Molecular identification of the diet of *Sardina pilchardus* larvae in the SW Mediterranean Sea

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Running page head: Sardine larval diet by multiplex PCR

Abstract: Molecular techniques are offering new insights on the feeding strategy and diets of planktonic organisms such as the larvae of marine fish. We applied multiplex PCR to provide the first estimates of the diets of the larvae of European sardine (*Sardina pichardus*) in the Alboran Sea (SW Mediterranean), where this species represents an important fishery resource. The feasibility of this technique was tested in a 26-hour continuous survey of a shoal of larvae (mean±SD, 10.80±0.73 mm in standard length). Multiplex PCR was designed to detect the presence of five copepod species, a microplanktonic dinoflagellate (*Gymnodinium*) and the picoeukaryote algae family Prasinophyceae in larval guts. We simultaneously sampled sardine larvae and their potential prey (pico- to mesoplankton) and compared diel variability of the prey field and ingested items. Microplankton was dominated by flagellates and copepods represented the most abundant mesozooplankton, reaching peak abundance at night. Prey DNA was detected throughout the entire diel cycle, despite no visible prey in the guts of larvae collected at night. Sardine larvae preyed on early life stages of the most abundant copepod species (*Oncaea waldemari, Paracalanus indicus* and *Temora stylifera*), suggesting an opportunistic foraging behavior. The use of multiplex PCR allowed species-level identification of ingested nauplii and protists, which otherwise would remain

27 unidentified.

28

29 **Keywords:** Alboran Sea, *Sardina pilchardus*, diel cycle, larval ecology, multiplex PCR

30 **Introduction**

31 Small pelagic fish (SPF) play a central role in the structuring of the marine food webs where they
32 can exert top-down control of mesozooplankton and bottom-up control on their predators (this
33 double role is known as wasp-waist control, Cury et al. 2000). Thus, small pelagic fish (SPF)
34 species, such as sardines and anchovies, play a critical role in transferring the energy from plankton
35 to large vertebrate predators in marine ecosystems.

36 In the Mediterranean Sea, the Bay of Málaga (located in the central part of the north Alboran Sea),
37 is the most important nursery site for the European sardine (*Sardina pilchardus*) and anchovy
38 (*Engraulis encrasicolus*) (García et al. 1988, García 2010, Giannoulaki et al. 2013). The bay is an
39 essential habitat for these SPF species as it fulfills the Bakun triad, a set of conditions needed for
40 larvae survival (Agostini & Bakun 2002), including high abundances of phytoplankton and
41 zooplankton (Mercado et al. 2007, Yebra et al. 2017). These conditions are promoted by mesoscale
42 hydrographic structures such as fronts and gyres caused by the influx of Atlantic water through the
43 Gibraltar Strait (Parrilla & Kinder 1987) and upwelling events induced by westerly winds (Sarhan
44 et al. 2000, Mercado et al. 2012). Sardine displays an extended spawning season in this region
45 (Rodríguez 1990, Tendero 2016) where its larvae are often dominant members of the
46 ichthyoplankton (Palomera et al. 2007). Despite its numerical abundance, no previous studies have
47 reported on the diet and feeding habits of sardine larvae in the Alboran Sea.

48 Although the diet of a fish larva depends on the abundance and diversity of prey encountered
49 (García et al. 2003), the larvae of most marine fish selectively feed on specific prey species (Peck et
50 al. 2012). To date, studies on the diet of various life stages of European sardine have shown that
51 mesozooplankton (>200 µm, mostly copepods and cladocerans) comprises the major food source
52 for larvae (e.g. Conway et al. 1994, Morote et al. 2010, Costalago & Palomera 2014). In the NW
53 Mediterranean Sea, tintinnids and copepod nauplii form the largest proportion of the gut content of
54 the smallest pre-flexion larvae while larger larvae preferentially consumed nauplii and copepodites
55 of calanoid copepods (Morote et al. 2010). However, important regional differences in diet and/or

56 omnivorous foraging may exist since Rasoanarivo et al. (1991) found sardine larvae consuming
57 exclusively phytoplankton, from 5 µm (*Chlorella spp.*) to 130 µm (*Synedra acus*) in the Gulf of
58 Lions (NW Mediterranean). Small microzooplankton (i.e. protozoan) might be important prey for
59 ichthyoplankton (Bils et al. 2017), however, the protozooplankton-ichthyoplankton link remains
60 largely unexplored, as most field studies employ microscopic analysis of gut contents of larvae
61 preserved in formaline (Peck et al. 2012). This traditional approach presents several limitations,
62 which include the difficulty of identifying early developmental stages (e.g. nauplii), soft body
63 organisms or partly digested items. Stable isotope analysis provides another technique to infer larval
64 feeding habits (Bode et al. 2004, Costalago et al. 2012, Laiz-Carrión et al. 2011) but this technique
65 provides no information on prey species or prey preference.
66 To overcome the limitations of using microscopic identification of gut contents to identify the diets
67 of marine fish larvae, molecular tools have been developed in recent years. These new techniques
68 not only complement to traditional microscopy counts, but are also useful tools that improve the
69 accuracy of identification of organisms at species level (even criptic ones or partly digested
70 remains) and increase the volume of samples that can be analysed in a cost-effective manner. Due to
71 their precision and sensitivity both PCR (Polymerase Chain Reaction) and quantitative PCR (qPCR)
72 have been applied to detect and quantify species from water samples (Vadopalus et al. 2006,
73 Miyaguchi et al. 2007, Pan et al. 2008), and have been successfully applied to examine the diet of
74 zooplankton (Nejstgaard et al. 2003, 2008, Troedsson et al. 2007, Simonelli et al. 2009). Moreover,
75 metabarcoding assays employed on gut contents of European sardine adults (Albaina et al. 2016)
76 and European eel (*Anguilla anguilla*) larvae (Ayala et al. 2018) identified the main taxonomic
77 groups, including protists and soft bodied organisms, not identifiable by microscopic examination.
78 The present study is the first to examine the diet of sardine larvae in the Alboran Sea and one of
79 only a handful of studies to apply molecular markers on larval fish gut contents. We tested two
80 hypotheses. First, sardine larvae feed on the most abundant prey at the beginning of the spawning
81 season to maximize rates of growth and development. Second, diel differences in the diet of sardine

82 larvae explain day/night differences in the nutritional condition of larval sardine (Conway et al.
83 1994, Cortés com. pers.). We quantified the taxonomic composition of the Alboran Sea plankton
84 community and designed and applied species-specific molecular markers to detect the presence of
85 selected target organisms within sardine larval guts (Hernández de Rojas et al. unpubl. data).
86 Based on i) the dominant phyto- and zooplankton in the Bay of Málaga during autumn, (e.g. small
87 flagellates (Mercado et al. 2005, 2007) and copepods (Rodríguez 1983, Sampaio de Souza et al.
88 2005)), and ii) gut contents of sardine larvae in other areas of the NW Mediterranean (Rasoanarivo
89 et al. 1991, Morote et al. 2010), molecular markers were developed for five mesozooplankton
90 copepod genus (*Oncaea, Acartia, Temora, Clausocalanus* and *Paracalanus*), a microplanktonic
91 dinoflagellate (*Gymnodinium*) and the picoeukaryote algae family Prasinophyceae, as representative
92 of Chlorophyta.
93
94 **Material and Methods**
95 *Sampling*
96 Sampling took place on board R/V Francisco de Paula Navarro, on 8-9th November 2014, during a
97 26 hours diel cycle within the Bay of Málaga (Fig. 1). Every 2 hours (T1 to T13, Table 1), Bongo
98 nets (60 cm diameter, 500 µm mesh) were deployed to collect sardine larvae by means of oblique
99 hauls down to 5 m above the seafloor. Sampling started at midday in shallow shelf waters (70-80 m
100 depth) where adult sardine are known to spawn, and we gradually moved towards nursery shallow
101 inshore waters (18-22 m depth), where larvae concentrate at nighttime (García et al. 1988). On
102 board, a subset of 351 individuals were sorted, identified, visually examined for gut contents and
103 photographed with a Leica EZ4HD, for later measurement of standard length (SL), and immediately
104 preserved in undenatured ethanol 96% for molecular assays. Larvae handling time between
105 collection and preservation did not exceed 5 minutes.
106 After each ichthyoplankton sampling, a CTD SBE-25 was used to obtain vertical profiles of
107 temperature and salinity at each sampling site. Then, Niskin bottles were used to collect seawater at

108 surface and close to the seafloor. Samples to determine the abundance and taxonomic composition

109 of phytoplankton >5 m were fixed in dark glass bottles with Lugolø s solution (2% final

110 concentration). Samples for determination of eukaryotic pico- and nanoplankton abundance were

111 fixed with glutaraldehyde (1% final concentration) and immediately frozen in liquid nitrogen

112 (Vaulot et al. 1989). Finally, a WP2-double net (200 µm mesh) was deployed vertically to collect

113 mesozooplankton, from 3 m above the bottom to the surface, at a speed of 0.5 $m \cdot s^{-1}$. Zooplankton

114 was carefully rinsed and preserved with 96% non-denatured ethanol for taxonomic analyses.

115

116 *Plankton community composition*

117 In the laboratory, 100 ml of each phytoplankton >5 m sample were sedimented in a composite

118 chamber for 24 h, following the technique developed by Utermöhl (1958). Cells were counted at

119 200× and 400× magnification with a Leica DMIL inverted microscope. The species nomenclature

120 was validated using Tomas (1997). Pico- and nanoplankton Samples for determination of eukaryotic

121 pico- and nanoplankton abundance were fixed with glutaraldehyde (1% final concentration) and

122 immediately frozen in liquid nitrogen (Vaulot et al. 1989). samples were analysed with a Becton

123 Dickinson FACScan flow cytometer. Cell counting was performed based on the forward-light

124 scatter and the orange and red fluorescence signals. BD TrueCOUNT Tubes were used to determine

125 absolute counts.Finally, a WP2-double net (200 µm mesh) was deployed vertically to collect

126 mesozooplankton, from 3 m above the bottom to the surface at a speed of 0.5 $m \cdot s^{-1}$. Zooplankton

127 was carefully rinsed and preserved with 96% non-denatured ethanol for taxonomic analyses.

128 Copepod abundance and taxonomic composition were determined using a stereomicroscope (Leica

129 M165C). Taxonomic identification was made to the lowest possible level according to Rose (1933),

130 Trégouboff & Rose (1957), and Razouls et al. (2005). Copepods identification to species level was

131 not always feasible, as some genera present cryptic species in the study area (e.g. Kasapidis et al.

132 2018). Thus, we report field copepod abundance data at genus level.

133

134 *Molecular analyses of larvae gut content*

135 In the laboratory, sardine larvae were dissected for gut DNA extraction. Prior to extraction,
136 individuals were washed three times with sterilized water and all the material, forceps and scalpels
137 were flame sterilized before and after each dissection. From each sampling (T1 to T13), the gut
138 content of 10 larvae were pooled together and total DNA was extracted using DNeasy Blood &
139 Tissue kit (Qiagen), following the manufacturerøs instructions, except for the proteinase K
140 incubation which was done overnight at 37 ºC. DNA pools were stored at -20 ºC until their assay,
141 and DNA purity and concentrations were assessed using NanoDrop 1000 (Thermo Scientific) in 1
142 µl of sample (DNA in each pool is shown in Table 3). In order to assess the presence/absence of
143 potential preys within the larval guts, 5 µl of total DNA from each pool were assayed in triplicate by
144 means of a species-specific multiplex PCR designed *ad hoc* for this purpose (Hernández de Rojas et
145 al. unpubl. data). In brief, a multiplex PCR was designed to detect, in a single assay, the DNA of the
146 5 most abundant copepod species found in the study area, by targeting short fragments (100 - 200
147 bp) of their mitochondrial COI (mtCOI) gene. The potential preys targeted were *Clausocalanus*
148 *parapergens*, *Acartia clausi*, *Paracalanus indicus*, *Temora stylifera* and *Oncaea waldemari*. PCR
149 melting temperatures (Tm) ranged from 42 to 50 ºC, and amplicon lengths varied from 104 to 193
150 bp. PCR products were separated and analysed using Bioanalyzer 2100 (Agilent), using the DNA
151 1000 kit (Agilent). Electropherograms were analysed with the 2100 Expert Software (Agilent), and
152 fragments of the expected length and that yielded ×1 fluorescent unit (FU) were counted as positive.
153 Likewise, phytoplankton gut content was studied by means of a second multiplex PCR. In this case,
154 group-specific primers were designed (Table 2) to detect the dinoflagellate genus *Gymnodinium*
155 (105 bp amplicon) and the picoeukaryote family Prasinophyceae (155 bp amplicon). The LSU
156 rDNA marker is preferentially used for dinoflagellates species identification due to its high
157 variability in some domains (Gomez et al. 2011). Thus, for *Gymnodinium* primer design, sequence
158 alignment of available (GenBank, October 2015) *G. catenatum* mitochondrial large subunit
159 ribosomal (LSU rDNA) gene sequences was performed. To ensure the detection of dinoflagellate

160 DNA partially digested, primers were designed for the amplification of a small fragment (between
161 100-200 pb) according to King et al. (2008) recommendations. Regarding to Prasinophyceae,
162 primers were designed for PCR amplification of the *rbcL* (ribulose-1,5-diphosphate carboxylase)
163 gene fragment, a core plant DNA barcode (Worden & Not 2008). All Prasinohyceae *sp*. sequences
164 available (GeneBank, October 2015) were aligned to design a family-specific primer following the
165 same procedure as for *Gymnodinium*. For sequence alignment Unigene software was used
166 (Okonechnikov et al. 2012) and primers were designed with Oligo 7 software (Molecular Biology
167 Insights, Inc.). As for copepods, three total DNA aliquots (5 µl) of each pool were assayed.
168 Amplicons were analysed as explained above.
169
170 *Larvae prey size estimations*
171 In order to estimate which copepod developmental stages could be potentially consumed by sardine
172 larvae within the size range collected in our study, we used the relationship by Morote et al. (2010)
173 between sardine larval standard length and prey width:
174 Prey width (µm) = 10.028·Larval SL (mm) + 5.747, $r^2 = 0.137$, $p<0.001$
175
176 **Results**
177 *Hydrography*
178 The mean±SD sea surface temperature was 15.9±0.2ºC, varying between 15.6 and 16.4ºC during
179 the diel cycle. The mean±SD sea surface salinity was 37.08±0.12, ranging from 36.92 to 37.22
180 (Table 1). The water column was not stratified and temperatures in bottom to surface waters was
181 similar by day (13.6-16.5 ºC) and night (14.5-16.3 ºC); as well as the salinity (36.9-38.2 by day and
182 37.1-37.7 at night).
183
184 *Plankton community composition*
185 Mesozooplankton was dominated by copepods, which accounted for 74.1±16.2% (45-94% range) of

186 the total abundance, followed by cladocerans (12.8±12.6%) and appendicularians (4.1±3.7%). The
187 most abundant copepod genus in the field was Oncaea (25.2±18.3%), with up to 2,880 ind·m^{-3} at
188 T5; while *Paracalanus, Temora, Acartia* and *Clausocalanus* adults represented from 7.9±4.9 to
189 5.0±3.5% of the total copepod abundance. The dominant species within these genera were: *Acartia*
190 *clausi* (99.93% of *Acartia* counts), *Temora stylifera* (96.92% of *Temora*), and *Paracalanus* cf.
191 *parvus* (88.37% of *Paracalanus*). *Clausocalanus* and *Oncaea* individuals were identified at genus
192 level, therefore comparison between field abundances and gut content contributions were done at
193 genus level. Unidentified copepodites and nauplii accounted for 20.1±15.1% and 4.0±3.3%,
194 respectively; and the remaining copepod species identified comprised a 25.5±10.9%. Copepods
195 were most abundant at night (T5-T7), when the community was dominated by *Oncaea* and *Acartia*;
196 also a diurnal increase was seen at T12-T13, except for *Acartia* whose abundance was very low by
197 day (Fig. 2a).The most abundant microplankton group was flagellates, followed by diatoms.
198 Dinoflagellates abundance was dominated by <20 µm cells, followed by *Gymnodinium catenatum*
199 which represented up to 58% of dinoflagellates counts. Abundance of cells <5 µm was dominated
200 by picoeukaryotes. Maximum abundances were observed at T10-T13 for microplankton, at T6 for
201 picoplankton and at T3-T4 for nanoplankton. All these fractions presented minimum abundances at
202 T5 (Fig. 2b), the beginning of the night period, coinciding with the highest copepod abundance.
203

204 *Sardine larval standard length*

205 The standard length (SL) of sardine larvae ranged from 5.9 to 20.8 mm, with a mean±SD of
206 10.64±2.15 mm (Fig. 3). There were no significant differences in the size distribution of the larvae
207 among sampling stations (Kolmogorov-Smirnov tests, $p > 0.05$), indicating that we were sampling
208 the same population. Only T5 distribution differed from some day and night stations probably due
209 to a larger contribution of larvae with SL >13 mm. The subset of sardine larvae selected for
210 molecular analyses showed a mean±SD SL of 10.80±0.73 mm. Of these, a 71% ranged between 9
211 and 13 mm (47% 9-11 mm, 24% 11-13 mm), 14% were <9 mm and 13% were >13 mm.

212

213 *Sardine larval gut contents*

214 We visually observed the presence of gut content in sardine larvae collected from mid-day (11:30 h)
215 until dusk (18:00 h). Feeding incidence estimated from photographs was 46% by day. However,
216 during the night and early morning hours guts seemed empty (Fig. A1). Larvae with visible gut
217 content were selected for molecular assays when available. Multiplex PCR results also showed day-
218 night differences in the presence/absence of the target copepod species within the guts of the sardine
219 larvae. *Temora stylifera* and *Oncaea waldemari* were detected during nearly the whole cycle;
220 whereas *Acartia clausi* was intermittently and poorly detected during the diel cycle (Table 3).
221 *Paracalanus indicus* and *Clausocalanus parapergens* were not found at night (T5 to T9), despite
222 their night abundances in the field accounting for up to 17.7 and 10.1% of total copepods,
223 respectively. Phytoplankton taxa were found during the entire cycle, although Prasinophyceae were
224 not detected at the end of the night (T9-T10).The relative contribution (ng DNA) of each prey
225 within the sardine larval guts was also different. *T. stylifera*, *P. indicus* and *O. waldemari* showed
226 the highest average contribution (42.1, 27.9 and 25.7 % of copepod DNA detected, respectively),
227 whereas *A. clausi* and *C. parapergens* represented a low percentage (2.6 and 1.7% of copepod DNA
228 detected, respectively, Fig. 4). Furthermore, we observed that peaks in *Oncaea* and *Acartia* relative
229 field abundance were significantly correlated to increases in their contribution to the DNA
230 concentration in larval gut contents 4 hours later (Fig. 4, $p<0.05$). *Temora* and *Clausocalanus* peaks
231 showed a positive, although not significant, relationship between field and gut contribution with a 2
232 hours delay ($p>0.05$). In the case of *Paracalanus* this coincidence was only observed during
233 daytime and the correlation was negative and not significant ($p>0.05$) during the diel cycle.

234

235 **Discussion**

236 Given that starvation is one of the main causes of mortality of the larvae of SPF and other marine
237 fish species (Hjort 1914), it is crucial to understand how plankton abundance and diversity affect

238 the diets of young fish larvae. In the Bay of Málaga, interactions among phytoplankton, ciliates and
239 zooplankton appear to play a central role in regulating the pelagic food web (Mercado et al. 2007)
240 and the role of sardine larvae as predators of these groups has not been previously investigated. This
241 study is the first to successfully combine traditional (microscopy) and molecular (multiplex PCR)
242 techniques to estimate the diet of sardine larvae.
243 The present study identified copepods as a main target of foraging by sardine larvae including four
244 calanoids (*Acartia clausi, Paracalanus indicus, Clausocalanus parapergens, Temora stylifera*) and
245 one poecilostomatoid (*Oncaea waldemari*). These copepods are cosmopolitan species distributed
246 across tropical and temperate oceans and seas (Razouls et al. 2005). The four calanoids are
247 epipelagic (Scotto di Carlo et al. 1984, Steinberg et al. 1994, Brugnano et al. 2012) and most
248 frequently sampled in the upper 200 m of the water column while *Oncaea waldemari* occurs across
249 a wider range in depths, from the deep-sea to the surface (Böttger-Schnack & Schnack 2013). As
250 hypothesized, we found that the three most frequently detected copepod within the guts of sardine
251 larvae (*Temora, Paracalanus* and *Oncaea*) were also the most abundant in the study area. However,
252 we cannot rule out preferential feeding on *Temora* whose DNA was the most abundant in guts (42%
253 *T. stylifera,* 26-28% *P. indicus* and *O. waldemari*) but was not the most numerically dominant
254 copepod in the field (25% *Oncaea*, 5-8% *Paracalanus*, *Acartia* and *Temora*). An important
255 limitation, however, is that the abundance of copepods in the field was based on adults identified to
256 the species or genus level using microscopy. Early copepodite and naupliar stages were not
257 identified (20 and 4% of total copepod counts, respectively) and the mesh of our sampling gear (200
258 µm) was not fine enough (e.g. 60 µm) to quantitatively sample these smaller life stages. Thus the
259 relative abundance of the adults of a species may not reflect the actual contribution of their nauplii
260 and copepodites to the suit of prey available to sardine larvae.
261 In the pelagic ecosystem, there is a tight relationship between the trophic position and the size of an
262 organism (Sharf et al. 2000). Also, the relationship between predator and prey sizes is the main
263 factor determining capture success (Hansen et al. 1994, Neubert et al. 2000). Hence, predation has

264 been considered opportunistic rather than taxon selective (Lundvall et al. 1999). Laboratory and
265 field studies on young European sardine larvae revealed that prey size significantly increased with
266 increasing larval length (Morote et al. 2010, Caldeira et al. 2014). Based on the model by Morote et
267 al. (2010) for sardine larvae in the NW Mediterranean and widths of potential prey estimated during
268 our field sampling, larvae up to 20 mm SL would be able to ingest adults of *O. waldemari* and early
269 copepodite stages and nauplii of the larger calanoid species detected in sardine gut contents (Table
270 4). Our results agree with microscopic observations of the gut contents of sardine larvae in the NW
271 Mediterranean and Cantabrian Seas, where 46-52% of the prey ingested by <10-13 mm sardine
272 larvae was copepod nauplii (Munuera Fernández & González-Quirós 2006, Morote et al. 2010).
273 Furthermore, The use here of a species-specific multiplex PCR assay has allowed, for the first time,
274 the identification to species level of the nauplii ingested by sardine larvae, which otherwise would
275 remain unidentified. However, apart from size, other factors such as nutritional quality or prey
276 motility can condition prey selection (Bautista & Harris 1992, Gragnani et al. 1999). Borme et al.
277 (2013) observed that post-flexion sardine larvae in the Adriatic Sea not only fed on the most
278 abundant copepods (*Temora longicornis* and *Paracalanus* spp.) but also *Temora stylifera, Acartia*
279 spp. and other copepod species which were rare in plankton samples. The positive selection of these
280 rare species was probably related to the poor alertness and weak escape response of these copepods
281 (Viitasalo et al. 2001). In the Bay of Málaga, despite the fact that *Oncaea* adults were more
282 abundant than other species, sardine larvae (as indicated by the DNA found in the guts) seemed to
283 prefer to prey on easier targets such as nauplii of *Temora* or *Paracalanus*, suggesting that motility
284 rather than nutritional quality was an important factor influencing prey selection by these larvae.
285 Phytoplankton DNA of bot taxa tested, *Gymnodinium* and Prasinophyceae, was also present in the
286 gut contents of sardine larvae. One previous study reported hervibory by sardine larvae in the NW
287 Mediterranean under a spring bloom situation (Rasoanarivo et al. 1991). However, several studies
288 in highly productive eastern boundary current systems have categorized larvae of SPF as passive
289 phytoplankton consumers, criticizing the assumption of phytophagy for this and other clupeid

290 species (Konchina et al. 1991, Van der Lingen 2002). In our study, we cannot ascertain whether
291 phytoplankton cells found in the guts were eaten directly by the sardine larvae or whether
292 phytoplankton DNA originated from the copepods ingested by these larvae. Sardine larvae lacked
293 the DNA of Prasinophyceae (<2 µm cells) at the end of the night (T9) when the lowest amounts of
294 copepod DNA were detected and when only *Oncaea* was detected (Table 3). Preliminary tests of the
295 phytoplankton primers showed that both phytoplankton taxa were not only detectable within the
296 guts of sardine larvae but also inside the copepod species preyed upon by larvae (data not shown).
297 Of the target copepods, all have been described as omnivores (Ohtsuka et al. 1993, Kouwenberg
298 1994, Mauchline 1998, Razouls et al. 2005, Benedetti 2016). However *A. clausi, P. indicus, C.*
299 *parapergens* and *T. stylifera* are predominantly herbivores (Wickstead 1962, Kouwenberg 1994,
300 Calbet & Saiz 2005) whereas *O. waldemari* is preferentially a detritivore (Wickstead 1962, Razouls
301 et al. 2005). The co-occurrence of the DNA of phytoplankton and herbivorous copepods in the guts
302 of sardine larvae, coupled with the weakest Prasinophyceae signal when the detritivore *Oncaea*
303 dominated gut contents, suggests that sardine larvae were most probably not consuming these
304 pigmented cells, but that we detected phytoplankton inside the guts of herbivore copepods eaten by
305 the larvae.
306 There was high day/night variability in the copepod field community as well as in the gut contents
307 of larvae; with a marked decrease at night in the number of species and DNA concentration
308 detected, suggesting a preferential diurnal feeding. This agrees with previous studies reporting that
309 the larvae of other clupeid species are visual predators (Arthur, 1976). Also, circadian variation in
310 the nutritional condition of sardine larvae (assessed as RNA:DNA) was suggested to be driven by
311 diel changes in larval diets (Conway et al. 1994, Cortés com. pers.). Total copepod DNA
312 concentration within the guts of sardine larvae did not match prey field abundance during the diel
313 cycle; we found higher DNA concentrations by day (T12-T13, >5 ng DNA·μl^{-1}), but highest
314 copepod numbers at night (T5-T6, >5,000 ind·m^{-3}). Further, *O. waldemari* and *T. stylifera* presented
315 a higher night signal within the guts along the diel cycle, whereas *P. indicus* and *C. parapergens*

316 were only detected by day. These differences might be explained by the diel variability of the
317 zooplankton community composition observed during the migration of the shoal of sardine larvae
318 towards shallow waters at dusk. Moreover, PCR is not quantitative and a high relative concentration
319 of DNA might be due to the presence of one entire (recently ingested) prey or the sum of several
320 heavily digested organisms. Nevertheless, it stands out that peaks of relative abundance of some
321 copepod species in the field were followed in time by increases in relative DNA concentration of
322 the same species within the guts of sardine larvae, irrespective of the time of the day (e.g. Acartia,
323 Fig. 4); supporting the idea of opportunistic feeding by these larvae.
324 The results of this molecular assessment of the diet of sardine larvae in the field support our initial
325 hypothesis, that sardine larvae have an opportunistic rather than selective feeding behavior.
326 Nevertheless, among these copepods, sardine larvae (mean SL of 10 mm) may select nauplii of
327 large copepods (i.e. *Temora*) likely because they are easier targets than adults of small-bodied
328 species (such as *Oncaea*). Also, in this work we studied predation on copepods, the most abundant
329 zooplankton group in the study area during autumn. In order to fully comprehend the trophic
330 ecology of sardine larvae, further molecular assays (e.g. metabarcoding) need to be conducted to
331 detect other potential planktonic prey, such as microplanktonic protists and gelatinous organisms.
332 The development and application of further genomic tools, such as the *ad hoc* designed multiplex-
333 PCR assays applied here, will facilitate the study of the autoecology of planktonic species and their
334 trophodynamimc role in the marine ecosystems. Furthermore, the species-specific multiplex PCR
335 used on sardine larvae can be applied, as a low cost, complementary or alternative tool to
336 microscopy, to detect a suit of 5 common copepods within guts of other SPF which are known to
337 prey on nauplii. These SPF would include species of commercial interest, such as anchovy
338 (*Engraulis encrasicolus*, Tudela et al. 2002, Morote et al. 2010), round sardinela (*Sardinella aurita*,
339 Morote et al. 2008) or European sprat (*Spratus spratus*, Conway et al. 1991); but also other fish
340 larvae for which there is no information on their diet, like the boarfish (*Capros caper*), which new
341 fishery has increasing commercial interest in the N Atlantic (Stange 2016).

342

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347

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543 **Tables**

544 Table 1. Sampling stations location (position of the zooplankton vertical haul) and bottom depth

545 (Depth, m), sea surface (5 m depth) temperature (SST, ºC) and salinity (SSS), and larvae and

546 zooplankton sampling time (local time: GMT+1). Bold font indicates night period.

Station	Latitude	Longitude	Depth	SST	SSS	Larvae	Zooplankton
T1	36° 38.37	-4° 21.18	80	15.78	37.01	11:30	12:38
T2	36° 39.47	-4° 22.00	64	16.44	36.93	13:30	14:51
T3	36° 38.79	-4° 20.71	73	15.90	36.93	16:00	17:07
T4	36° 40.74	-4° 23.32	47	16.24	36.97	18:00	**18:36**
T5	**36° 42.60**	**-4° 24.14**	**20**	**15.67**	**37.22**	**19:50**	**20:26**
T6	**36° 42.76**	**-4° 24.16**	**18**	**16.13**	**37.10**	**22:10**	**22:34**
T7	**36° 42.52**	**-4° 24.28**	**21**	**15.74**	**37.20**	**24:10**	**00:34**
T8	**36° 42.53**	**-4° 24.19**	**22**	**15.90**	**37.13**	**02:15**	**02:44**
T9	**36° 42.47**	**-4° 23.75**	**22**	**16.04**	**37.07**	**04:15**	**04:39**
T10	**36° 42.33**	**-4° 24.11**	**22**	**15.75**	**37.20**	**06:10**	**06:40**
T11	36° 40.88	-4° 22.89	48	15.84	36.92	08:30	09:08
T12	36° 40.76	-4° 22.94	46	15.58	37.18	10:30	10:58
T13	36º 40.54	-4º 22.93	47	15.70	37.20	12:15	12:49

547

548 Table 2. Sequences of the phytoplankton primers designed for this study. Tm: primer melting

549 temperature (ºC).

Taxa	Target gene	Primer name	Primer sequence (5ø-3ø)	Amplicon size (bp)	Tm
Gymnodinium catenatum	LSU rDNA	Gymno-F	TGT GAA ACC GAT AGC AAA CAA GT	105	51.7
		Gymno-R	ATC CTT CGC TTC CAG TTC AGC		54.3
Prasinophyceae	rbcL	Cloro-F	CCA GCT CTA GTT GAG ATC TTC G	155	55.3
		Cloro-R	CGA AGC TAA GTC ACG TCC TTC		56.5

550

551

552 Table 3. Presence/absence of prey detected by multiplex PCR within guts of sardine larvae during

553 the diel cycle. +: positive replicates, -: non detected, [DNA]: DNA concentration (ng μL^{-1}) of each

554 sardine gut pool, assessed using NanoDrop 1000. Bold font indicates night period.

Sample	*Oncaea*	*Temora*	*Paracal.*	*Acartia*	*Clausocal.*	*Gymno.*	Prasino.	[DNA]
T2	+++	+++	+++	+	-	+++	+++	200.95
T3	-	+++	+++	-	+	+++	+++	134.35
T4	-	+++	+++	+	++	+++	+++	185.60
T5	**++**	**+++**	**-**	**++**	**+**	**+++**	**+++**	**287.65**
T6	**+**	**+++**	**-**	**-**	**-**	**+++**	**+++**	**257.10**
T7	**+**	**+**	**-**	**-**	**-**	**+++**	**+++**	**247.00**
T8	**++**	**+++**	**-**	**+**	**-**	**+++**	**++**	**303.75**
T9	**+**	**-**	**-**	**-**	**-**	**+++**	**-**	**137.55**
T10	**+**	**+++**	**++**	**++**	**++**	**+++**	**-**	**398.45**
T11	++	+++	+	+	+	+++	+++	188.10
T12	+++	+++	+++	-	-	+++	+++	175.45
T13	-	+++	+++	-	+	+++	+++	184.90

555 Image analyses of the larvae pools revealed that 2 larvae in T1 were *Engraulis encrasicolus*, thus
556 we discarded T1 gut content results.
557

558

559 Table 4. Mean length (mm) and width (mm) of the five target copepod species. Corresponding

560 standard length (SL, mm) of potential predator (*Sardina pilchardus*) was calculated as in Morote et

561 al. 2010. Bold font indicates developmental stages falling within the expected prey size for the

562 sardine larvae in our study (SL <20 mm).

Species		Length	Width	Sardine SL	Reference
Acartia clausi	**CI**	0.48	0.14	13.39	(1)
	CII	0.58	0.16	15.38	(1)
	CIII	0.70	0.19	18.37	(1)
	CIV	0.93	0.23	22.36	(1)
	CIV	0.88	0.24	23.36	(1)
	CV	1.04	0.28	27.35	(1)
	CV	1.06	0.26	25.35	(1)
	CVI	1.16	0.28	27.35	(1)
	CVI	1.13	0.28	27.35	(1)
Clausocalanus parapergens	CVI	1.10	0.38	37.78	(2)
	CVI	1.31	0.27	26.02	(2)
Paracalanus indicus	CVI	0.85-1.02	0.29	28.35	(2)(3)
	CVI	0.85-0.95	0.26	25.35	(3)
Temora stylifera	**CI**	0.29	0.19	18.57	(4)
	CII	0.41	0.24	23.36	(4)
	CIII	0.51	0.32	31.24	(4)
	CIV	0.59	0.34	33.23	(4)
	CV	0.69	0.34	33.43	(4)
	CVI	0.88	0.42	41.51	(4)
	CVI	0.93	0.48	47.29	(4)
Oncaea waldemari	**CVI**	0.37-0.58	-	-	(2)
	CVI	0.49-0.76	0.16	15.58	(2)

563 [1]Conway 2012, [2]Razouls et al. 2005, [3]Bradford 1978, [4]Shmeleva 1965. *C. parapergens* and *O.*

564 *waldemari* female sizes were extracted from taxonomical plates.

565

566 **Figure legends**

567 Figure 1. Sampling stations location and Alboran Sea surface circulation. WAG: West anticyclonic
568 gyre, EAG: East anticyclonic gyre.

569 Figure 2. Trophic conditions during the diel cycle. Field abundance of a) copepods ($ind \cdot m^{-3}$), b)
570 microplankton ($cells \cdot mL^{-1}$, left axis), picoeukaryotes ($10^3 \cdot cells \cdot mL^{-1}$, right axis) and
571 nanoeukaryotes ($cells \cdot mL^{-1}$, right axis). Grey shadow indicates night period.

572 Figure 3. Frequency distribution of sardine larval size classes (standard length, mm).

573 Figure 4. Diel variation of relative copepod field abundance (%, left axis, closed circles) and
574 relative prey concentration within sardine larvae guts (%, right axis, open circles). Grey shadow
575 indicates night period.

576 Appendix figure A1. Photographs of *Sardina pilchardus* larvae caught during a 26 hours diel cycle
577 within the Bay of Málaga. Larva caught at day showing gut content (a) and larva caught at night
578 void of gut content (b).

579

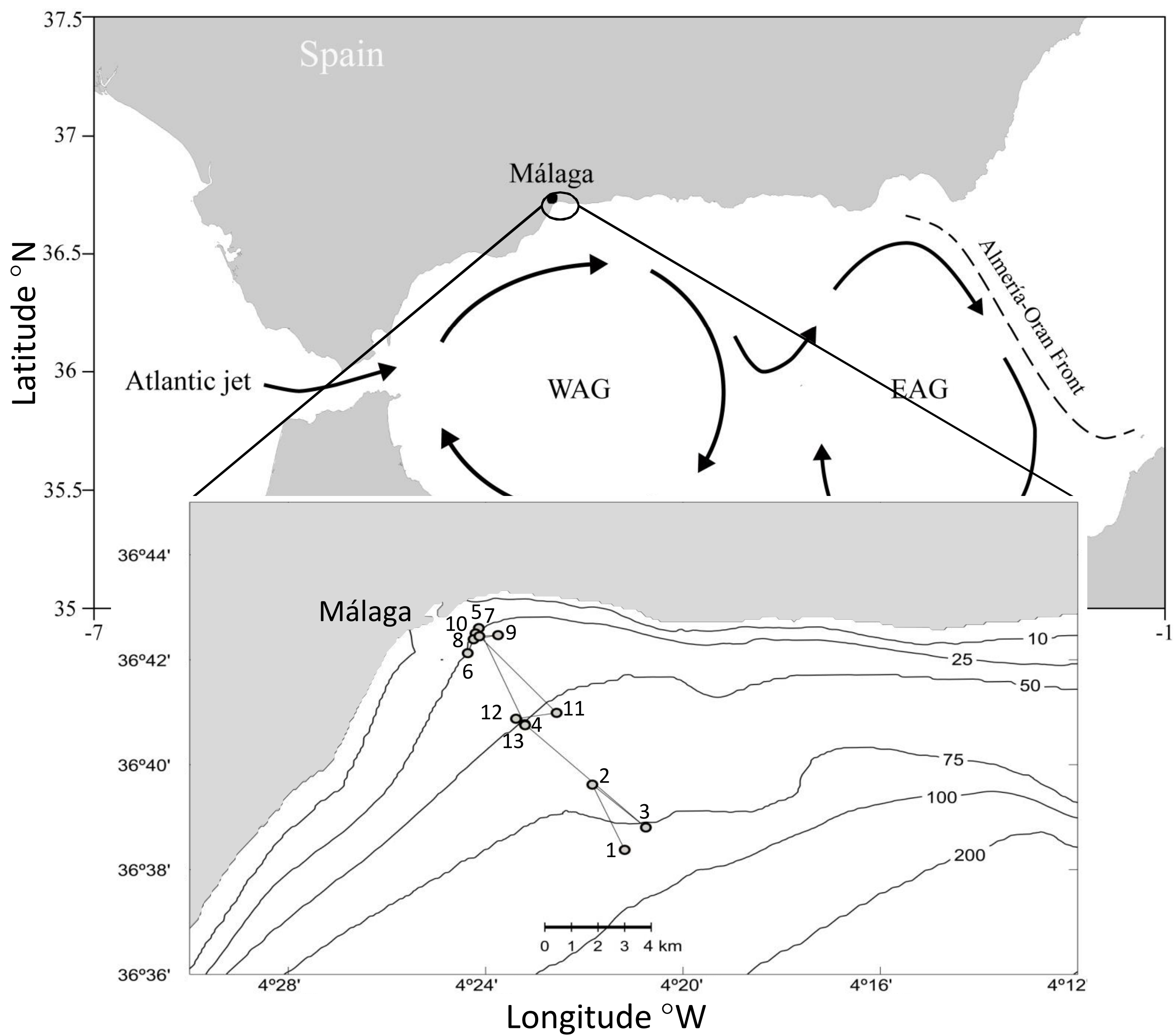

Fig.1

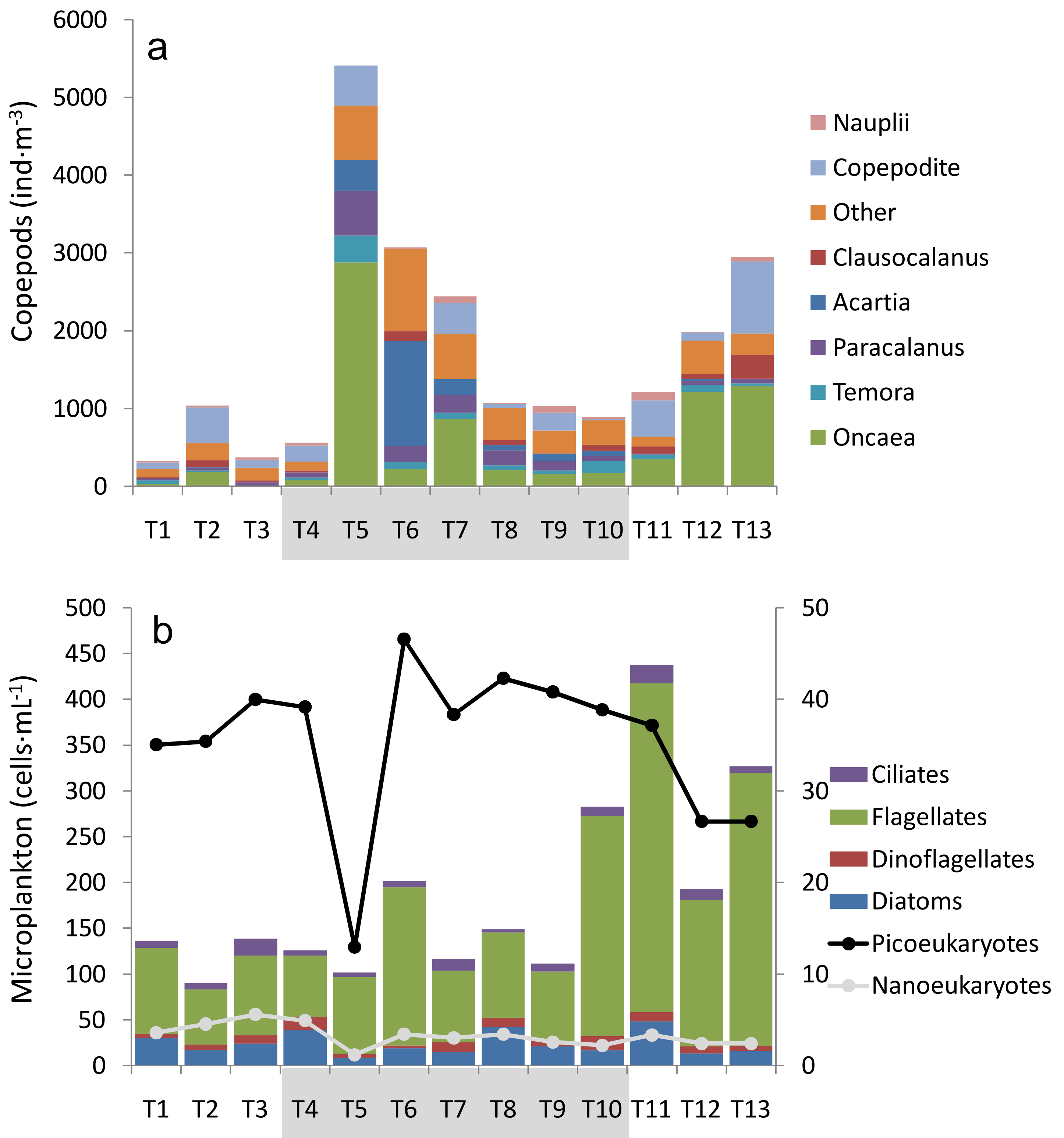

Fig.2

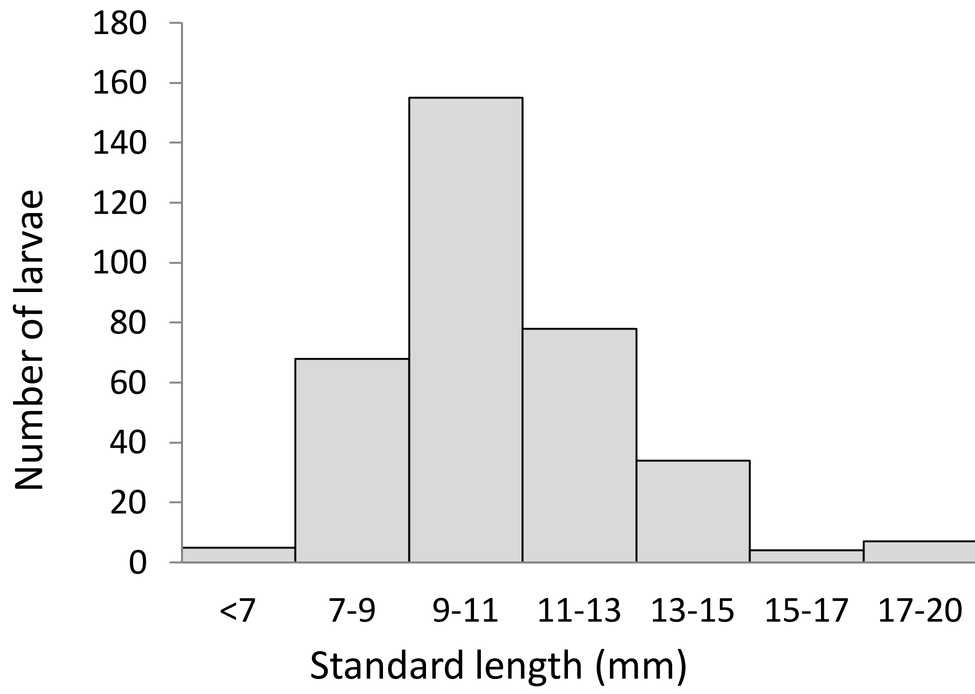

Fig.3

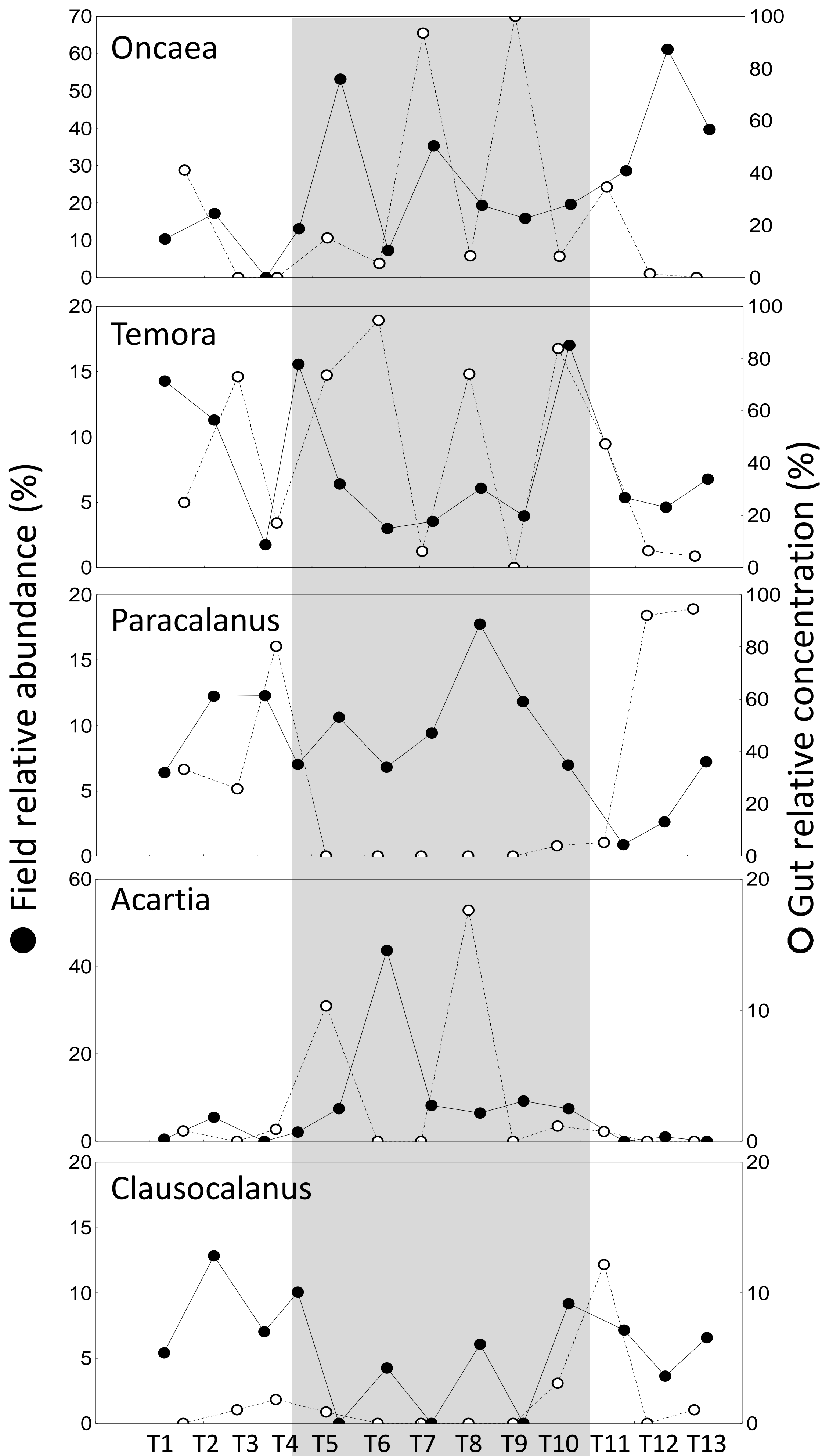

Fig.4

Appendix.

Figure A1. Photographs of *Sardina pilchardus* larvae caught during a 26 hours diel cycle within the Bay of Málaga. Larva caught at day showing gut content (a) and larva caught at night void of gut content (b).