**Advance View**
https://doi.org/10.3354/meps12833

**MARINE ECOLOGY PROGRESS SERIES**
**Mar Ecol Prog Ser**

**Available online:**
**January 16, 2019**

*Contribution to the Theme Section 'Drivers of dynamics of small pelagic fish resources: biology, management and human factors'*

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

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ABSTRACT: Molecular techniques provide new insights into the feeding strategies and diets of planktonic organisms such as the larvae of marine fish. We applied multiplex PCR to obtain the first estimates of the diets of larval European sardine *Sardina pilchardus* in the Alboran Sea (SW Mediterranean), where this species represents an important fishery resource. The feasibility of this technique was tested in a 26 h continuous survey of a shoal of larvae (10.80 ± 0.73 mm standard length, mean ± SD). Multiplex PCR was designed to detect the presence of 5 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 behaviour. The use of multiplex PCR allowed species-level identification of ingested nauplii and protists, which otherwise would remain unidentified.

KEY WORDS: Alboran Sea · *Sardina pilchardus* · Diel cycle · Larval ecology · Multiplex PCR



## 1. INTRODUCTION

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

In the Mediterranean Sea, the Bay of Málaga in the central part of the north Alboran Sea is the most important nursery site for the European sardine *Sardina pilchardus* and anchovy *Engraulis encrasicolus* (García et al. 1988, García 2010, Giannoulaki et al. 2013). The bay is an essential habitat for these SPF species as it fulfills the Bakun triad, a set of conditions needed for larvae survival (Agostini & Bakun 2002), including high abundances of phytoplankton and zooplankton (Mercado et al. 2007, Yebra et al. 2017). These conditions are promoted by mesoscale hydrographic structures such as fronts and gyres caused by the influx of Atlantic water through the Strait of Gibraltar (Parrilla & Kinder 1987) and upwelling events induced by westerly winds (Sarhan et al. 2000, Mercado et al. 2012). Sardines display an

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extended spawning season in this region (Rodríguez 1990, Tendero 2016) where their larvae are often dominant members of the ichthyoplankton (Palomera et al. 2007). Despite this numerical abundance of sardine larvae, no previous studies have reported on their diet and feeding habits in the Alboran Sea.

Although the diet of a fish larva depends on the abundance and diversity of prey encountered (García et al. 2003), the larvae of most marine fish selectively feed on specific prey species (Peck et al. 2012). To date, studies on the diet of various life stages of European sardine have shown that mesozooplankton (>200 µm, mostly copepods and cladocerans) comprises the major food source for larvae (e.g. Conway et al. 1994, Morote et al. 2010, Costalago & Palomera 2014). In the NW Mediterranean Sea, tintinnids and copepod nauplii form the largest proportion of the gut contents of the smallest pre-flexion larvae, while larger larvae preferentially consume nauplii and copepodites of calanoid copepods (Morote et al. 2010). However, important regional differences in diet and/or omnivorous foraging may exist, as Rasoanarivo et al. (1991) found sardine larvae consuming exclusively phytoplankton, from 5 µm (*Chlorella* spp.) to 130 µm (*Synedra acus*) in the Gulf of Lions (NW Mediterranean). Small microzooplankton (i.e. protozoans) might be important prey for ichthyoplankton (Bils et al. 2017); however, the protozooplankton–ichthyoplankton link remains largely unexplored, as most field studies employ microscopic analysis of gut contents of larvae preserved in formalin (Peck et al. 2012). This traditional approach presents several limitations, which include the difficulty of identifying early developmental stages (e.g. nauplii), soft-bodied organisms or partly digested items. Stable isotope analysis provides another tool to infer larval feeding habits (Bode et al. 2004, Laiz-Carrión et al. 2011, Costalago et al. 2012) but this technique provides no information on prey species or prey preference.

To overcome the limitations of using microscopic identification of gut contents to identify the diets of marine fish larvae, molecular tools have been developed in recent years. These new techniques not only complement traditional microscopy counts, but are also useful tools that improve the accuracy of identification of organisms at the species level (even cryptic ones or partly digested remains) and increase the volume of samples that can be analysed in a cost-effective manner. Due to their precision and sensitivity, both PCR and quantitative PCR (qPCR) have been applied to detect and quantify species from water samples (Vadopalas et al. 2006, Miyaguchi et al. 2008, Pan et al. 2008), and have been successfully applied to examine the diet of zooplankton (Nejstgaard et al. 2003, 2008, Troedsson et al. 2007, Simonelli et al. 2009). Moreover, metabarcoding assays employed on gut contents of adult European sardine (Albaina et al. 2016) and larval European eel *Anguilla anguilla* (Ayala et al. 2018) identified the main taxonomic groups, including protists and soft-bodied organisms, not identifiable by microscopic examination.

The present study is the first to examine the diet of sardine larvae in the Alboran Sea and is one of only a handful of studies to apply molecular markers to larval fish gut contents. We tested 2 hypotheses: (1) sardine larvae feed on the most abundant prey at the beginning of the spawning season to maximize rates of growth and development and (2) diel differences in the diet of sardine larvae explain day/night differences in the nutritional condition of the larvae (Conway et al. 1994, D. Cortés unpubl. data). We quantified the taxonomic composition of the Alboran Sea plankton community and designed and applied species-specific molecular markers to detect the presence of selected target organisms within sardine larval guts (A. Hernández de Rojas et al. unpubl. data).

Based on (1) the dominant phyto- and zooplankton in the Bay of Málaga during autumn (e.g. small flagellates, Mercado et al. 2005, 2007; copepods, Rodríguez 1983, Sampaio de Souza et al. 2005) and (2) gut contents of sardine larvae in other areas of the NW Mediterranean (Rasoanarivo et al. 1991, Morote et al. 2010), molecular markers were developed for 5 mesozooplankton copepod genera (*Oncaea, Acartia, Temora, Clausocalanus* and *Paracalanus*), a microplanktonic dinoflagellate genus (*Gymnodinium*) and the picoeukaryote algae family Prasinophyceae, as a representative of the Chlorophyta.

## 2. MATERIALS AND METHODS

### 2.1. Sampling

Sampling took place onboard the RV 'Francisco de Paula Navarro', on 8–9 November 2014, during a 26 h diel cycle within the Bay of Málaga (Fig. 1). Every 2 h (T1 to T13, Table 1), bongo nets (60 cm diameter, 500 µm mesh) were deployed to collect sardine larvae by means of oblique hauls down to 5 m above the seafloor. Sampling started at midday in shallow shelf waters (70–80 m depth) where adult sardine are known to spawn, and we gradually moved towards nursery shallow inshore waters (18–22 m

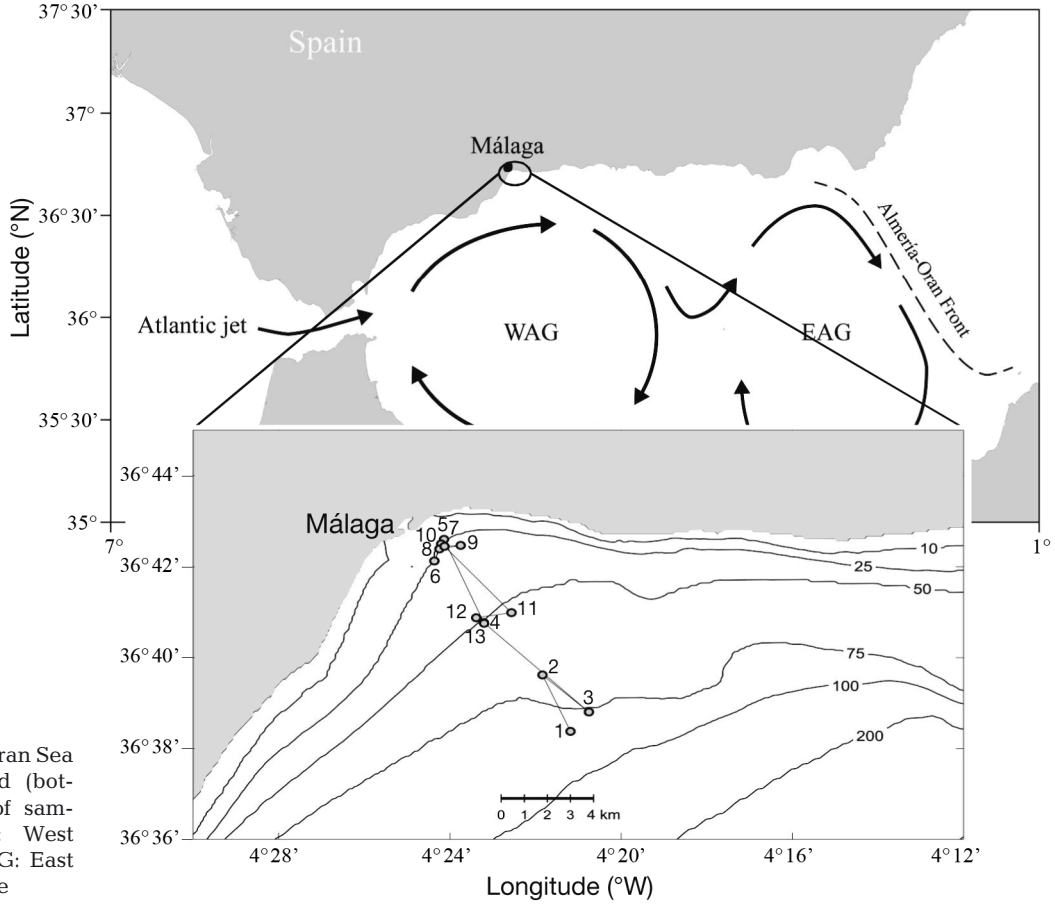


Fig. 1. (Top panel) Alboran Sea surface circulation and (bottom panel) locations of sampling stations. WAG: West Anticyclonic Gyre, EAG: East Anticyclonic Gyre

depth), where larvae concentrate at nighttime (García et al. 1988). On board, a subset of 351 ind. were sorted, identified, visually examined for gut contents and photographed with a Leica EZ4HD, for later measurement of standard length (SL), and immediately preserved in undenatured ethanol (96%) for molecular assays. Larvae handling time between collection and preservation did not exceed 5 min.

After each ichthyoplankton sampling, a CTD SBE-25 was used to obtain vertical profiles of temperature and salinity at each sampling site. Niskin bottles were then used to collect seawater at the surface and close to the seafloor. Samples to determine the abundance and taxonomic composition of phytoplankton >5 µm were fixed in dark glass bottles with Lugol's solution (2% final concentration). Samples for determination of eukaryotic pico- and nanoplankton abundance were fixed with glutaraldehyde (1% final concentration) and immediately frozen in liquid nitrogen (Vaulot et al. 1989). Finally, a WP2-double net (200 µm mesh) was deployed vertically to collect mesozooplankton, from 3 m above the bottom

Table 1. Details of sampling stations in the Alboran Sea: location (position of the zooplankton vertical haul), bottom depth (m), sea surface (5 m depth) temperature (SST, °C) and salinity (SSS), and larvae and zooplankton sampling +time (local time: GMT+1). **Bold** font indicates the night period

| Station | Latitude (N) | Longitude (W) | Depth (m) | SST (°C) | SSS | Sampling time (h) 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 | 36° 40.54′ | 4° 22.93′ | 47 | 15.70 | 37.20 | 12:15 | 12:49 |

to the surface, at a speed of 0.5 m $s^{-1}$. Zooplankton was carefully rinsed and preserved with 96% non-denatured ethanol for taxonomic analyses.

### 2.2. Plankton community composition

In the laboratory, 100 ml of each phytoplankton >5 µm sample were sedimented in a composite chamber for 24 h, following the technique developed by Utermöhl (1958). Cells were counted at 200× and 400× magnification with a Leica DMIL inverted microscope. The species nomenclature was validated using Tomas (1997). Pico- and nanoplankton samples for determination of eukaryotic pico- and nanoplankton abundance were fixed with glutaraldehyde (1% final concentration) and immediately frozen in liquid nitrogen (Vaulot et al. 1989). Samples were analysed with a Becton Dickinson FACScan flow cytometer. Cell counting was performed based on the forward-light scatter and the orange and red fluorescence signals. BD TrueCOUNT Tubes were used to determine absolute counts. Copepod abundance and taxonomic composition were determined using a stereomicroscope (Leica M165C). Taxonomic identification was made to the lowest possible level according to Rose (1933), Trégouboff & Rose (1957) and Razouls et al. (2005). Copepod identification to species level was not always feasible, as some genera include cryptic species in the study area (e.g. Kasapidis et al. 2018). Thus, we report field copepod abundance data at the genus level.

### 2.3. Molecular analyses of larval gut content

In the laboratory, sardine larvae were dissected for gut DNA extraction. Prior to extraction, individuals were washed 3 times with sterilized water, and all the material, forceps and scalpels were flame sterilized before and after each dissection. From each sampling, the gut contents of 10 larvae were pooled together and total DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's instructions, except for the Proteinase K incubation, which was done overnight at 37°C. DNA pools were stored at −20°C until their assay, and DNA purity and concentrations were assessed using NanoDrop 1000 (Thermo Scientific) in 1 µl of sample (for DNA in each pool, see Table 3). In order to assess the presence/absence of potential prey within the larval guts, 5 µl of total DNA from each pool were assayed in triplicate by means of a species-specific multiplex PCR designed ad hoc for this purpose (A. Hernández de Rojas et al. unpubl. data). In brief, a multiplex PCR was designed to detect, in a single assay, the DNA of the 5 most abundant copepod species found in the study area, by targeting short fragments (100–200 bp) of their mitochondrial cytochrome c oxidase subunit I (mtCOI) gene. The potential prey targeted were *Clausocalanus parapergens*, *Acartia clausi*, *Paracalanus indicus*, *Temora stylifera* and *Oncaea waldemari*. PCR melting temperatures (Tm) ranged from 42 to 50°C, and amplicon lengths varied from 104 to 193 bp. PCR products were separated and analysed with Bioanalyzer 2100 (Agilent), using the DNA 1000 kit (Agilent). Electropherograms were analysed with 2100 Expert Software (Agilent), and fragments of the expected length which also yielded ≥1 fluorescent unit (FU) were counted as positive.

Likewise, phytoplankton content of the gut was studied by means of a second multiplex PCR. In this case, group-specific primers were designed (Table 2) to detect the dinoflagellate genus *Gymnodinium* (105 bp amplicon) and the picoeukaryote family Prasinophyceae (155 bp amplicon). The large subunit ribosomal DNA (LSU rDNA) marker is preferentially used for dinoflagellate species identification due to its high variability in some domains (Gomez et al. 2011). Thus, for *Gymnodinium* primer design, sequence alignment of available (GenBank, October 2015) *G. catenatum* mitochondrial LSU rDNA gene sequences was performed. To ensure the detection of partially

Table 2. Sequences of the phytoplankton primers designed for this study. Tm: primer melting temperature (°C)

| Taxon | Target gene | Primer name | Primer sequence (5′-3′) | 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 | Chloro-F | CCA GCT CTA GTT GAG ATC TTC G | 155 | 55.3 |
| | | Chloro-R | CGA AGC TAA GTC ACG TCC TTC | | 56.5 |

digested dinoflagellate DNA, primers were designed for the amplification of a small fragment (between 100 and 200 bp) according to the recommendations of King et al. (2008). For the Prasinophyceae, primers were designed for PCR amplification of the ribulose-1,5-diphosphate carboxylase (*rbcL*) gene fragment, a core plant DNA barcode (Worden & Not 2008). All Prasinophyceae sp. sequences available in GenBank (October 2015) were aligned to design a family-specific primer following the same procedure as for *Gymnodinium*. For sequence alignment, we used Unigene software (Okonechnikov et al. 2012), and primers were designed with Oligo 7 software (Molecular Biology Insights). In the same way as for copepods, 3 total DNA aliquots (5 µl) of each pool were assayed. Amplicons were analysed as explained above.

### 2.4. Prey size estimations

In order to estimate which copepod developmental stages could be potentially consumed by sardine larvae within the size range collected in our study, we used the relationship given by Morote et al. (2010) between sardine larval SL and prey width:

$$\text{Prey width (µm)} = 10.028 \times \text{Larval SL (mm)} + 5.747 \quad (r^2 = 0.137,\ p < 0.001) \quad (1)$$

## 3. RESULTS

### 3.1. Hydrography

The mean ± SD sea surface temperature was 15.9 ± 0.2°C, varying between 15.6 and 16.4°C during the diel cycle. Sea surface salinity was 37.08 ± 0.12, ranging from 36.92 to 37.22 (Table 1). The water column was not stratified, and temperatures in bottom to surface waters were similar by day (13.6–16.5°C) and night (14.5–16.3°C), as was salinity (36.9–38.2 by day and 37.1–37.7 at night).

### 3.2. Plankton community composition

Mesozooplankton was dominated by copepods, which accounted for 74.1 ± 16.2% (45–94% range) of the total abundance, followed by cladocerans (12.8 ± 12.6%) and appendicularians (4.1 ± 3.7%). The most abundant copepod genus in the field was *Oncaea* (25.2 ± 18.3%), with up to 2880 ind. $m^{-3}$ at T5, while *Paracalanus, Temora, Acartia* and *Clausocalanus* adults represented from 7.9 ± 4.9 to 5.0 ± 3.5% of the total copepod abundance. The dominant species within these genera were: *A. clausi* (99.93% of *Acartia* counts), *T. stylifera* (96.92% of *Temora*) and *P.* cf. *parvus* (88.37% of *Paracalanus*). *Clausocalanus* and *Oncaea* individuals were identified to genus, so comparison between field abundances and gut content contributions were also made at the genus level. Unidentified copepodites and nauplii accounted for 20.1 ± 15.1% and 4.0 ± 3.3%, respectively, and the remaining copepod species identified comprised 25.5 ± 10.9%. Copepods were most abundant at night (T5–T7), when the community was dominated by *Oncaea* and *Acartia*. A diurnal increase was also seen, at T12–T13, except for *Acartia* whose abundance was very low by day (Fig. 2a). The most abundant microplankton group was flagellates, followed by diatoms. Dinoflagellate abundance was dominated by <20 µm cells, followed by *Gymnodinium catenatum*, which represented up to 58% of dinoflagellate counts. Abundance of cells <5 µm was dominated by picoeukaryotes. Maximum abundances were observed at T10–T13 for microplankton, at T6 for picoplankton and at T3–T4 for nanoplankton. All of these fractions presented minimum abundances at T5 (Fig. 2b), the beginning of the night period, coinciding with the highest copepod abundance.

### 3.3. Sardine larval SL

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

### 3.4. Sardine larval gut contents

We visually observed the presence of gut contents in sardine larvae collected from mid-day (11:30 h) until dusk (18:00 h). Feeding incidence estimated from photographs was 46% by day. However, during the night and early morning hours, guts seemed empty (Fig. A1 in the Appendix). Larvae with visible

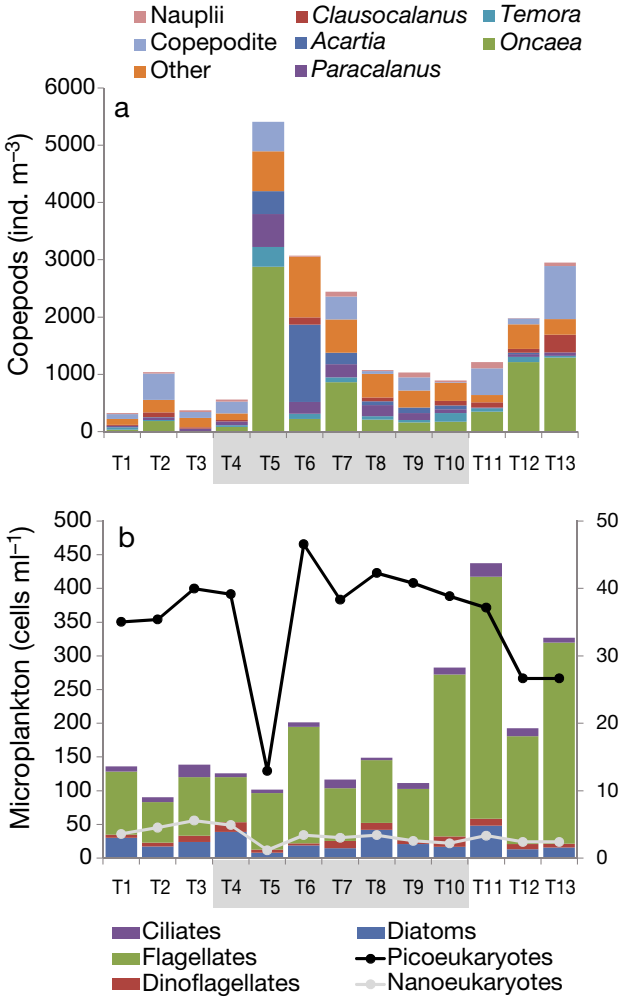


Fig. 2. Trophic conditions during the diel cycle. Field abundance of (a) copepods (ind. $m^{-3}$), (b) microplankton (cells $ml^{-1}$, left axis), picoeukaryotes ($10^3$ cells $ml^{-1}$, right axis) and nanoeukaryotes (cells $ml^{-1}$, right axis). Grey shading indicates the night period

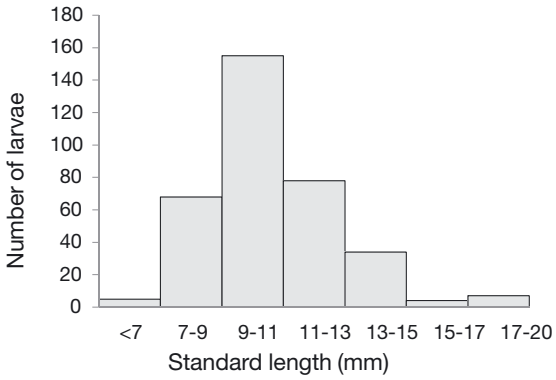


Fig. 3. Frequency distribution of sardine larval size classes (standard length, mm)

gut contents were selected for molecular assays when available. Multiplex PCR results also showed day–night differences in the presence/absence of the target copepod species in the guts of the sardine larvae. *T. stylifera* and *O. waldemari* were detected during nearly the whole cycle, whereas *A. clausi* was intermittently and poorly detected during the diel cycle (Table 3). *P. indicus* and *C. parapergens* were not found at night (T5–T9), despite their night abundances in the field accounting for up to 17.7 and 10.1% of total copepods, respectively. Phytoplankton taxa were found during the entire cycle, although Prasinophyceae were not detected at the end of the night (T9–T10). The relative contribution (ng DNA) of each prey in the sardine larval guts also varied. *T. stylifera*, *P. indicus* and *O. waldemari* showed the highest average contribution (42.1, 27.9 and 25.7% of copepod DNA detected, respectively), whereas the percentage of *A. clausi* and *C. parapergens* was low (2.6 and 1.7% of copepod DNA

Table 3. Presence/absence of prey detected by multiplex PCR within guts of sardine larvae during the diel cycle. +: positive replicates, –: not detected, [DNA]: DNA concentration (ng $\mu l^{-1}$) of each sardine gut pool, assessed using NanoDrop 1000. **Bold** font indicates the night period. Image analyses of the larvae pools revealed that 2 larvae in T1 were *Engraulis encrasicolus*; thus we discarded the T1 gut content results

| Station | *Oncaea* | *Temora* | *Paracalanus* | *Acartia* | *Clausocalanus* | *Gymnodinium* | Prasinophyceae | [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 |

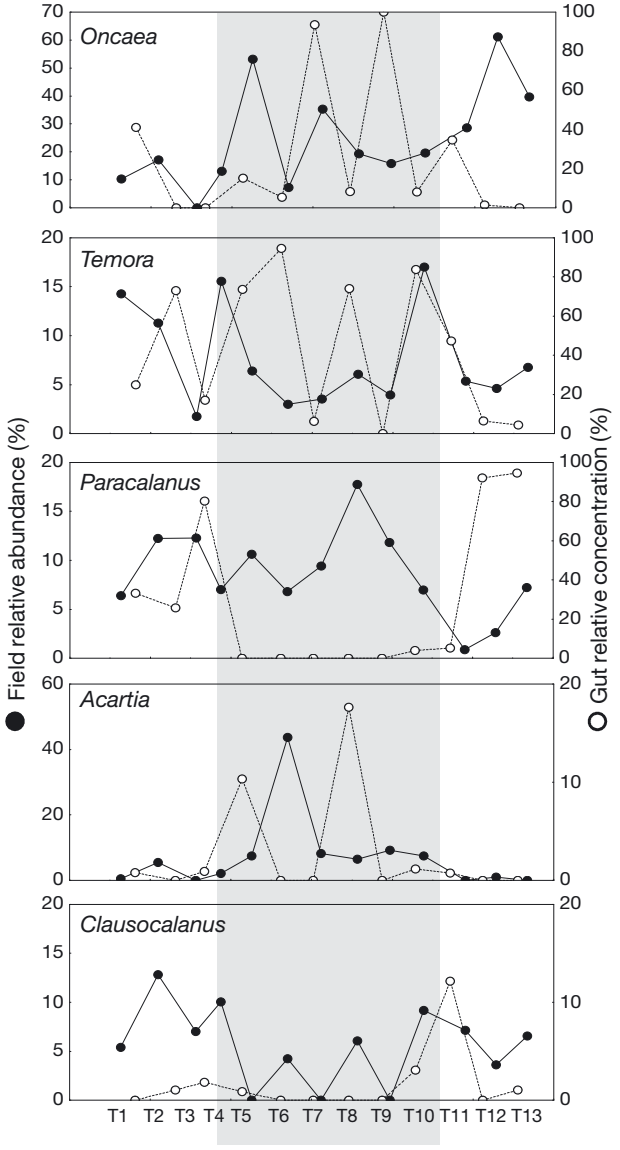


Fig. 4. Diel variation of relative copepod field abundance (%, left axis, closed circles) and relative prey concentration within sardine larvae guts (%, right axis, open circles). Grey shading indicates the night period

detected, respectively, Fig. 4). Furthermore, we observed that peaks in the relative field abundance of *Oncaea* and *Acartia* were significantly correlated to increases in their contribution to the DNA concentration in larval gut contents 4 h later (Fig. 4, $p < 0.05$). *Temora* and *Clausocalanus* peaks showed a positive, although not significant, relationship between field and gut contribution with a 2 h delay ($p > 0.05$). In the case of *Paracalanus*, this coincidence was only observed during daytime, and the correlation was negative and not significant ($p > 0.05$) during the diel cycle.

## 4. DISCUSSION

Given that starvation is one of the main causes of mortality in the larvae of SPF and other marine fish species (Hjort 1914), it is crucial to understand how plankton abundance and diversity affect the diets of young fish larvae. In the Bay of Málaga, interactions among phytoplankton, ciliates and zooplankton appear to play a central role in regulating the pelagic food web (Mercado et al. 2007), and the role of sardine larvae as predators of these groups has not been previously investigated. This study is the first to successfully combine traditional (microscopy) and molecular (multiplex PCR) techniques to estimate the diet of sardine larvae.

The present study identified copepods as the main target of foraging by sardine larvae, including 4 calanoids (*Acartia clausi, Paracalanus indicus, Clausocalanus parapergens, Temora stylifera*) and 1 poecilostomatoid (*Oncaea waldemari*). These copepods are cosmopolitan species distributed across tropical and temperate oceans and seas (Razouls et al. 2005). The 4 calanoids are epipelagic (Scotto di Carlo et al. 1984, Steinberg et al. 1994, Brugnano et al. 2012) and most frequently sampled in the upper 200 m of the water column, while *O. waldemari* occurs across a wider range of depths, from the deep-sea to the surface (Böttger-Schnack & Schnack 2013). As hypothesized, we found that the 3 most frequently detected copepods within the guts of sardine larvae (*Temora, Paracalanus* and *Oncaea*) were also the most abundant in the study area. However, we cannot rule out preferential feeding on *Temora*, whose DNA was the most abundant in guts (42% *T. stylifera,* 26–28% *P. indicus* and *O. waldemari*), although it was not the most numerically dominant copepod in the field (25% *Oncaea*, 5–8% *Paracalanus*, *Acartia* and *Temora*). An important limitation, however, is that the abundance of copepods in the field was based on adults identified to the species or genus level using microscopy. Early copepodite and naupliar stages were not identified (20 and 4% of total copepod counts, respectively) and the mesh of our sampling gear (200 µm) was not fine enough (e.g. 60 µm) to quantitatively sample these smaller life stages. Thus, the relative abundance of the adults of a species may not reflect the actual contribution of their nauplii and copepodites to the suite of prey available to sardine larvae.

In the pelagic ecosystem, there is a tight relationship between the trophic position and the size of an organism (Scharf et al. 2000). Also, the relationship between predator and prey sizes is the main factor

determining capture success (Hansen et al. 1994, Neubert et al. 2000). Hence, predation has been considered opportunistic rather than taxon selective (Lundvall et al. 1999). Laboratory and field studies on young European sardine larvae revealed that prey size significantly increased with increasing larval length (Morote et al. 2010, Caldeira et al. 2014). Based on the model by Morote et al. (2010) for sardine larvae in the NW Mediterranean and widths of potential prey estimated during our field sampling, larvae up to 20 mm SL would be able to ingest adults of *O. waldemari* and early copepodite stages and nauplii of the larger calanoid species detected in sardine gut contents (Table 4). Our results agree with microscopic observations of the gut contents of sardine larvae in the NW Mediterranean and Cantabrian Seas, where 46–52% of the prey ingested by <10–13 mm sardine larvae were copepod nauplii (Munuera Fernández & González-Quirós 2006, Morote et al. 2010). Furthermore, the use here of a species-specific multiplex PCR assay has allowed, for the first time, the identification to species level of the nauplii ingested by sardine larvae, which otherwise would remain unidentified. However, apart from size, other factors such as nutritional quality or prey motility can condition prey selection (Bautista & Harris 1992, Gragnani et al. 1999). Borme et al. (2013) observed that post-flexion sardine larvae in the Adriatic Sea not only fed on the most abundant copepods (*T. longicornis* and *Paracalanus* spp.) but also *T. stylifera, Acartia* spp. and other copepod species which were rare in plankton samples. The positive selection of these rare species was probably related to the poor alertness and weak escape response of these copepods (Viitasalo et al. 2001). In the Bay of Málaga, despite the fact that *Oncaea* adults were more abundant than other species, sardine larvae (as indicated by the DNA found in the guts) seemed to prefer to prey on easier targets such as nauplii of *Temora* or *Paracalanus*, suggesting that motility rather than nutritional quality was an important factor influencing prey selection by these larvae.

DNA of both phytoplankton taxa tested, *Gymnodinium* and Prasinophyceae, was also present in the gut contents of sardine larvae. One previous study reported herbivory by sardine larvae in the NW Mediterranean under a spring bloom situation (Rasoanarivo et al. 1991). However, several studies in highly productive eastern boundary current systems have categorized larvae of SPF as passive phytoplankton consumers, criticizing the assumption of phytophagy for this and other clupeid species (Konchina 1991, Van der Lingen 2002). In our study, we cannot ascertain whether phytoplankton cells found in the guts were eaten directly by the sardine larvae or whether phytoplankton DNA originated from the copepods ingested by these larvae. Sardine larvae lacked the DNA of Prasinophyceae (<2 µm cells) at the end of the night (T9) when the lowest amounts of copepod DNA were found and when only *Oncaea* was detected (Table 3). Preliminary tests of the phytoplankton primers showed that both phytoplankton taxa were not only detectable within the guts of sardine larvae but also inside the copepod species preyed upon by larvae (data not shown). Of the target copepods, all have been described

Table 4. Mean length (mm) and width (mm) of the 5 target copepod species. Corresponding standard length (SL, mm) of the potential predator (*Sardina pilchardus*) was calculated as in Morote et al. (2010). **Bold** font indicates developmental stages falling within the expected prey size for the sardine larvae in our study (SL <20 mm). References: (1) Conway (2012), (2) Razouls et al. (2005), (3) Bradford (1978), (4) Shmeleva (1965). –: no data available

| Species | Stage | Sex | 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 | ♀[a] | 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** | ♀[a] | 0.49–0.76 | 0.16 | 15.58 | 2 |

[a]*C. parapergens* and *O. waldemari* female sizes were extracted from taxonomical plates

as omnivores (Ohtsuka et al. 1993, Kouwenberg 1994, Mauchline 1998, Razouls et al. 2005, Benedetti 2015, 2016). However, *A. clausi, P. indicus, C. parapergens* and *T. stylifera* are predominantly herbivores (Wickstead 1962, Kouwenberg 1994, Calbet & Saiz 2005), whereas *O. waldemari* is preferentially a detritivore (Wickstead 1962, Razouls et al. 2005). The co-occurrence of the DNA of phytoplankton and herbivorous copepods in the guts of sardine larvae, coupled with the weakest Prasinophyceae signal when the detritivore *Oncaea* dominated gut contents, suggests that sardine larvae were most probably not consuming these pigmented cells, but that we detected phytoplankton inside the guts of herbivore copepods eaten by the larvae.

We found high day/night variability in the copepod field community as well as in the gut contents of larvae, with a marked decrease at night in the number of species and DNA concentration detected, suggesting a preferential diurnal feeding. This agrees with previous studies reporting that the larvae of other clupeid species are visual predators (Arthur 1976). Also, circadian variation in the nutritional condition of sardine larvae (assessed as RNA:DNA) was suggested to be driven by diel changes in larval diets (Conway et al. 1994, D. Cortés unpubl. data). Total copepod DNA concentration within the guts of sardine larvae did not match prey field abundance during the diel cycle; we found higher DNA concentrations by day (T12–T13, >5 ng DNA $\mu l^{-1}$), but highest copepod numbers at night (T5–T6, >5000 ind. $m^{-3}$). Further, *O. waldemari* and *T. stylifera* presented a higher night signal within the guts along the diel cycle, whereas *P. indicus* and *C. parapergens* were only detected by day. These differences might be explained by the diel variability of the zooplankton community composition observed during the migration of the shoal of sardine larvae towards shallow waters at dusk. Moreover, PCR is not quantitative, and a high relative concentration of DNA might be due to the presence of one entire (recently ingested) prey or the sum of several heavily digested organisms. Nevertheless, it is noteworthy that peaks of relative abundance of some copepod species in the field were followed in time by increases in relative DNA concentration of the same species within the guts of sardine larvae, irrespective of the time of the day (e.g. *Acartia*, Fig. 4), supporting the idea of opportunistic feeding by these larvae.

The results of this molecular assessment of the diet of sardine larvae in the field support our initial hypothesis that sardine larvae have an opportunistic rather than selective feeding behaviour. Nevertheless, among these copepods, sardine larvae (mean SL of 10 mm) may select nauplii of large copepods (i.e. *Temora*) likely because they are easier targets than adults of small-bodied species (such as *Oncaea*). Also, in this work we studied predation on copepods, the most abundant zooplankton group in the study area during autumn. In order to fully comprehend the trophic ecology of sardine larvae, further molecular assays (e.g. metabarcoding) need to be conducted to detect other potential planktonic prey, such as microplanktonic protists and gelatinous organisms. The development and application of further genomic tools, such as the ad hoc designed multiplex-PCR assays applied here, will facilitate the study of the autecology of planktonic species and their trophodynamic role in marine ecosystems. Furthermore, the species-specific multiplex PCR used on sardine larvae can be applied as a low-cost, complementary or alternative tool to microscopy, to detect a suite of 5 common copepods within guts of other SPF which are known to prey on nauplii. These SPF would include species of commercial interest, such as anchovy *Engraulis encrasicolus* (Tudela et al. 2002, Morote et al. 2010), round sardinella *Sardinella aurita* (Morote et al. 2008) or European sprat *Sprattus sprattus* (Conway et al. 1991), but also other fish larvae for which there is no information on their diet, like the boarfish *Capros caper*, a new fishery species of increasing commercial interest in the North Atlantic (Stange 2016).

*Acknowledgements*. This work is dedicated to the memory of our beloved colleague, Luis F. Quintanilla Hervás. We thank the crew on board the RV 'Francisco de Paula Navarro' for their help during sampling, and ICES WGIMT members for stimulating discussions. We also thank Prof. M.A. Peck and 3 anonymous referees for their comments on the manuscript. This work was funded by the Andalusian Government through project MOLDIALB (P11-RMN-7354).

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**Appendix**

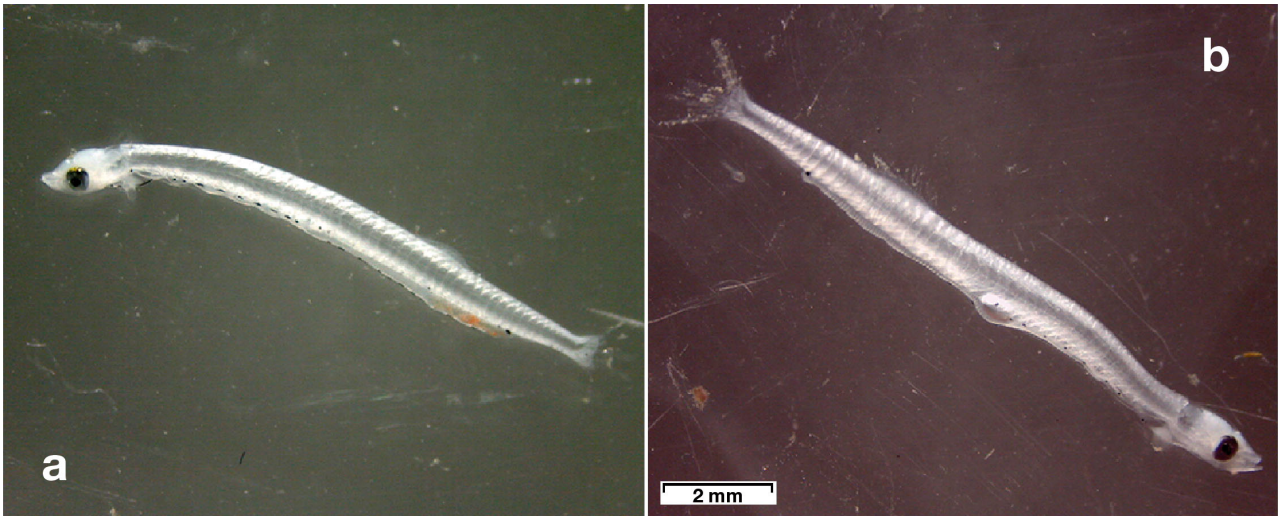


Fig. A1. *Sardina pilchardus* larvae caught during a 26 h diel cycle within the Bay of Málaga. (a) Larva caught during the day showing gut content and (b) larva caught at night void of gut content

*Editorial responsibility: Myron Peck, Hamburg, Germany*

*Submitted: July 12, 2018; Accepted: November 28, 2018*
*Proofs received from author(s): December 21, 2018*