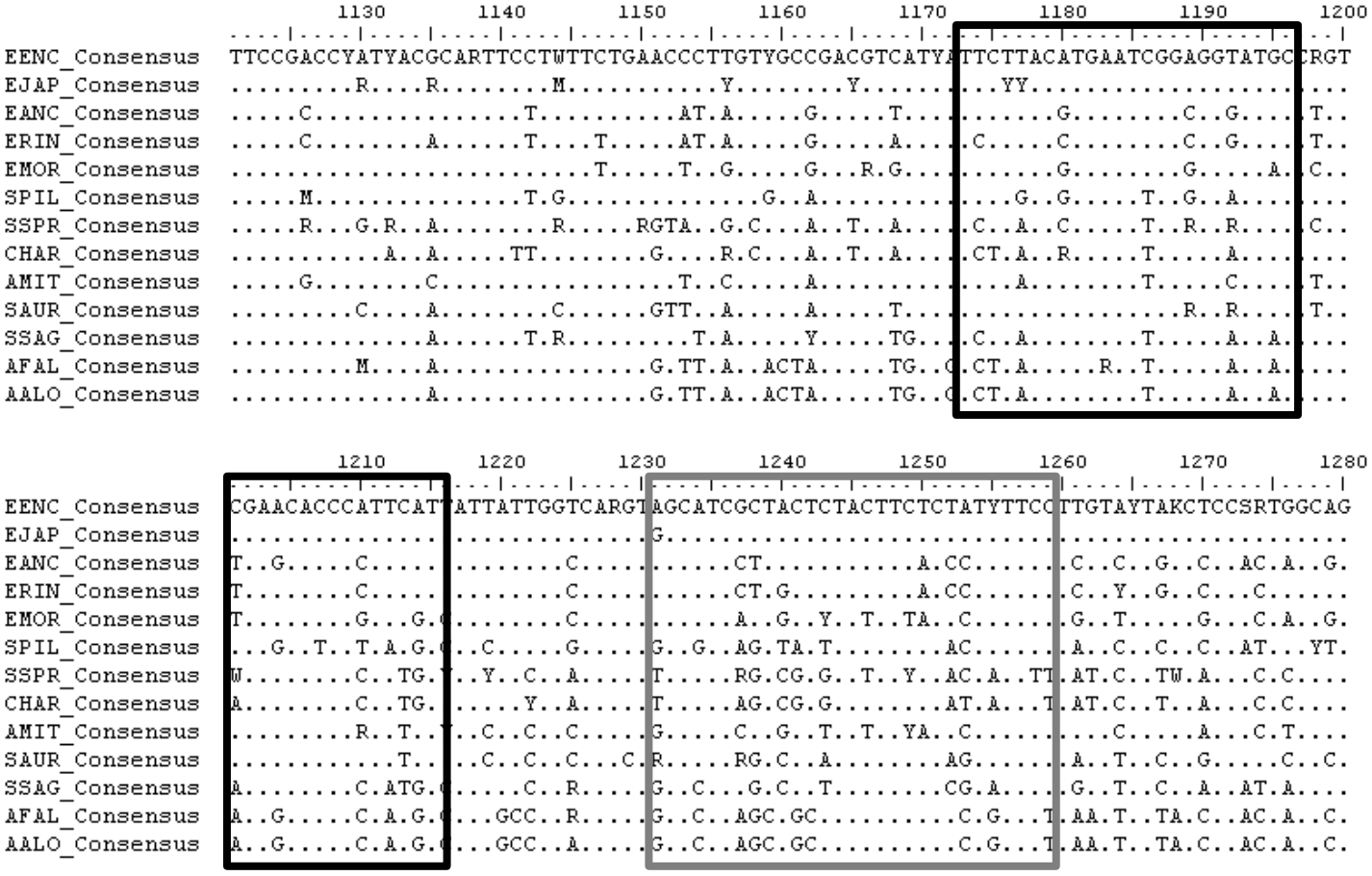

Figure 1. *Engraulis encrasicolus* DNA assay design.

Figure2

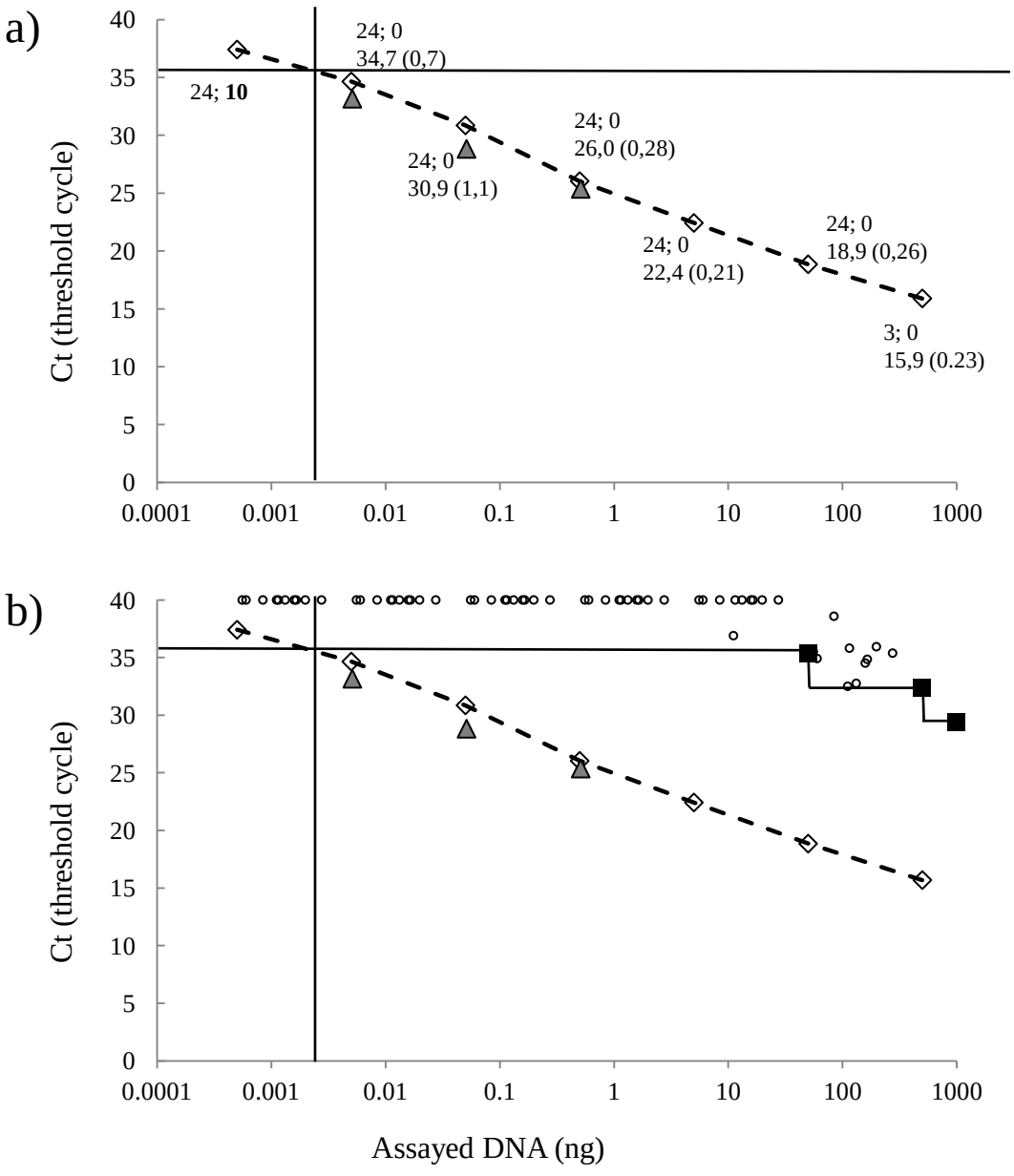

Figure 2. *E. encrasicolus* DNA assay validation.

Figure3

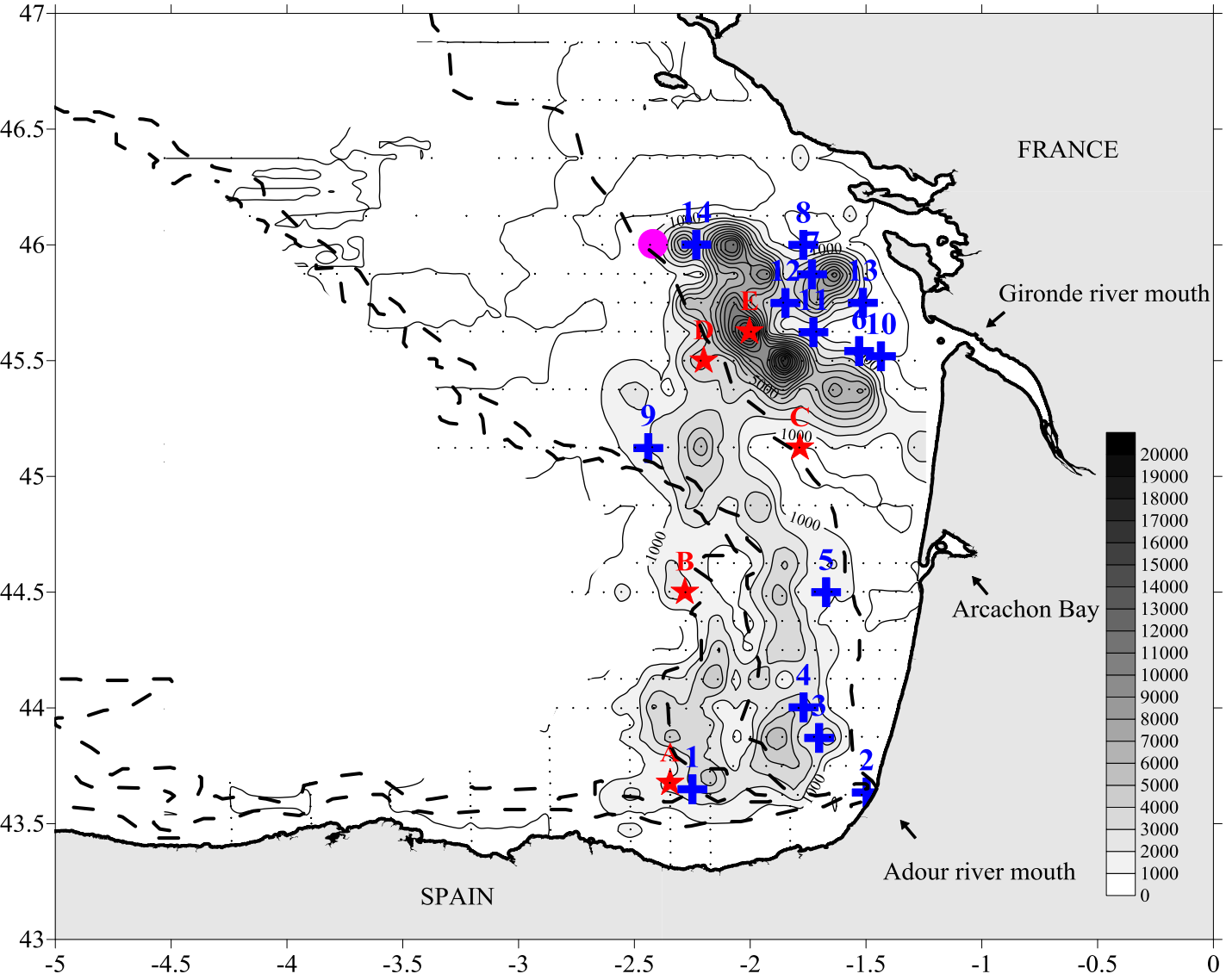

Figure 3. Prey and predators´ spatial location.

Figure4

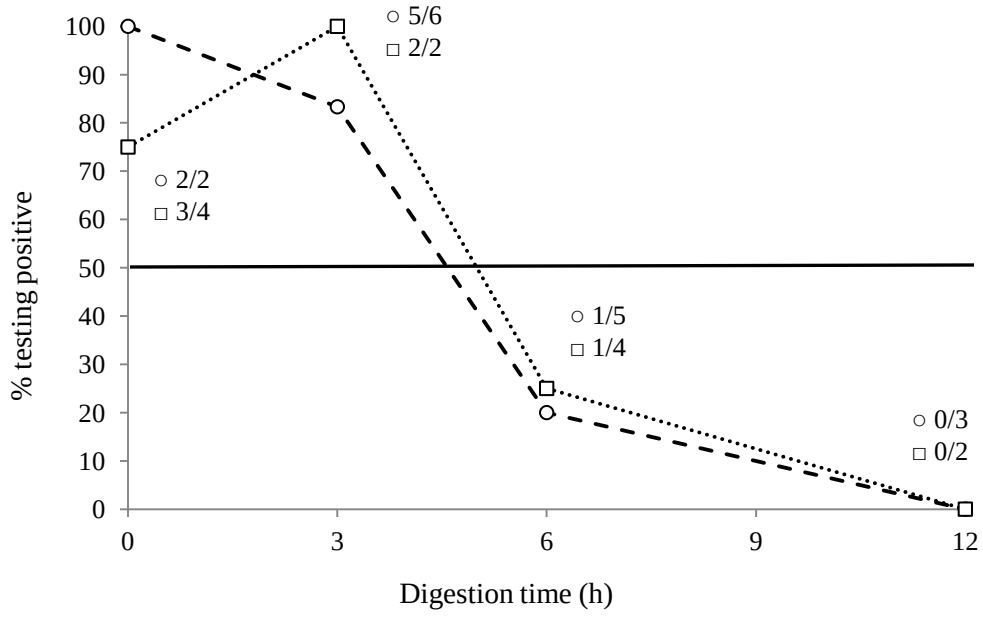

Figure 4. Detectability curves for *Liocarcinus spp.* onboard digestion experiment.

Figure5

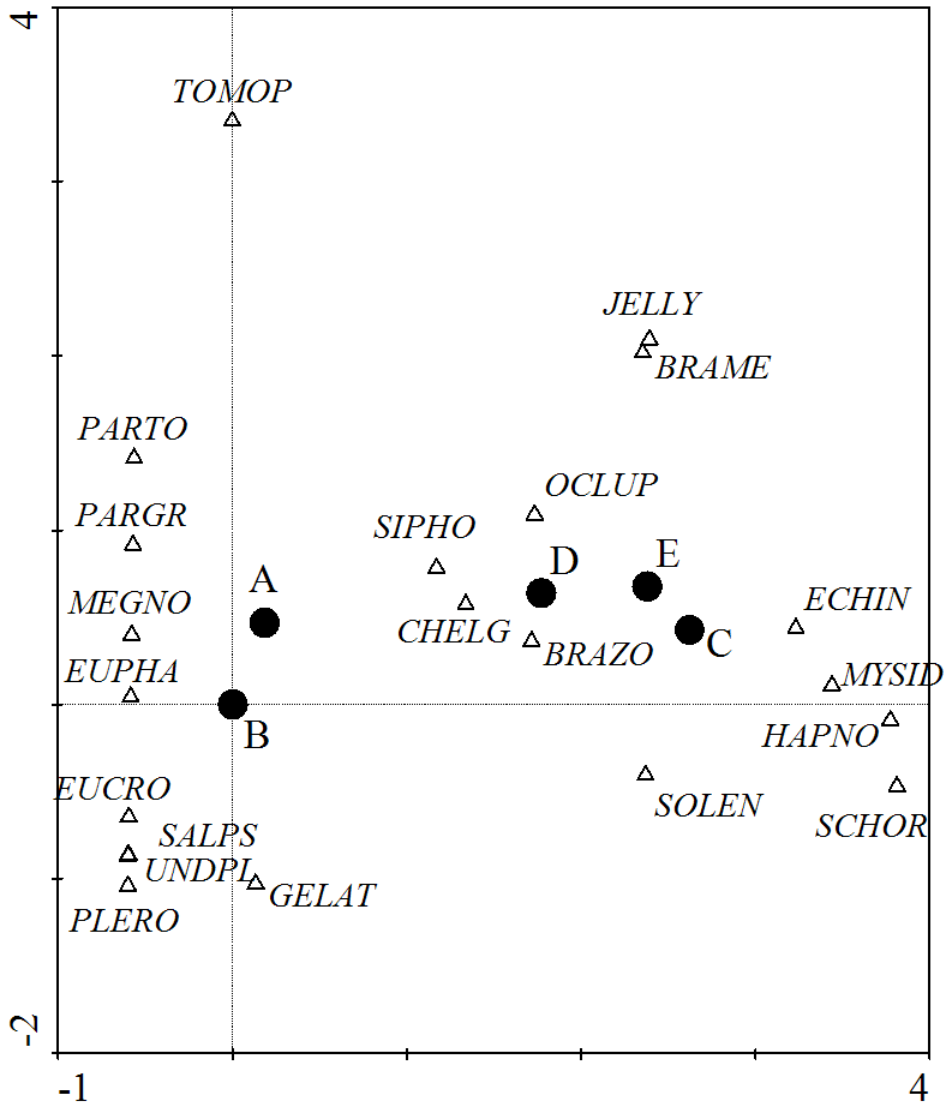

Figure 5. Macrozooplankton community.

Figure6

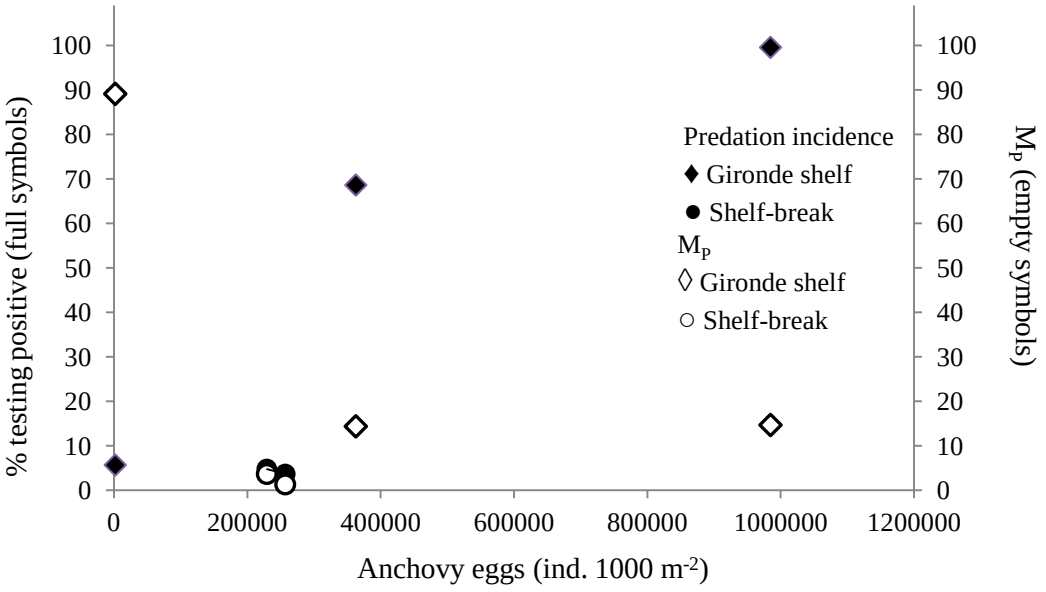

Figure 6. Macrozooplankton predation on anchovy eggs.

Figure7

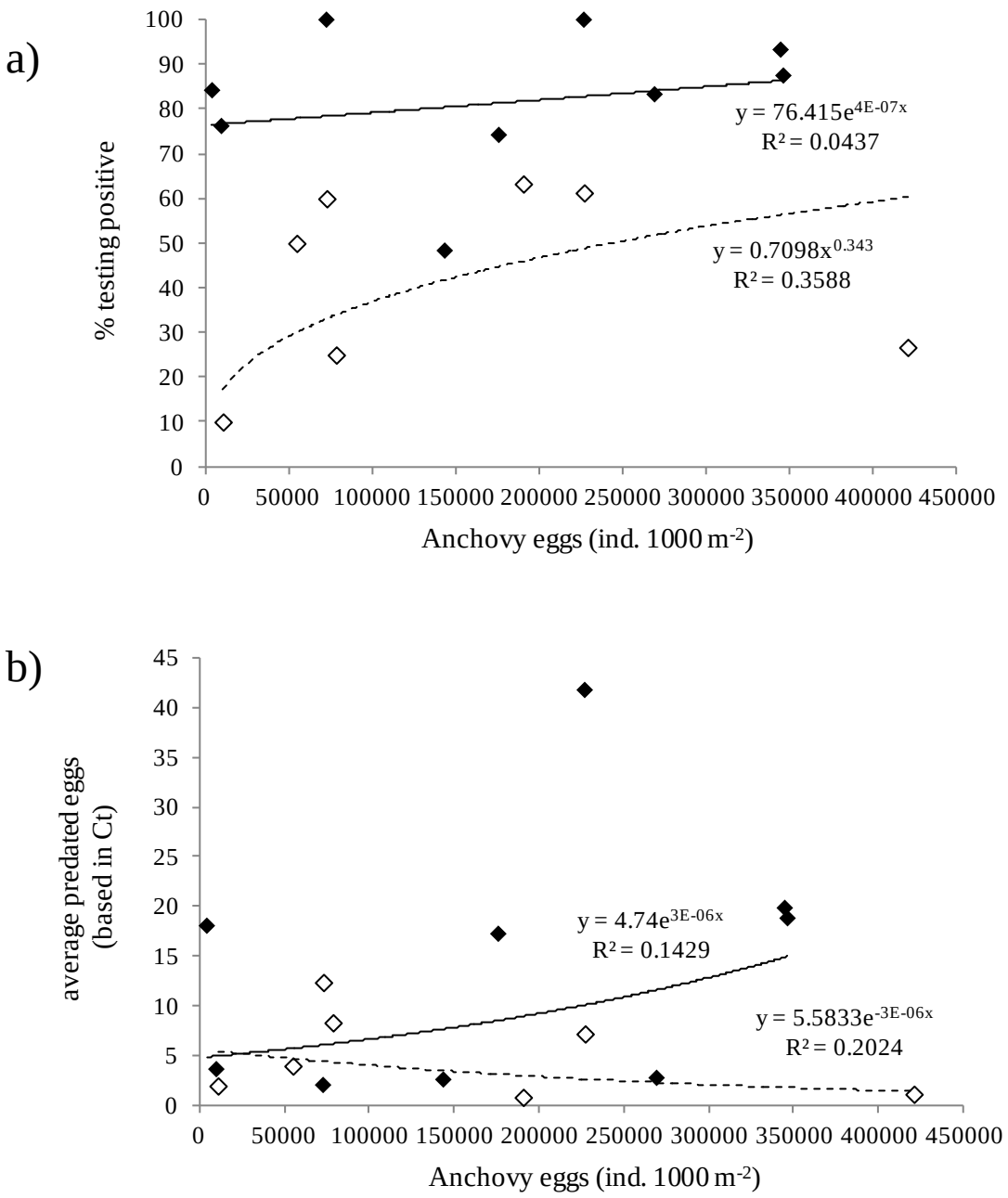

Figure 7. Sardine and sprat predation on anchovy eggs.