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Macrozooplankton predation impact on anchovy (*Engraulis encrasicolus*) eggs mortality at the Bay of Biscay shelf-break spawning center.

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Abstract

A real-time PCR based method involving a species-specific probe was applied to detect *Engraulis encrasicolus* eggs predation by the macrozooplankton community during the 2011 spawning season. Three locations along the shelf-break presenting contrasting but high prey densities were sampled. A total of 840 individuals from 38 taxa of potential macrozooplankton predators were assayed for *E. encrasicolus* DNA presence and 27 presented at least one positive signal. Carnivorous copepods were responsible for the majority of predation events (66%) followed by euphausiids (16%), chaetognaths (5%) and myctophid fish (4%). Macrozooplankton predation on anchovy eggs followed a type-I functional response with daily mortalities below 4% of available prey abundance suggesting a negligible impact on the species recruitment at the shelf-break spawning center.

Introduction

Disentangling predator/prey relationships with the aim of resolving complete food webs is crucial for the desired Ecosystem Based Fisheries Management (EBFM; e.g. Gallego *et al.*, 2012). Furthermore, efforts to rebuild fisheries can be undermined by not incorporating ecological interactions into fisheries models and management plans (Richardson *et al.*, 2011). In this context, methods capable of yielding a reliable, fast and cost-effective direct estimation of fish early life stages (ELS) mortality by predation are demanded as this factor has been traditionally either ignored or grossly estimated, based in indirect data, in fisheries management resulting in limited or null value in standard fisheries recruitment models (Kenchington, 2013). The technical limitations related to traditional visual assessment of contents could explain the relative scarcity of field studies devoted to predation of fish eggs (Heath, 1992; Houde, 2008). However, nowadays, molecular methods offer an alternative to measure predation in the field (Symondson, 2002; King *et al.,* 2008; Pompanon et al., 2012).

In this sense, while predation by fish, including other clupeids and cannibalism, is known to be responsible of a significant part of anchovies´ ELS mortality (e.g. Szeinfeld, 1991), studies applying traditional (visual) methods to invertebrate predators of anchovy ELSs are scarce (e.g. Terazaki, 2005). Applying immunoassays, two studies revealed the importance of invertebrate predation on anchovy ELS mortality. While Krautz *et al.* (2007) showed that predation by the euphausiid *Euphausia mucronata* accounted for 24 to 27% of eggs´ natural mortality in the Chilean anchoveta (*Engraulis ringens*), Theilacker *et al.* (1993) reported that euphausiids accounted for between 47 - 78% of the natural mortality on northern anchovy (*Engraulis mordax*) eggs and yolk-sac larvae.

In order to characterize the range of predators of anchovy ELS in the Bay of Biscay a DNA based method was developed and applied to both invertebrate and vertebrate potential predators during the 2010 spawning season (Albaina *et al.*, submitted). These authors reported that < 5 % of the macrozooplankton predators presented anchovy DNA remains within their gut contents when sampling two SE Biscay offshore stations. These results pointed to a reduced impact on anchovy eggs mortality (respectively 1.3 and 3.6 %) corresponding to ~250 eggs m^{-2} prey abundances. However, to clarify the impact of macrozooplankton predation on anchovy eggs survival at the shelf-break spawning center a wider range of prey densities needs to be assessed. Furthermore, ideally, the whole potential spawning area of the species should be queried. It is known that Bay of Biscay anchovy is capable of spawning along the whole shelf-break but this takes place only at years of high species abundance (e.g. Motos *et al.* 1996; ICES, 2011). In this sense, in 2011, for the first time after a decade of low recruitments, the Bay of Biscay anchovy recovered to historical maximum levels of both adults and egg production allowing collecting macrozooplankton predators at areas of high anchovy egg abundances along the whole Bay shelf-break area. By assaying the presence of anchovy DNA in these specimens we expect to give insights on the role of macrozooplankton predation on anchovy recruitment.

Materials and Methods

Prey and predators sampling

Macrozooplankton was collected during the BIOMAN 2011 survey (6-27 May) onboard the research vessel `Investigador´. Briefly the BIOMAN survey applies the DEPM (Daily Egg Production Method; Lasker, 1985) to estimate fishable anchovy biomass based in the amount of eggs produced during the peak spawning period of the species and adult anchovy information. In 2011, anchovy egg abundance was measured for a grid of 699 stations by means of vertical hauls of a 150 µm PairoVET net with 0.1 m^2 of mouth opening area (Figure 1; ICES, 2011). Sampled stations covered the whole species spawning area from 47º23'N to 3º54'W. The net was lowered to 100 m or 5 m above the bottom at shallower stations. Apart from PairoVET samples, the Continuous Underway Fish Egg Sampler (CUFES, Checkley *et al.*, 1997) was used to record the eggs found at 3 m depth with a net mesh size of 350µm. CUFES sampling device collect eggs along 1.5 nm ship tracks at both sides of the PairoVET location. Anchovy eggs were identified and counted onboard for both sampling devices and abundances were computed.

Three MIK (Methot Isaac Kidd) net samples, with a mesh size of 1 mm and a mouth area of 1 m^2, were collected along the shelf-break as to sort potential predators for assay testing (Figure 1). MIK hauls were performed from 70-75 meters depth to surface (ship at 2 knots, cable retrieved at ~6 meters min^{-1} speed), during the night and at areas of high but contrasting anchovy eggs. Immediately after collection, samples were preserved in 100% ethanol. This ethanol was changed at least two times including one after 24 h (onboard). 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 1). Gelatinous organisms, mainly siphonophores and salps but also jellyfish and ctenophores, were grouped together due to relatively damaged condition, caused by an inappropriate sampling device, preventing identification. Because of this, potential predators did not include gelatinous zooplankton. For the remaining groups, only taxa reported as carnivorous or, at least, omnivorous in the literature were sorted for assay testing. While every large animal was sorted from the whole sample (mainly juvenile fish, salps > 20 mm total length and pteropods and malacostracans over 7 mm cephalothorax length) the rest of the sample was aliquoted using a Motoda plankton splitter and aliquots were sorted until a minimum of 150 individuals for assay testing were sorted. Every individual to be assayed was transferred to a 2 ml microtube (Sarstedt) with fresh ethanol until DNA extraction.

Beside this, the acoustic data recorded onboard during the three MIKS hauls were analyzed. Acoustic data were recorded with a Simrad EK60 split-beam scientific echosounder at 38 and 120 kHz frequencies (Kongsberg Simrad AS). The echosounder was calibrated in accordance with Foote et al. (1987). The acoustic data were selected, classified and analyzed with Echoview Myriaxand MATLAB (MathWorks) software. Data analyzed were restricted to the depth sampled by the net, from 10 m depth from surface to MIK maximum depth as recorded by the mounted CTD. Data from the first 10 meters were discarded to avoid the near field of the 38 kHz transducer as it is usually recommended (Simmonds and Maclennan, 2005). Acoustic echoes were discriminated with a bi-frequency acoustic method developed by Ballón *et al.* (2011); the method was applied directly with few modifications as in Lezama-Ochoa *et al.* (2011). This method uses the 38 and 120 kHz frequencies to split, based on their scattering models, acoustic

signals in three categories: (1) “fish”, (2) “fluid-like zooplankton” and (3) “other plankton”. According to authors the “fluid-like” group includes euphausiids, copepods, salps, siphonophores (without gas inclusion) and other large crustacean zooplankton while the “other plankton” group included all targets other than fluid-like zooplankton and fish. For each of these broad taxonomic categories, the acoustic backscattering was integrated to provide an acoustic abundance index, nautical area scattering coefficient (NASC; m^2 nm^{-2}), an acoustic biomass index determined according to MacLennan *et al.* (2002).

Finally 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.).

Egg predation detection assay

The DNA based assay described and validated in Albaina *et al.* (submitted) was applied to the 38 macrozooplankton taxa sorted in 2011 for anchovy predation detection. Briefly, this assay, that includes an *E. encrasicolus* species-specific TaqMan probe (15bp long; located within an 87bp amplicon of the cytochrome-b gene), measures the amount of anchovy DNA within the stomach contents of potential predators by means of the real-time PCR technique. This assay was capable of detecting 0.005 ng of anchovy DNA (roughly 1/100 of the DNA extracted from a single egg) in a reliable way and had a 90% success in detecting predation events occurred in the last 3h for an experiment performed with the megalopae stages of two swimming crab (genus *Liocarcinus*) species. Anchovy DNA was not detectable after > 6 h of digestion.

Detection of anchovy DNA within predators´ stomach contents

Both DNA extraction protocol and real-time PCR assay settings followed Albaina *et al.*, (submitted). DNA was extracted in 1.5 ml Eppendorf tubes using a modified salt extraction protocol (Aljanabi and Martinez, 1997) including a mechanical homogenization step, using a plastic pestle treated with bleach and UV radiation after each use, for malacostracans. For every juvenile/adult myctophid fish and other large organisms, at least partial dissection of the stomach contents was performed to facilitate the DNA extraction process. Prior to extraction, individual organisms were placed over a highly absorbent wiper and washed with distilled water using a Pasteur pipette. Dissection tools were flamed with ethanol after each sample. Two types of extraction blanks (EBs), negative controls where no tissue is added to the extraction buffer prior to DNA extraction protocol, were included every 10 samples to prevent cross-contamination: including or not the introduction of a plastic pestle. Following extraction, DNA was resuspended in 100 µl ultrapure H_2O and stored at−20 °C. The DNA yield (ng μl^{-1}) was determined using a ND-1000 Spectrophotometer (NanoDrop). Assays were run on an Applied Biosystems 7900 real-time sequence detection system in 384-well reaction plates including 20 no template controls (NTCs; another negative control) and 12 positive controls (DNA extracted from anchovy muscle tissue) per plate. After 3 min at 95 °C, the run comprised 40 cycles of 5 s at 95 °C followed by 15 s at 60 °C. Each 10 µl volume 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.

After the real-time PCR run, each well´s threshold cycle value (Ct; the number of PCR cycles at which a significant exponential increase in the signal is detected) was computed using the Sequence Detection Software version 2.3 (Applied Biosystems). The Ct value is directly correlated with the number of copies of target DNA present in the reaction (see e.g. Albaina *et al.*, 2010). The thresholds defined in Albaina *et al.*, (submitted) for the unambiguous detection of anchovy DNA within predators´ extracted DNA were applied. While Ct values over 35.4 units were required for calling a positive when less than 50 ng of DNA extracted from stomach contents was tested, for values between 50 - 500 and 500 - 5000 ng, a threshold of, respectively, 32.4 and 29.4 Ct units was applied. Finally, the percentage of positive signals was computed per taxa and MIK haul.

Anchovy egg mortality estimations

We made the following assumption: each assay positive signal corresponded to one anchovy egg killed in the last 24h. Although the detectability experiment performed in *Liocarcinus* megalopae showed that predation events were detectable during ~3h (Albaina et al., submitted) and, 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 zooplankton feeding behaviour and digestion times (e.g. Durbin et al., 2011) make 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. Beside this, the risk of positive signals arising from predation events dated > 24 h ago is discarded by the *Liocarcinus* digestion experiment and the available literature on marine invertebrates detectability experiments using real-time PCR assays targeting short mtDNA regions (Albaina et al., 2010; Durbin et al., 2011). Although the DNA based assay cannot distinguish between the anchovy egg and larval stages, we restrict to anchovy egg distribution data to compute mortality as these are the only available prey abundances. However, at this early stage of the species´ spawning season anchovy eggs would undoubtedly represent the bulk of anchovy ELS and thus, a significant bias due to the previous simplification is not to be expected (e.g. Motos *et al.*, 1996). Furthermore, due to the quantitative nature of real-time PCR, we can estimate the number of anchovy eggs corresponding to a certain Ct value (Albaina *et al.,* submitted); applying this we found only 5 cases (out of 140 positive assays) where measured Ct values could corresponded to the amount of DNA of > 1 anchovy egg thus giving further support to the "1 positive assay = 1 egg killed in the last 24h" assumption. Then daily egg mortality at the sampled locations was computed as the fraction of anchovy eggs eaten in the last 24h (equation 1 and 2). 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. D_P was estimated based in CUFES data due to the high discrepancy between CUFES and PairoVET records (Table 2). While PairoVET hauls are more sensitive to patchiness due to the small area sampled (0.1 m^2), CUFES data integrate egg abundances along 1.5 nm at both sides of the PairoVET location (where approximately the MIK net tow starts). CUFES data were transformed to eggs m^{-2} by applying a CUFES/PairoVET ratio of 6 (SD = 4-6; consistent along 2011 sampling depth and abundances ranges).

Results

Prey and predator distribution

Anchovy eggs were distributed in two main areas in the BIOMAN 2011 campaign reaching up to 47.5ºN and 5.7ºW (Figure 1). While spawning on the inner shelf (0-100 m depth) was present only along the French coast, the second spawning band, at shelf-break location, also included the Spanish area. In between, in waters with 100-200 m depth, the presence of anchovy eggs was rare. The same patterns are kept when plotting CUFES device abundances (data not shown). Regardless of the discrepancy between CUFES and PairoVET sampling devices (see Materials and Methods), the three MIK samples were collected at areas of relatively high anchovy egg abundances along the shelf-break (Figure 1 and Table 2). Samples were collected at the onset of the stratification period and in waters with a primary production cline developed at around 30 m depth for MIK-II and MIK-III stations and at 50m for MIK-I (Figure 2). The vertical distribution of pelagic biomass during the haul is shown by means of acoustic biomass profiling. Maximum acoustic biomasses corresponded to (swimbladder-bearing) "fish" category. Regarding distribution along the analyzed depth strata (10-25 m, 25–45 m and 45-70/75 m), while acoustic signals corresponding to fish always peaked at shallower waters (with values in MIK-I being one order of magnitude higher than those in MIK-II and III), both plankton categories presented highest abundances in the shallowest strata (10-25 m depth) at MIK-I location but at the deepest strata at MIK-II and III ones (Figure 2). Taxonomic identification of the net collected individuals included 58 distinct taxa (Table 1) and abundances from 5.2 to 8.4 ind. m^{-3}. Apart from gelatinous organisms (58 % of total abundance), the remaining taxa showing relative

abundances ≥ 1% included copepods (22%), euphausiids (10%), decapods larvae (4%) and chaetognaths (1%). A total of 38 taxa, including mollusks, annelids, crustaceans, chaetognaths and fish, were sorted for assay testing (Table 1). Considering only the assayed taxa their abundances were 1, 2.5 and 2.8 ind. m^{-3} for, respectively, MIK-I, MIK-II and MIK-III hauls. The number of assayed specimens was related with their field abundance and because of this, copepods and euphausiids comprised 82 % of the assayed organisms (respectively 56 and 26%; Table 2).

Detection of anchovy DNA within macrozooplankton taxa

A total of 17% of the assayed organisms yield a positive signal for anchovy DNA (140 out of 840). Among these, the majority of positive reactions corresponded to copepods (66%) followed by euphausiids (16%), chaetognaths (5%) and myctophids (4%). However, considering only abundant taxa, those with at least 25 assayed individuals (13 taxa; Table 2), only five presented a predation incidence over 20% and four of them were copepods: *Paraeuchaeta gracilis* (52%), *P. tonsa* (40%), *Undeuchaeta plumosa* (31%) and *U. major* (24%), followed by chaetognaths (21%). For the abundant euphausiids and myctophids, only 10% of the assayed individuals presented anchovy DNA remains within their stomach contents. When all the assayed taxa are considered together a total of 48, 5 and 9 % of positive signals corresponded to, respectively, MIK-I, MIK-II and MIK-III hauls. Plotting these values against the estimated anchovy egg densities a positive relationship between prey abundance and predation incidence is shown (Figure 3). Apart from this, none of the 190 negative controls tested positive for anchovy DNA (respectively 102 EBs and 88 NTCs; see Materials and Methods).

Anchovy eggs mortality due to macrozooplankton predation

Daily anchovy eggs mortality due to macrozooplankton predation (M_P; see Materials and Methods) was 1.6, 3 and 4% for, respectively, MIK-I, II and III (Figure 3). The range of prey abundances was 268 - 2122 eggs m^{-2}. No relationship between prey abundance and M_P was evident.

Discussion

Twenty five years after the seminal paper of Baily and Houde (1989) on the fate of predation on fish ELSs mortality, "*detailed knowledge and understanding of the sources and stage-specific rates mortality, and of the relative roles of density-independent versus density-dependent processes, remains elusive"* (Browman and Skiftesvik, 2014). However, nowadays, molecular identification of prey in the stomachs of predators allows obtaining important information on trophic interactions that may be difficult if not impossible to obtain in any other way. In this sense, applying a real-time PCR based assay capable of detecting European anchovy (*Engraulis encrasicolus*) DNA traces we have provided insights on the generally neglected role of macrozooplankton predation on anchovy eggs mortality. The target species spawns along two main areas in the Bay of Biscay: the shelf around the Gironde river mouth and the shelf-break, from a core region at the SE edge of the Bay up to the whole shelf-break area in years of high anchovy abundance (e.g. Motos *et al.*, 1996; ICES, 2011). In 2011, for the first time in a decade, we were able to study macrozooplankton predation along the whole shelf-break spawning area. The main results from the application of our molecular method are that (1) macrozooplankton predation impact is low, with daily egg mortalities (M_P) below 4% for a broad range of prey abundances and that, (2) both M_P and predation incidence patterns suggest macrozooplankton predation on anchovy ELSs following a functional response I (Figure 3). Although a value up to ~50 % of positive signals was recorded for the macrozooplankton predators´ community in MIK-I station, this corresponded to the third highest prey abundance record for the whole BIOMAN 2011 campaign (2122 eggs m^{-2}). Present results point to a low and density-independent impact and, therefore, suggest that macrozooplankton predation exert a negligible effect on anchovy egg

survival at the shelf-break spawning center. However a range of factors potentially affecting this conclusion need to be discussed.

On one hand, other factors, apart from prey abundance, could be contributing to the observed patterns; these include vertical match/mismatch of prey and predators, alternative prey availability and the relative abundance of competing predators (the amount of prey available per predator; e.g. Arditi and Ginzburg 2012). The bulk of positive signals corresponded to large species of carnivorous calanoid copepods (mainly Aetideidae and Euchaetidae families) characterized by performing relatively large amplitude diel vertical migrations (DVM) and feeding at night in shallower waters (e.g. Hays *et al.*, 1994; Mauchline, 1998). Apart from these, only chaetognaths, myctophid fish and euphausiids exerted a significant impact in anchovy eggs mortality. These organisms also perform large DVM (e.g. Kaartvedt *et al.*, 2002; Irigoien *et al.*, 2004; Dypvik *et al.*, 2012) and due to the permanent shallow location of fish eggs (mainly in the first 20 m; Boyra *et al.*, 2003; Coombs *et al.*, 2004) the putative predatory impact of these species is limited both in the time and space. In this sense, the higher percentage of animals having ingested anchovy DNA at MIK-I could also be partially explained by the shallower location of plankton as estimated acoustically (Figure 2). However the reduced taxonomic resolution of the existing algorithms prevents further testing of this hypothesis and depth-stratified plankton sampling would be required. Interestingly, the location of the Chl-*a* cline was deeper at the former station (~ 50 m compared with 30 m for MIK-II and MIK-III). Although we lacked actual measurements of alternative prey abundances, this cline generally coincides with the center of distribution for herbivorous plankton (e.g. Longhurst, 1976). A distant location regarding anchovy eggs strata could favour a vertical mismatch for predation as small-medium sized copepods

are typical foods of the above cited predators. As an example, switching from carnivorous to herbivorous feeding modes during the spring phytoplankton bloom has been documented for the abundant *Meganyctiphanes norvegica* (Kaartvedt et al., 2002). However, the above commented higher predation incidence in MIK-I, including the 71% of the *M. norvegica* positive assays in 2011, make us reject this hypothesis. Finally, the reported patterns could be affected by the relative abundance of predators. The fact that assayed predator abundance in MIK-I was around one third of those measured for the remaining hauls could imply a reduced competence for the existing prey resource. Nevertheless, this is confused by the fact that prey abundance at this particular location was five to eight times higher than in the remaining hauls. Finally, while typically, predation studies are focused in one or few predators, the high-throughput character of the molecular method allows an holistic approach to the predation impact on anchovy eggs reducing the bias potentially associated with the omission of competing macrozooplankton predators to a minimum. Beside this, the fate of false positive signals in the reported results is unlikely due to the included negative controls´ results. However, false negatives can arise from the conservative nature of the assay and thus results are to be considered as minimum values (see Albaina *et al.*, submitted for further discussion).

On the other hand, other predators apart from the assayed ones might be exerting a mortality pressure on anchovy eggs. For example, gelatinous organisms were not sorted for assay testing, but these organisms can be important predators of fish eggs worldwide (e.g. Purcell and Arai, 2001). However, to our knowledge, no work addressing the role of gelatinous organism in anchovy eggs mortality has been performed in the Bay of Biscay and thus this question remains undetermined. Beside

this, zooplanktivorous fish are another important source of anchovy ELSs mortality worldwide (e.g. Szeinfeld, 1991; Krautz *et al.*, 2007). Regarding the Bay of Biscay, recently, two studies have measured the fish predation impact on anchovy eggs mortality. While Bachiller (2013), using visual identification of contents in 8 fish species including cannibalism by anchovy, reported that zooplanktivorous fish were responsible of 16-57% of the anchovy eggs mortality in the whole Bay of Biscay (for respectively, the 2008 and 2009 BIOMAN campaigns), a ~7 % was reported by Albaina *et al.* (submitted) when applying the present molecular method to sprats and sardines in the BIOMAN 2010 campaign. The latter reduced to a mere 2% when considering solely the shelf-break spawning area (Albaina *et al.*, submitted). Interestingly, based on the combination of sufficient food fields for larvae and juveniles and the fact that fish predators of anchovy ELSs are relatively scarce at Bay of Biscay offshore waters, Irigoien *et al*. (2007) proposed that anchovy could be recruited through a spatial loophole (*sensu* Bakun and Broad, 2003). In this sense, present results, regarding macrozooplankton predation on anchovy eggs, along with those on anchovy larvae growth by Cotano *et al.* (2008), where higher survival was reported at offshore waters, support the consideration of shelf-break spawning area as a a predation refuge for anchovy ELSs. Although present data were based on three stations for a sole survey, data from another two macrozooplankton hauls in the 2010 BIOMAN campaign (Albaina *et al.*, submitted) allow further testing of the reported pattern. Figure 4 shows that 2010 M_P data corresponded well with 2011 ones where a broader density field and spatial area were sampled. Shelf-break macrozooplankton communities were dominated by the same taxa in both campaigns with just the appearance, in low numbers, of the euphausiid *Euphausia krohnii* and the myctophid *Myctophum punctatum* and, a higher presence of the copepod *Pleuromamma robusta* and the euphausiid *Nematoscelis*

megalops, corresponding to the northernmost located hauls, in 2011. However, regarding the other Bay of Biscay anchovy spawning center, the shelf between Gironde and Adour river mouths (Figure 1), 2010 results indicated that macrozooplankton alone, dominated mainly by mysids and decapods larvae instead of copepods and euphausiids, could control anchovy recruitment at low abundances and that predation followed a functional response II pattern (Albaina *et al.*, submitted). While 63 and 66 % of the positive assays in the shelf-break area corresponded to copepods in, respectively, 2010 and 2011 surveys (followed by euphausiids with another 11 and 16 % of the predation events, respectively), 23 and 70 % corresponded to mysids and decapods in the 2010 shelf one. A combination of feeding behavior (shelf-break vs. shelf macrozooplankton community) and prey availability would explain the reported patterns for anchovy egg predation in the Bay of Biscay.

Finally, a reduced mortality due to low predation pressure and enough food availability does not necessarily imply a higher survival in the shelf-break spawning center. Along with disease, parasitism and pollutants, a mortality source of special relevance at offshore spawning areas is the advection of eggs and larvae to unsuitable habitats. In this sense, models predicting minimum or no survival off the shelf due to unfavorable winds/currents have been proposed for the Bay of Biscay anchovy eggs and larvae (Allain *et al.*, 2007) and this could counterbalance the reduced predation impact at this domain. In this sense, based in otolith microchemistry analyses for a reduced number (n = 40) of anchovy juveniles collected along the Bay of Biscay, Aldanondo *et al.* (2010) reported that all of those juveniles had been spawn at low salinity waters suggesting low survival at the shelf-break spawning area. Beside this, both research groups reported the highest survival for anchovy eggs laid after the peak spawning season (Allain *et al.*,

2007; Aldanondo *et al.*, 2010) where BIOMAN campaigns take place. Because of this, further analysis of a higher number of anchovy juvenile otoliths along with a broader temporal coverage of predation studies is needed as to resolve the role of the shelf-break spawning center in the Bay of Biscay anchovy recruitment.

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Tables legends

Table 1. Macrozooplankton species list. Average taxa abundances (individuals 1000 m^{-3}) is shown for the three MIK hauls along with total relative abundance. Last two columns show, respectively, the taxa selected for *E. encrasicolus* DNA assay testing and, those with at least one positive reaction (shaded).

Table 2. Detection of anchovy eggs/larvae predation by macrozooplankton taxa. MIK hauls data are shown along with the number of predators assayed per species and the percentage of the assays testing positive for *E. encrasicolus* DNA. Prey abundance (egg 1000 m^{-3}) based in both PairoVET net and CUFES device are shown (see Materials and Methods).

Figure legends

Figure 1. Prey and predators´ spatial location in BIOMAN 2011 campaign. The three MIK hauls location (large stars) along with anchovy egg abundance based in PairoVET net vertical hauls (small crosses) is shown. Egg abundance (log_{10} ind. 1000 m^{-3}, scale superimposed) was interpolated using kriging method (SURFER 10; Golden Software). Isobaths of 100, 200, 1000 and 2000 m are shown (bold lines) along with the spatial limits of anchovy spawning area in the 2010 campaign (the two empty polygons).

Figure 2. MIK hauls´ acoustic and CTD vertical profiles. Top row graphs show the acoustic biomasses corresponding to the MIK haul towed distance (a, b and c graphs for, respectively, MIK-I, MIK-II and MIK-III), expressed as NASC values (log_{10} values; m^2 nm^{-2}). The three different lines correspond to the "fish" (solid line with full triangles; top axis), "fluid-like zooplankton" (broken line with full squares, bottom axis) and "other plankton" (broken line with empty squares; bottom axis) defined categories (see Materials and Methods for further information). Data are shown by depth strata, from 10 meters depth to 25 m, from 25 to 45 m and, from 45 m to maximum MIK haul depth (left axis). Bottom row graphs show the vertical (haul depth in meters; left axis) profiles of density (sigma-t, top axis; solid bold line) and fluorescence (relative units, bottom axis; broken line) from the CTD data of the 3 MIK hauls (from left to right MIK I, II and III). Sigma-t (kg m^{-3}) is the density anomaly of a water sample when the total pressure on it has been reduced to atmospheric pressure (i.e. zero water pressure), but the temperature and salinity are *in situ* values.

Figure 3. Macrozooplankton predation on anchovy eggs. Full circles represent the relationship between the macrozooplankton predation incidence (percentage of positive

signals; left axis) and the abundance of anchovy eggs at the MIK haul location (as estimated from CUFES device, see Materials and Methods). Empty circles correspond to the relationship between egg abundance and daily mortality due to macrozooplankton (M_P, see Materials and Methods; right axis).

Figure 4. Anchovy eggs daily mortality due to macrozooplankton (M_P) in the Bay of Biscay (2010 and 2011 data). Present work data (BIOMAN 2011 campaign) are plotted along with those in Albaina *et al.* (submitted; BIOMAN 2010 campaign). Bottom axis represents the abundance of *E. encrasicolus* eggs at the MIK haul location. While empty circles correspond to the stations sampled in 2011, squares refer to MIK stations located at the two spawning centers in 2010 (see Figure 1), respectively, shelf-break (empty squares) and shelf (full squares) stations. Note that the full square at the upper left has a different scale.

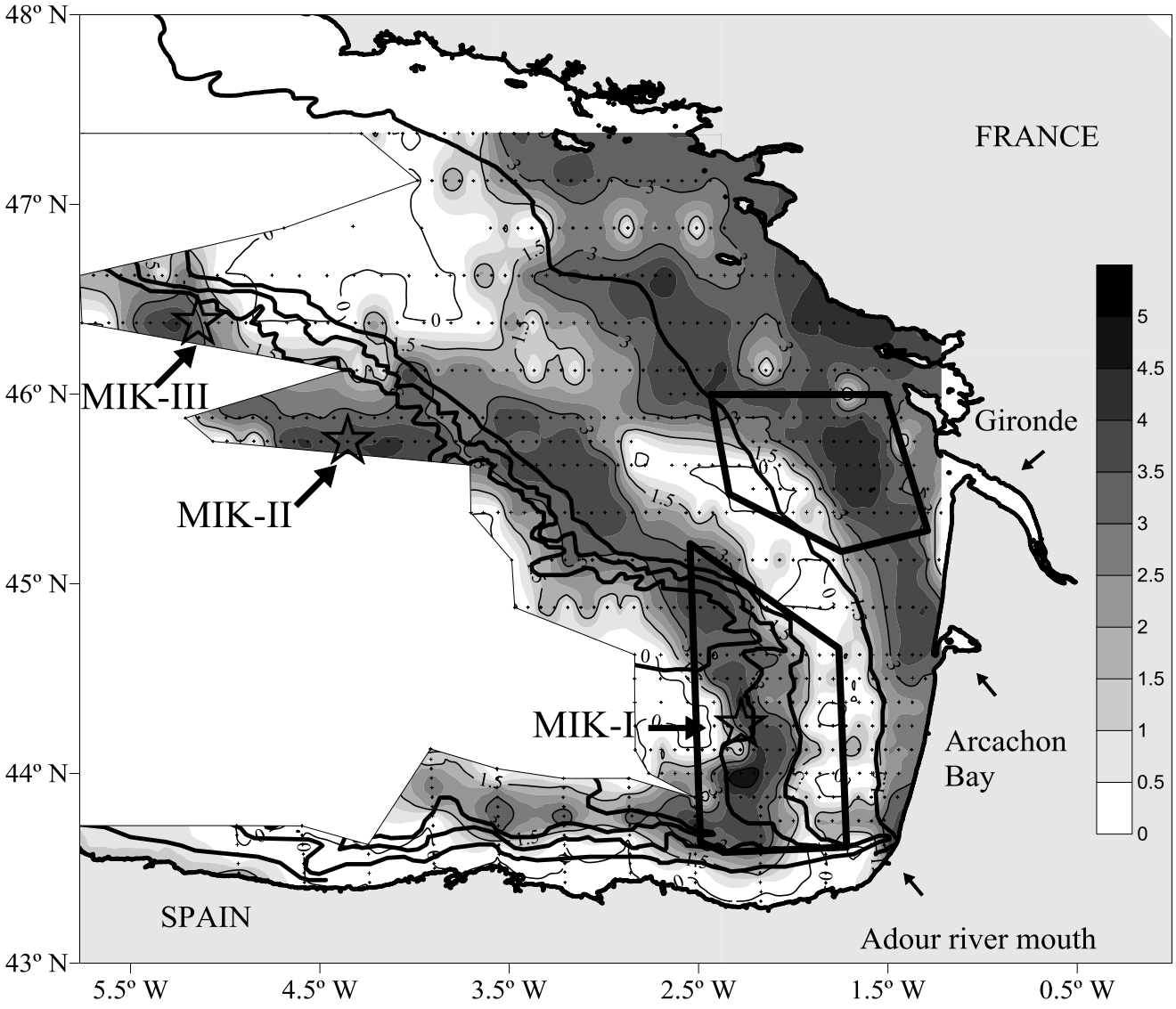
48º N
47º N
46º N
45º N
44º N
43º N
5.5º W
4.5º W
3.5º W
2.5º W
1.5º W
0.5º W
FRANCE
SPAIN
MIK-III
MIK-II
MIK-I
Gironde
Arcachon Bay
Adour river mouth
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0

Figure 1

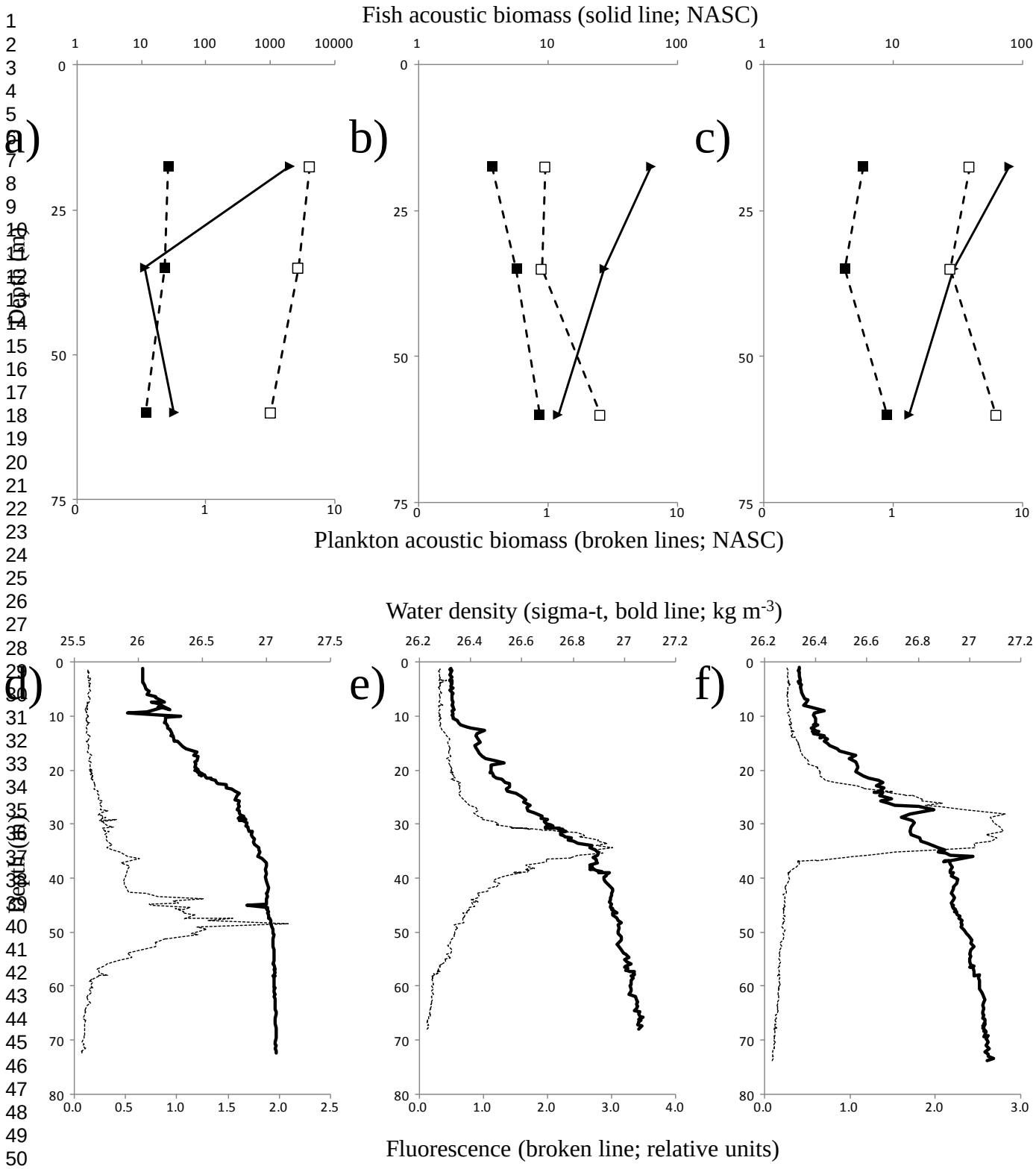
Fish acoustic biomass (solid line; NASC)
a)
b)
c)
Depth (m)
Plankton acoustic biomass (broken lines; NASC)
Water density (sigma-t, bold line; kg m-3)
d)
e)
f)
Depth (m)
Fluorescence (broken line; relative units)

Figure 2

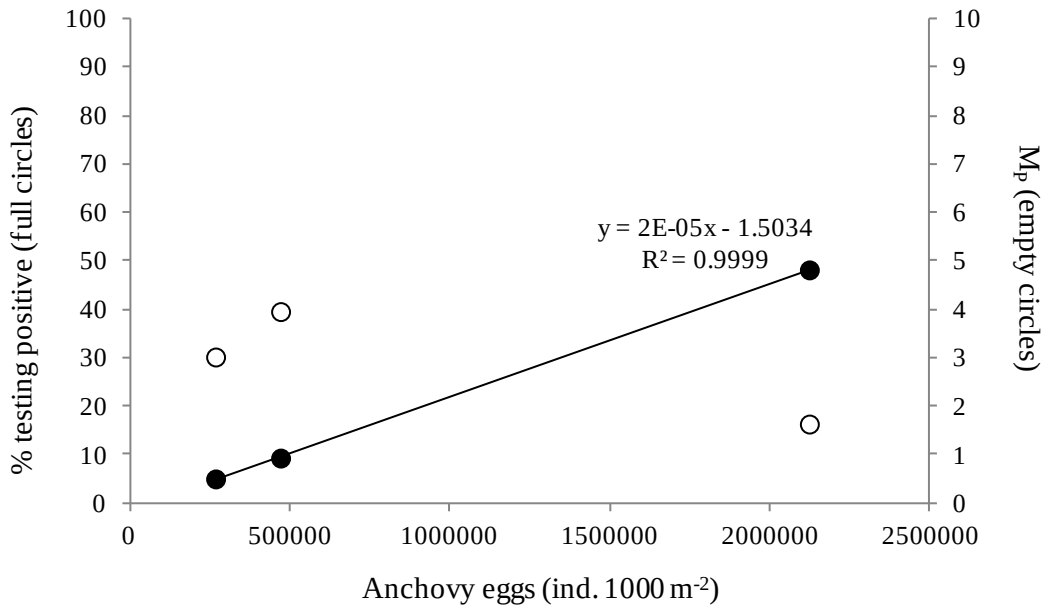
100
90
80
70
60
50
40
30
20
10
0
% testing positive (full circles)
10
9
8
7
6
5
4
3
2
1
0
M_p (empty circles)
y = 2E-05x - 1.5034
R² = 0.9999
0
500000
1000000
1500000
2000000
2500000
Anchovy eggs (ind. 1000 m-2)

Figure 3

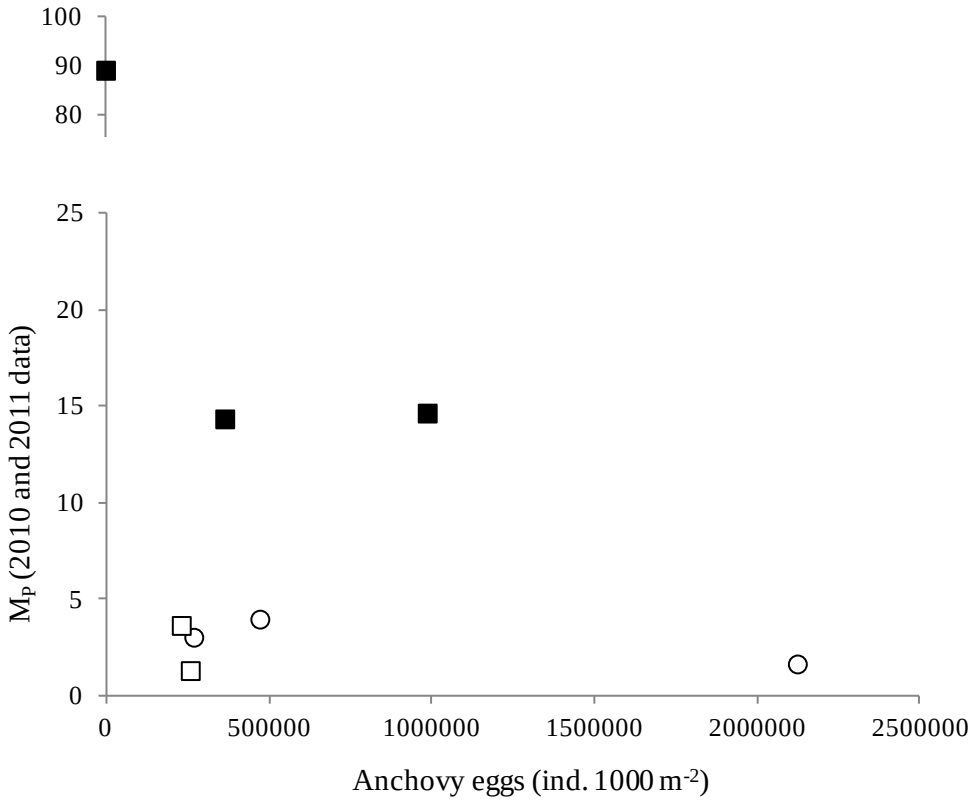
100
90
80
25
20
15
10
5
0
M_P (2010 and 2011 data)
0
500000
1000000
1500000
2000000
2500000
Anchovy eggs (ind. 1000 m^{-2})

Figure 4

	Abundance (ind. 1000 m^{-3})			%	rtPCR	
	I	II	III	Average	Assayed	Positive
Total	5206.4	8405.0	6932.3	100.00		
Gelatinous	3747.4	4059.2	3719.8	57.98		
Non-Gelatinous	1459.0	4345.7	3212.5	42.02		
Cephalopoda (paralarvae)	1.3	0.0	8.3	0.05	+	+
Tomopteris spp.	15.3	0.0	0.0	0.10	+	+
Polychaeta larvae	10.2	0.0	0.0	0.07		
Cymbulia peroni	12.7	1.0	0.0	0.09	+	+
Clio spp.	20.4	15.2	33.4	0.35	+	+
Pteropod spp.	0.0	0.0	16.7	0.08	+	
Calanus helgolandicus	15.3	639.7	83.5	3.04		
Rhincalanus nasutus	40.8	0.0	8.3	0.30		
Eucalanus elongatus	76.5	0.0	0.0	0.49		
Centropages typicus	0.0	22.8	0.0	0.09		
Candacia armata	20.4	30.5	58.4	0.53	+	
Euchirella rostrata	5.1	76.2	41.7	0.54	+	+
Euchirella curticauda	20.4	106.6	58.4	0.83	+	+
Euchirella spp.	5.1	0.0	0.0	0.03	+	+
Metridia lucens	15.3	7.6	0.0	0.13	+	+
Pleuromamma robusta	117.2	441.7	559.4	5.19	+	+
Pleuromamma xiphias	0.0	0.0	16.7	0.08	+	
Pleuromamma spp.	0.0	0.0	8.3	0.04		
Euchaeta acuta	0.0	60.9	33.4	0.40	+	
Euchaeta hebes	15.3	53.3	217.1	1.35	+	
Euchaeta spp.	20.4	114.2	217.1	1.63	+	+
Paraeuchaeta gracilis	40.8	106.6	33.4	0.84	+	+
Paraeuchaeta norvegica	0.0	15.2	0.0	0.06		
Paraeuchaeta tonsa	193.7	198.0	200.4	2.99	+	+
Paraeuchaeta spp.	0.0	22.8	0.0	0.09		
Undeuchaeta major	71.4	129.5	175.3	1.81	+	+
Undeuchaeta plumosa	66.3	83.8	91.8	1.20	+	+
Undeuchaeta spp.	10.2	15.2	16.7	0.21	+	+
Other/damaged Copepods	5.1	76.2	25.0	0.46		
Conchoecilla daphnoides	20.4	0.0	8.3	0.17	+	
Parathemisto abyssorum	5.1	0.0	0.0	0.03	+	
Diastylidae	0.0	7.6	0.0	0.03	+	+
Meganyctiphanes norvegica	103.2	392.2	229.6	3.32	+	+
Nematoscelis megalops	20.4	38.1	179.5	1.14	+	+
Euphausia krohnii	0.0	22.8	0.0	0.09	+	
Damaged Euphausiacea (eye bilobed	25.5	243.7	451.9	3.30	+	+
Euphausiacea spp. (eye simple)	56.1	220.9	108.5	1.76		
Pasiphaea sivado	6.4	1.0	3.1	0.06	+	+
Pasiphaea spp.	5.1	0.0	0.0	0.03	+	+
Solenocera larvae	10.2	0.0	0.0	0.07	+	+
Zoea Porcellana	0.0	799.7	8.3	3.21		
Brachyuran zoeae	66.3	30.5	25.0	0.67		
Porcellana megalopa	0.0	7.6	0.0	0.03		
Other brachyuran megalopae	5.1	15.2	0.0	0.09	+	
Other decapod larvae	25.5	7.6	33.4	0.35	+	+
Chaetognatha	25.5	228.5	50.1	1.31	+	+
Echinodermata larvae	71.4	0.0	0.0	0.46		
Oikopleura spp.	5.1	0.0	0.0	0.03		
Benthosema glaciale	10.2	37.1	57.4	0.49	+	+
Myctophum punctatum	1.3	0.0	1.0	0.01	+	+
Damaged myctophid (juvenile/adult)	3.8	0.0	0.0	0.02		
Myctophidae larvae	15.3	22.8	41.7	0.39	+	+
Saccopharyngiformes	0.0	0.0	2.1	0.01	+	
E. encrasicolus larvae	20.4	0.0	0.0	0.13		
Clupeid larvae damaged	20.4	0.0	0.0	0.13		
Other fish larvae	45.9	7.6	8.3	0.36	+	
Fish egg ≠ Anchovy	0.0	0.0	83.5	0.40		
Others (non-gelatinous)	91.7	45.7	16.7	0.85		

	MIK-I		MIK-II		MIK-III		ALL	
Date	5/12/2011		5/19/2011		5/22/2011			
Time of haul (local time)	3:56		2:41		4:20			
Haul depth (m)	75.1		69.5		75.6			
Bottom depth (m)	1070		3000		2944			
Anchovy eggs (PairoVET)	2589.3		7165.1		642.8			
Anchovy eggs at 3m depth (CUFES)	127312.6		16053.9		28228.5			
	% + assays	n assayed	% + assays	n assayed	% + assays	n assayed	% + assays	n assayed
Cephalopoda (paralarvae)	100.0	1			0.0	1	50.0	2
Tomopteris spp.	66.7	3					66.7	3
Cymbulia peroni	11.1	9	0.0	1			10.0	10
Clio spp.	25.0	4	0.0	2	0.0	4	10.0	10
Pteropod spp.					0.0	1	0.0	1
Candacia armata	0.0	4	0.0	4	0.0	7	0.0	15
Euchirella rostrata	100.0	1	0.0	10	0.0	5	6.3	16
Euchirella curticauda	50.0	4	7.1	14	0.0	7	12.0	25
Euchirella spp.	100.0	1					100.0	1
Metridia lucens	50.0	2	0.0	1			33.3	3
Pleuromamma robusta	36.4	22	2.1	47	5.0	60	9.3	129
Pleuromamma xiphias					0.0	1	0.0	1
Euchaeta acuta			0.0	7	0.0	4	0.0	11
Euchaeta hebes	0.0	3	0.0	7	0.0	23	0.0	33
Euchaeta spp.	0.0	3	0.0	9	8.0	25	5.4	37
Paraeuchaeta gracilis	100.0	8	15.4	13	75.0	4	52.0	25
Paraeuchaeta tonsa	68.4	38	4.0	25	33.3	24	40.2	87
Undeuchaeta major	50.0	14	12.5	16	15.0	20	24.0	50
Undeuchaeta plumosa	76.9	13	0.0	11	9.1	11	31.4	35
Undeuchaeta spp.	50.0	2	0.0	3	0.0	2	14.3	7
Total copepods	**56.5**	**115**	**4.2**	**167**	**10.4**	**193**	**19.4**	**475**
Conchoecilla daphnoides	0.0	4			0.0	1	0.0	5
Parathemisto abyssorum	0.0	1					0.0	1
Diastylidae			100.0	1			100.0	1
Meganyctiphanes norvegica	50.0	20	5.0	60	3.2	31	12.6	111
Nematoscelis megalops	50.0	4	0.0	2	0.0	25	6.5	31
Euphausia krohnii			0.0	3			0.0	3
Damaged Euphausiacea (eye bilobed)	0.0	3	5.0	20	10.4	48	8.5	71
Total euphausiids	**44.4**	**27**	**4.7**	**85**	**5.8**	**104**	**10.2**	**216**
Pasiphaea sivado	50.0	2	0.0	1	33.3	3	33.3	6
Pasiphaea spp.	100.0	1					100.0	1
Solenocera larvae	100.0	2					100.0	2
Other brachyuran megalopae	0.0	1	0.0	2			0.0	3
Other decapod larvae					66.7	3	66.7	3
Chaetognatha	100.0	5	9.1	22	0.0	6	21.2	33
Benthosema glaciale	0.0	8	5.6	18	10.0	20	6.5	46
Myctophum punctatum	100.0	1			100.0	1	100.0	2

Myctophidae larvae	50.0	2	0.0	3	0.0	5	10.0	10
Total myctophids	**18.2**	**11**	**4.8**	**21**	**11.5**	**26**	**10.3**	**58**
Saccopharyngiformes					0.0	2	0.0	2
Other fish larvae	0.0	7			0.0	1	0.0	8
Total	**48.2**	**193**	**5.0**	**302**	**9.3**	**345**	**16.7**	**840**

Dear Editor,

Please find in this document the answers (***bold & italics***) to every reviewers´ comment.

We hope that the new, revised, version of our manuscript, will fulfill ICES Journal of Marine Science´s criteria for acceptance.

We would like to thank the four anonymous reviewers as the quality of the paper has increased substantially due to their comments/suggestions.

The authors

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author
I have reviewed the manuscript Macrozooplankton predation impact on anchovy (Engraulis encrasicolus) eggs mortality at the Bay of Biscay shelf-break spawning center, from Albaina et al. and I would like to recommend its approval with minor corrections.
However, I suggest to make some few modifications in tables and figures to improve the manuscript before its approval.
In the Results section, authors reports grouped values of predation incidence/positive response but table 2 shows only data by specie. This is very confused to the reader. I suggest include a new column which includes data from three stations considered or a new row which incluide percentage/number of positive signal for each group of species (e.g. copepods).

ACTION: Done; in the revised Table 2 we have included a new column for the three stations together and 3 new rows grouping together copepods, euphausiids and myctophids (the most represented categories and the ones cited grouped in the MS).

2. Considering that this manuscript compares data with a previous study (performed during 2010 spawning season), I suggest including a new figure that compares predation incidence by each group (copepods, euphausiids, chaetognats/jellyfish,myctophids) during these two years.

ACTION: We have created a new table (see below) showing the required information (the number of assayed individuals and the percentage of positive assays for the most abundant macrozooplankton categories putting together 2010 and 2011 studies).

While the MP and total predation incidence values for these two studies already appeared in the MS´s Figure 4 (related to prey abundance at each station), the number of assayed individuals per category is related to their field abundance and a distinct shelf-break (dominated by large copepods and euphausiids) and shelf (dominated by mysids and decapods) communities are evident; however, this has been already detailed in the MS discussion section.

In this sense, we think that this new table, which would fit within the discussion, would not ease the interpretation of the MS but the contrary.

Because of this, we think that adding the following sentence "While 63 and 66 % of the positive assays in the shelf-break area corresponded to copepods in, respectively, 2010 and 2011 surveys (followed by euphausiids with another 11 and 16 % of the predation events, respectively), 23 and 70 % corresponded to mysids and decapods in the 2010 shelf one. A combination of feeding behavior (shelf-break vs. shelf macrozooplankton community) and prey availability would explain the reported patterns for anchovy egg predation in the Bay of Biscay." to the discussion (lines 399-405 in the revised version) clarifies that: 1) the patterns for 2010 and 2011 shelf-break areas are similar due to a similar plankton community while 2) the higher predation impact at the 2010 shelf stations is related to a distinct community (dominated by mysids and decapods, that are outnumbered by large copepods and euphausiids in the shelf-break area).

	2011		2010			
	2011 shelf-break		**2010 shelf-break**		**2010 shelf**	
MIK stations	3		2		3	
Date	5/12-22/2011		5/8-11/2010		5/13-15/2010	
Time of haul (local time)	2:41-4:20		3:28-4:46		1:12-5:33	
Haul depth (m)	69-76		64-66		46-55	
Bottom depth (m)	1070-3000		1153-1600		73-94	
Anchovy eggs (PairoVET)	643-7165		2292-2568		25-14482	
Anchovy eggs at 3m depth (CUFES)	16054-127313		22405-28791		166-60851	
M_P (%)	**1.6-4**		**1.3-3.6**		**14.3-89.1**	
	% + assays	n assayed				
Total copepods (A)	**19.4**	**475**	**5.6**	**215**	**66.7**	**3**
Total mysids (B)	**0.0**	**0**	**0.0**	**0**	**25.1**	**303**
Total euphausiids (C)	**10.2**	**216**	**1.6**	**128**	**0.0**	**0**
Total decapods (D)	**46.7**	**15**	**13.6**	**22**	**83.3**	**281**
Chaetognatha (E)	**21.2**	**33**	**0.0**	**7**	**66.7**	**3**
Total myctophids (F)	**10.3**	**58**	**2.7**	**37**	**0.0**	**0**
Total (A+B+C+D+E+F)	**16.8**	**797**	**4.4**	**409**	**53.2**	**590**
Total	**16.7**	**840**	**4.2**	**451**	**54.0**	**618**

Minor questions:

1. Why some very abundant copepods was not included in DNA Assays? (e.g Calanus helgonlandicus, Rhyncalanus nasutus, others).

ACTION: This was already detailed in the Methods section: "For the remaining groups, only taxa reported as carnivorous or, at least, omnivorous in the literature were sorted for assay testing." Although we agree that it would have been interesting to test all the available macrozooplankton taxa this was logistically and economically unaffordable (we already tested 840 individuals).

2. Why do you assume that the decay of positive signal (DNA concentration) in copepods/euphauisiids guts is similar to Liocarcinus megalopae?

ACTION: We do not assume this but we agree that it was somewhat confusing in the original Methods section. We have clarified this now.

More in detail, it is true that the only experimental data on the detectability of the ingested anchovy DNA along the digestion process of a macrozooplankton taxa was that of Liocarcinus megalopae (performed in the 2010 spawning season study, Albaina et al,. submitted) and, because of this, we cited it. But, due to the lack of comparable data for the remaining taxa, when computing mortality (M_P) we followed the conservative assumption already described in the Methods section ("We made the following assumption: each assay positive signal corresponded to one anchovy egg killed in the last 24h."). As detailed there the Ct values from the assayed taxa supported this conservative assumption.

In this sense, the available experimental data for Liocarcinus megalopae give further support to this assumption by showing that the detection of predation events that took place >24h ago would not be possible. This assures us that we are not overestimating predation mortality but the contrary. This was already discussed in the 2010 study paper but we have added this to the revised MS (line 153) to clarify this: "Anchovy DNA was not detectable after > 6 h of digestion".
Finally, this is also supported by the available data on detectability experiments in the literature regarding other marine invertebrates (either mesozooplankton or macroinvertebrates) and applying a similar rtPCR assay (Albaina et al. 2010; Durbin et al. 2011). This have been also included in the revised MS (lines 203-207): "Beside this, the risk of positive signals arising from predation events dated > 24 h ago is discarded by the Liocarcinus digestion experiment and the available literature on marine invertebrates detectability experiments using real-time PCR assays targeting short mtDNA regions (Albaina et al., 2010; Durbin et al., 2011)."

Reviewer: 2

Comments to the Author
This an interesting study. The paper is well written and I only have minor comments.
How were gelatinous zooplankton processed for DNA stomach contents?

ACTION: Gelatinous organisms were not sorted for assay testing due to their damaged condition. This was already mentioned in the Discussion "For example, gelatinous organisms were not sorted for assay testing, but these organisms can be important predators of fish eggs worldwide (e.g. Purcell and Arai, 2001). " and in the methods section "Gelatinous organisms, mainly siphonophores and salps but also jellyfish and ctenophores, were grouped together due to relatively damaged condition preventing identification. For the remaining groups, only taxa reported as carnivorous or, at least, omnivorous in the literature were sorted for assay testing."

However we agree with the referee that the methods section is not clear enough and we have improved the previous paragraph to (lines 106-111 in the revised version): "Gelatinous organisms, mainly siphonophores and salps but also jellyfish and ctenophores, were grouped together due to relatively damaged condition, caused by an inappropriate sampling device, preventing identification. Because of this, potential predators did not include gelatinous zooplankton. For the remaining groups, only taxa reported as carnivorous or, at least, omnivorous in the literature were sorted for assay testing." We think it is clear now.

Is there any anchovy DNA on the predator exoskeleton?

ACTION: This is something that can´t be totally discarded and this, among other potential biases for the molecular detection of predation has been discussed in detail in the 2010 spawning season paper (as already mentioned in the Discussion section).

However, the process we followed from MIK haul collection to DNA extraction was designed to avoid this. More in detail, following sample collection, the ethanol was changed at least twice prior to predator sorting; then, each individual predator to be assayed was transferred to individual tubes with 2 ml fresh ethanol. Finally prior to extraction each organism was placed over a disposable piece of highly absorbent wiper (Kimberly Clark WYPALL* X60 Wipers) and washed with several drops of distilled water with a Pasteur pipette. The above detailed serial washes should reduce the risk of detecting target DNA from the animal surface to a minimum. We have added (lines 163-164 in the revised section) "Prior to extraction, individual organisms were placed over a highly absorbent wiper and washed with distilled water using a Pasteur pipette." to the Methods section.

What is the digestion rate of anchovy egg DNA? If it is very rapid and substantially less than 24 hr (as I suspect) then your mortality estimates would be far too low. Are there any estimates of prey DNA digestion rates? You need to discuss this issue. It has an important bearing on your results.

ACTION: This has been partially discussed also within the previous referee´s last comment.
In this sense, we followed a conservative assumption to estimate mortality (M_P) and therefore we agree with the referee that the actual MP values could be somewhat higher, however the lack of knowledge on the assay detectability rate and feeding behavior (e.g. DVM, etc) for the majority of macrozooplankton taxa prevents yielding a more refine calculation. Moreover, available data from the literature on feeding

experiments support the previously cited assumption of 1 positive signal = 1 predated egg/larvae at least in some taxa: 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).

-Coston-Clements L, Wagget RJ, Tester PA (2009) Chaetognaths of the United States South Atlantic Bight: distribution, abundance and potential interactions with newly spawned larval fish. J Exp Mar Biol Ecol 373 (2): 111-123.

We have included this in the Methods section (lines 195-203 in the revised version): "Although the detectability experiment performed in Liocarcinus megalopae showed that predation events were detectable during ~3h (Albaina et al., submitted) and, 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 zooplankton feeding behaviour and digestion times (e.g. Durbin et al., 2011) make 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."