The unexpected occurrence of late *Sardina pilchardus* (Walbaum, 1792) (Osteichthyes: Clupeidae) larvae in a temperate estuary

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Abstract: The presence of sardine larval stages inside estuaries has generally been regarded as accidental and restricted to the lower part of estuaries. Sampling done with a zooplankton net in the Guadiana estuary corroborated this hypothesis. Late sardine larvae (32.8 ± 2.0 mm) were, however, unexpectedly collected with an otter trawl, 14 km upstream from the mouth of the estuary. Thus, this work intends to: (1) discriminate and compare the abiotic characteristics of the sites where maximum abundance of sardine larval stages were registered, when captured with a zooplankton net, with the characteristics of the sites where late sardine larvae were collected with an otter trawl; (2) demonstrate that the presence of the late sardine larvae inside the Guadiana estuary was not accidental. The average salinity of the site were late larvae where collected was 4.2 ± 2.3, i.e. 87.3% lower than the average salinity where maximum abundance of early larvae was registered. We suggest that the presence of the late sardine larvae inside the Guadiana estuary was not accidental. In order to migrate to that location and remain in the estuary, counteracting river inflow, these late larvae must have employed active migration and retention strategies. New methodologies need to be implemented for routine sampling of all larval stages of sardine, in order to achieve a complete understanding of their life cycle.

Résumé : *Présence inattendue de larves âgées de* Sardina pilchardus *(Walbaum, 1792) (Osteichthyes : Clupeidae) dans un estuaire tempéré.* La présence des stades larvaires de sardine dans les estuaires est considérée comme accidentelle et limitée à sa partie inférieure. Dans l'estuaire du Guadiana, les récoltes faites dans un filet à plancton confirment cette hypothèse. Toutefois, des larves de sardine plus développées ($32,8 \pm 2,0$ mm) ont été recueillies de façon inattendue, avec un chalut à panneaux, à 14 km en amont de la côte. Le présent travail vise à : (1) discriminer et comparer les caractéristiques abiotiques des sites où l'abondance maximum des stades larvaires de sardine, capturées à l'aide d'un filet à plancton, a été enregistrée, avec les larves de sardine plus développés, capturées avec un chalut à panneaux ; (2) démontrer que la présence des larves de sardine plus développées à l'intérieur de l'estuaire du Guadiana n'était pas accidentel. La salinité moyenne du site où les larves plus développées ont été récoltées était de $4,2 \pm 2,3$, c'est-à-dire 87,3% inférieur à la moyenne maximale de salinité où l'abondance des larves plus jeunes a été enregistrée. Les larves plus développées doivent migrer activement pour arriver à cette place et utiliser des stratégies de rétention pour rester dans l'estuaire, pour contrer le courant de la rivière. De nouvelles méthodes doivent être mises en œuvre pour échantillonner tous les stades larvaires de sardines, afin de parvenir à une compréhension complète de son cycle de vie.

Keywords: *Sardina pilchardus* ● Late larvae ● Distribution record ● Migration strategies ● Guadiana estuary

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Introduction

The fishery of sardine, *Sardina pilchardus* (Walbaum, 1792) (Osteichthyes: Clupeidae), represented in 2006 the most important landing for the Moroccan (452,180 t) and Portuguese (74,113 t) fishing fleets and the third most important for the Spanish (70,103 t) and French (40,295 t) fishing fleets (FAO, 2008). Hence, numerous studies are focused on stock assessment (e.g. Borges et al., 2003; Stratoudakis et al., 2003; Silva, 2007) and on different aspects of the biology and ecology of this species (e.g. Meneses & Ré, 1991; Borges et al., 1996; Chícharo, 1997; Coombs et al., 2006; Garrido et al., 2007).

Along the distribution range of sardine, the main spawning grounds have been identified near coasts or in the continental platform (e.g. Škrivanić & Zavodnik, 1973; Ettahiri et al., 2003; Stratoudakis et al., 2003). Sardine larvae can also be collected in the edge of the platform and beyond, due to offshore transport (Santos et al., 2007). The location of spawning grounds may be linked to local productivity patterns (Chícharo, 1998), such as upwelling areas (Regner et al., 1987; Mercado et al., 2007), shelf break fronts or coastal areas adjacent to important rivers and estuarine systems (John et al., 1996; Silva, 2007). Indeed, river inflow increases the potential survival of sardine larvae developing in the open ocean (Santos et al., 2007) and its fluctuations might explain the inter-annual variability of coastal fisheries landings (Erzini, 2005).

Despite the importance of estuaries as nursery areas for many fish species (e.g. Fujita et al., 2002; Cabral et al., 2007), the presence of sardine eggs and larvae in estuaries has been classified as accidental and restricted to the mouth of estuaries, due to tidal transport from the adjacent coastal areas (Ré, 1999). Nevertheless, we have collected late sardine larvae 14 km upstream from the mouth of the Guadiana estuary (SE-Portugal/SW-Spain) using an otter trawl. Therefore, this work intends to: (1) discriminate and compare the abiotic characteristics of the sites where maximal abundances of sardine larvae were registered, when captured with a zooplankton net and with an otter trawl; (2) demonstrate that the presence of late sardine larvae far inside the Guadiana estuary was not accidental.

Material and methods

Study site

This study was done in the Guadiana estuary and in the adjacent coastal area (SE-Portugal/SW-Spain) (Fig. 1). This estuary is approximately 70 km in length, with the lower 50 km constituting the southern border of Portugal and Spain (Iberian Peninsula, Europe). It is a mesotidal estuary having an average depth of 6.5 m, occupying an area of 22 km^2, with tidal amplitudes that range from 1.3 to 3.5 m. The Guadiana river flow varies substantially among and within years, as is characteristic in regions of Mediterranean climate. The average annual rainfall fluctuates between 561 and 600 mm in the Portuguese basin, with considerable variation among years. This variability in rainfall is reflected in the Guadiana river flow, which oscillates between 8 and 63 m^3 s^{-1} during dry years, between 170 and 190 m^3 s^{-1} in typical years, and between 412 and 463 m^3 s^{-1} in wet years (Bettencourt et al., 2003).

Sampling strategy & field methodology

Monthly samples were collected from March 2002 to February 2003 in nine stations during new moon spring tides, at low and high tides. This strategy allows inferring the maximum tidal transport of plankton along the estuary. Sampling started in station 1 (coastal area), which is away from the direct influence of the river outflow, at the beginning of the flood tide. Sampling ended 3 to 4 hours later, in the station further upstream (station 9). The same procedure was done at the beginning of the ebb, except in January and February 2003, due to technical problems.

In each station, zooplankton samples were collected with a 250 μm mesh net, equipped with a Hydro-Bios flow-meter. Zooplankton trawls generally took between 5 to 10 minutes, tending to be longer in the coast and decreasing in duration towards the upper estuary. During periods of high abundance of cnidarians, zooplankton trawls did not exceed 3 minutes. Immediately after collection, the samples were preserved with buffered formaldehyde (4% v/v).

Vertical profiles of water temperature and salinity were recorded with an YSI 6600 probe. Water samples for the determination of chlorophyll *a*, seston and suspended organic matter concentration were collected at 1 meter depth and stored cooled until laboratorial processing.

Sardine late larvae were collected 14 km from the river mouth (station 5- Posto do Cinturão) during a trawling done to capture adult fish, in February 6th 2003, as part of a two year survey along the Guadiana estuary (January 2001 to February 2003). The larvae were preserved with buffered formaldehyde (4% v/v). The abundance of larvae was not calculated because the escape through the net was high. The net is conical, it has a stretched mouth of 3 m and an overall length of 25 m. It is composed of two panes; the outer one is made from 30 mm stretched mesh, protecting the inner pane, made from 10 mm stretched mesh. This otter trawl is equipped with two otter boards, each weighting 12 kg.

Laboratory and data analysis

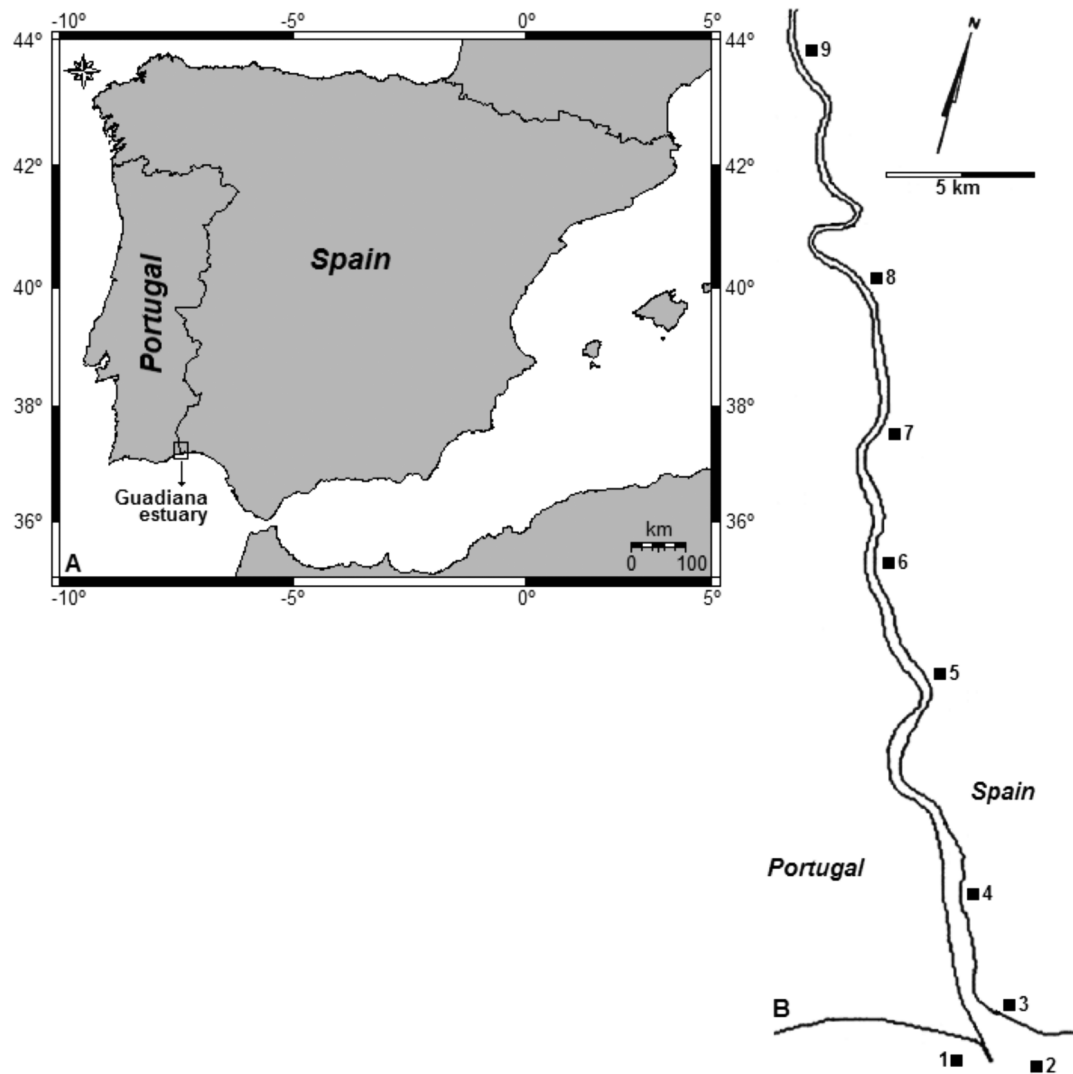At the arrival to the laboratory, water samples for the determination of chlorophyll *a* concentration were filtered

Figure 1. *Sardina pilchardus.* Location of the Guadiana estuary (A) and of the sampling stations in the estuary and in the adjacent coastal area (B).

Figure 1. *Sardina pilchardus.* Localisation de l'estuaire du fleuve Guadiana (A) et des stations d'échantillonnage dans l'estuaire et dans les zones côtières adjacentes (B).

through 0.7 µm glass fibre filters (Whatman) and stored at -20°C until fluorimetric analysis in Turner Designs 10-AU fluorometer (Welschemeyer, 1994). Water samples used to determine seston and suspended organic matter concentration were filtered through ignited pre-weighted 0.7 µm glass fibre filters (Whatman) (Greenberg et al., 1992). Both analyses were done in triplicates and filtration pressure did not exceed 100 mmHg, in order to avoid cell disruption.

Sardine eggs and larvae were sorted from zooplankton samples. All sardine eggs were graded in 11 stages (Gamulin & Hure, 1955). The total length of the larvae was measured and the gut content analysed. The Frequency of Occurrence (FO) and Relative Abundance (RA) of preys was calculated for each prey item and plotted according to Costello (1990).

T-tests were used to: (1) evaluate the differences between the mean lengths of larvae captured with the zooplankton net against those captured in station 5 with the otter trawl, (2) determine if the average of each parameter determined in the sampling stations where the abundance of eggs was maximum, in each month, was different from those where maximum abundance of larvae was collected. Moreover, a t-test and a one-way ANOVA, followed by an *a posteriori* Tukey HSD test, were used to assess spatial differences of larvae length, along the studied area, in the

month of their higher abundance and widest distribution at low and high tide (November 2002). In all the mentioned analyses, whenever the assumptions of data normality (p < 0.01) (Kolmogorov-Smirnov test) and homogeneity of variances (p < 0.01) (Cochran test) were not met, data were transformed. Ultimately, when data transformation failed to achieve the assumptions, the t-test was replaced by the Mann-Whitney test (U-test) and the one-way ANOVA by the Kruskal-Wallis one-way analysis of variance on ranks. In this case, Dunn's Method was chosen as the *a posteriori* test (Sokal & Rohlf, 1995). Standard deviation were used as a measure of dispersion.

The Guadiana river inflow was measured in Pulo do Lobo hydrometric station (code: 27L/01) (INAG, 2006).

Results

A total of 879 eggs and 75 larvae of sardine were collected with the zooplankton net, during two broad periods: (1) March to June 2002 (no larvae were collected in May); (2) October 2002 to February 2003 (no larvae were collected in January and no eggs in February). They were collected between stations 1 and 5, but mainly in the coastal area adjacent to the estuary (stations 1 and 2) and in the low estuary (stations 3 and 4). The abundance of eggs and larvae was higher during the high tide and generally decreased towards the estuary. The maximum values were 254.8 eggs.100 m^{-3} (st. 3, high tide, November 2002) (Fig. 2) and 15.8 larvae.100 m^{-3} (st. 4, low tide, June 2002) (Fig. 3).

The majority of the sardine eggs were at stages V, VI and VII, while few were at stages VIII, IX and X. On December 2002, the collected sardine eggs corresponded to 49.7% of the total collected eggs. During this month, at the low tide, the eggs were mainly at stages VI and VII, while at stage V during the high tide (Fig. 4).

The average total length of sardine larvae captured with the zooplankton net and with the otter trawl was 5.0 ± 2.0 mm (minimum 2.2 mm, maximum 12.6 mm, n = 75) and 32.8 ± 2.0 mm (minimum 28.3 mm, maximum 38.9 mm, n = 43), respectively. There was a significant difference in the average length of the sardine captured with each type of gear (U = 2967, p < 0.001). In November 2002, when 9.3% of the total larvae were collected, it was not observed a significant difference on the total length of sardine larvae among sampling stations, either at low tide (U = 111.000, p = 0.224) or at high tide ($F_{2.14} = 0.669$, p = 0.528).

The range of the parameters determined in all sites, where sardine eggs and larvae were collected, is summarized in Table 1. On the sites where maximum abundances of eggs were registered, salinity ranged between 33.8 and 36.9, with an average of 35.3 ± 0.7. On the sites with maximum abundances of larvae, it varied from 25.6 to 36.0
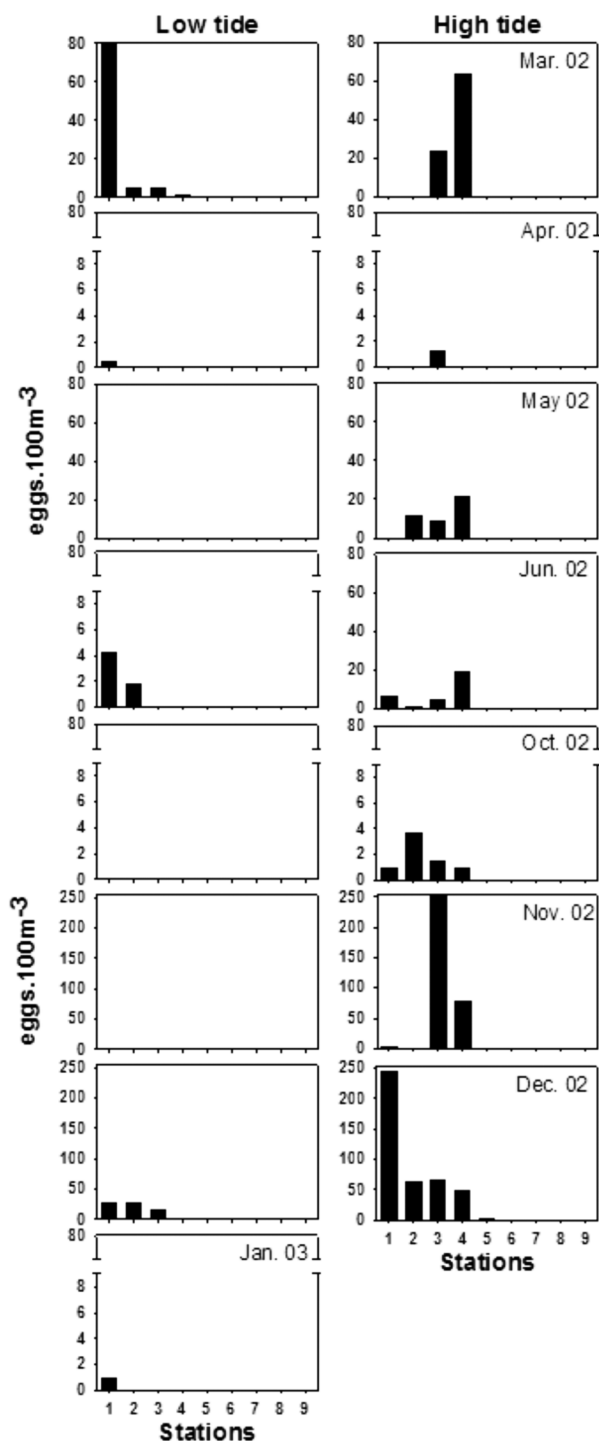

Figure 2. *Sardina pilchardus.* Abundance and distribution of sardine eggs collected with the zooplankton net in the Guadiana estuary and in the adjacent coastal area at low (A) and high (B) tide.

Figure 2. *Sardina pilchardus.* Abondance et distribution des œufs de sardine récoltés au filet à plancton dans l'estuaire du Guadiana et dans la zone côtière adjacente à marée basse (A) et haute (B).
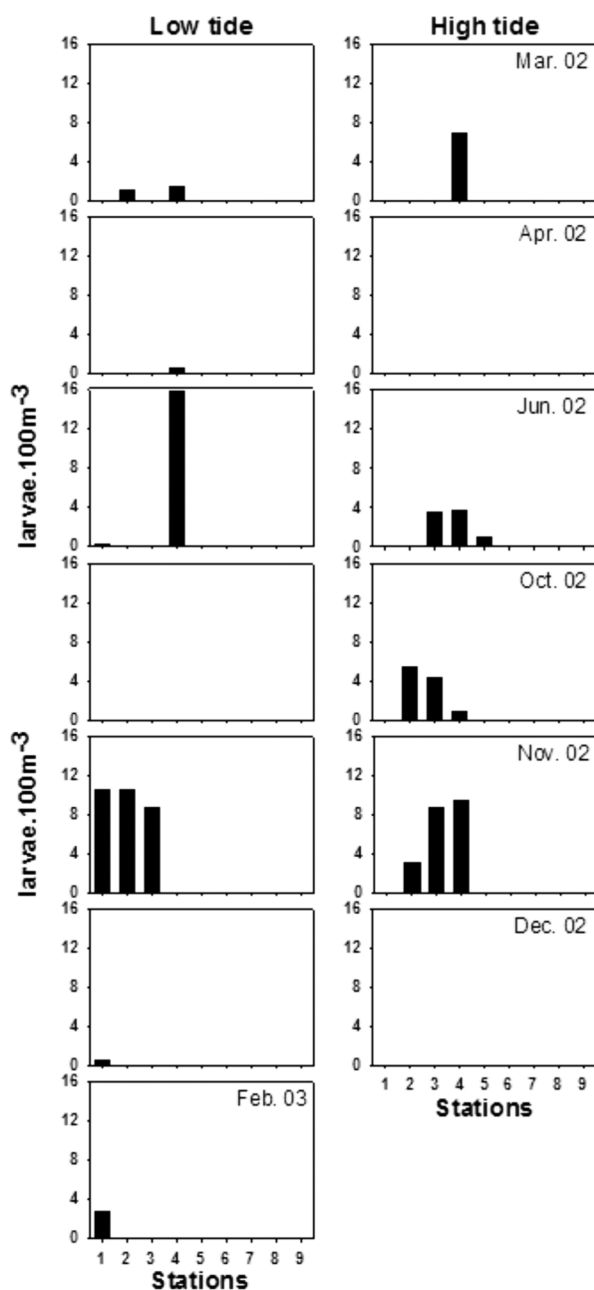
Figure 3. *Sardina pilchardus.* Abundance and distribution of sardine larvae collected with the zooplankton net in the Guadiana estuary and in the adjacent coastal area at low (A) and high (B) tide.

Figure 3. *Sardina pilchardus.* Abondance et distribution des larves de sardine récoltées au filet à plancton dans l'estuaire du Guadiana et dans la zone côtière adjacente à marée basse (A) et haute (B).

and averaged 33.2 ± 4.1. This average value is 87.3% higher than the average salinity registered in the location where late sardine larvae were collected (4.2 ± 2.3, Table 2). None of the abiotic and biotic parameters considered revealed significant differences between the sites where

maximum abundances of eggs and larvae were registered, when using a zooplankton net (Table 3).

When late sardine larvae were collected with the otter trawl, a slight salt edge was observed until station 5. The average salinity in the first 2 metres depth was 3.0 ± 0.2 and in the last 2 metres was 8.1 ± 0.9 (Fig. 5). According to Dulčić (1995), the late larvae had an average age of 60 days. The average river inflow, in the 60 days prior to the capture of these larvae, was 53.7 ± 76.0 m^3.s^{-1} (Fig. 6).

There was no gut content in any of the larvae collected with the zooplankton net and in 51.3% of the late larvae collected with the otter trawl. The three main prey items identified in the remaining 48.7% late larvae were: ostracods (FO = 0.32, RA = 0.18), crustacean nauplii (FO = 0.26, RA = 0.18) and eggs of cladocerans (FO = 0.21, RA = 0.15) (Fig. 7).

Discussion

During this study, two peaks in the abundance of sardine eggs and larvae were registered in the Guadiana estuary. The spawning period observed during this study coincided with the findings of Coombs et al. (2006; see area 4 in figure 4), but contrary to the general description of Stratoudakis et al. (2007), which stated that off southern Iberia there is a single long spawning period from autumn to spring.

The abundance of sardine eggs in the low Guadiana estuary (stations 3 & 4) was higher during high tide, which is in agreement with the work of Chícharo (1988) in the Guadiana estuary and with studies done in other estuaries (Ré, 1984; Ribeiro, 1991; Duarte, 1993). In the Tagus estuary (Portugal), few sardine eggs were collected and all of those were in an advanced stage of development (Ré, 1984). In the present study, all the eggs were found to be in stage V or above. These results suggest that sardine eggs were displaced from the coast into the estuaries during the flood, where spawning might have occurred around 21:00 to 23:00 GMT (Ré et al., 1988). The data on egg development stages and the absence of sardine adults in the estuary (Chícharo et al., 2006) exclude the possibility of sardine spawning inside the estuary. In the Guadiana estuary, the distribution pattern of sardine eggs is substantially different from that of anchovy eggs (Faria et al., 2006). The eggs of anchovy are always present in the estuary, during low and high tides, suggesting that anchovy spawns in the estuary and sardine in the coast. Moreover, the maximum abundance of sardine eggs collected in the Gulf of Cádiz (between the coastal area off the Guadiana and Guadalquivir estuaries, from March 2002 till March 2003) was 14.9 times higher (Baldó et al., 2006) than the maximum abundance of sardine eggs collected in this study.
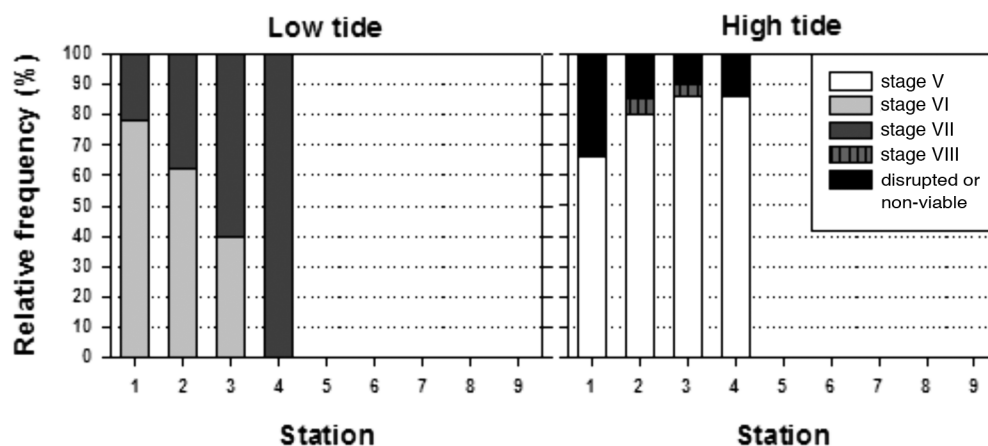
Figure 4. *Sardina pilchardus.* Stages of sardine eggs collected in the Guadiana estuary and in the adjacent coastal area in December 2002. Number of eggs collected in each station. *Low tide*: station 1, n = 55; station 2, n = 30; station 3, n = 23. *High tide*: station 1, n = 184; Station 2, n = 56; Station 3, n = 39; Station 4, n = 54. NV: non-viable eggs.

Figure 4. *Sardina pilchardus.* Stades des œufs de sardine récoltés dans l'estuaire du Guadiana et dans les zones côtières adjacentes en décembre 2002. Nombre d'œufs récoltés dans chaque station. *Marée basse*: station 1, n = 55 ; station 2, n = 30 ; station 3, n = 23. *Marée haute*: station 1, n = 184 ; station 2, n = 56 ; station 3, n = 39 ; station 4, n = 54. NV: œufs non viables.

Table 1. *Sardina pilchardus.* Range of the parameters registered in the sampling stations where sardine eggs and larvae where collected with the zooplankton net.

Tableau 1. *Sardina pilchardus.* Gamme des paramètres enregistrés aux stations d'échantillonnage des oeufs et des larves récoltés au filet à plancton.

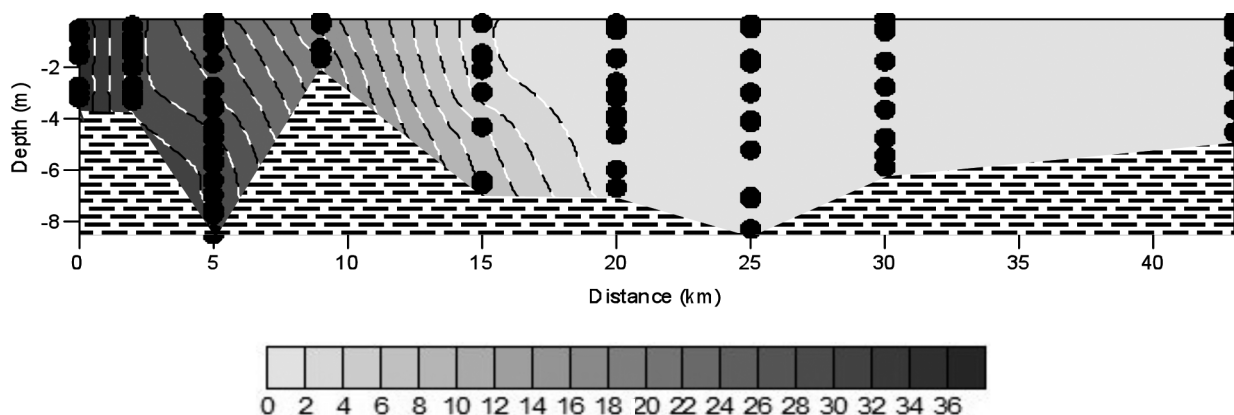|	N (Mar02- Feb03)	Temperature (ºC)	Salinity	Chlorophyll a ($\mu g.L^{-1}$)	Seston ($mg.L^{-1}$)	Organic Matter ($mg.L^{-1}$)
Eggs	879	14.7-22.8	19.8-36.0	0.4-3.0	3.0-34.0	2.0-7.6
Larvae	75	13.0-22.3	7.6-36.0	0.3-2.2	3.0-21.5	2.0-7.0

Figure 5. *Sardina pilchardus.* Vertical profile of salinity along the Guadiana estuary and adjacent coastal area during the low tide sampling of February 2003. Station 1 is located in far left of the x-axis.

Figure 5. *Sardina pilchardus.* Profil vertical de salinité le long de l'estuaire du Guadiana et à proximité des zones côtières au cours de la marée basse en février 2003. La station d'échantillonnage 1 est sur le côté gauche de l'axe des abscisses.

Table 2. *Sardina pilchardus.* Value of each parameter registered in the sampling stations where the abundance of eggs and larvae was maximum in each month and tide (LT- low tide, HT- high tide). Data concerning to the location where sardine larvae were collected with the otter trawl is also shown.

Tableau 2. *Sardina pilchardus.* Valeur de chaque paramètre enregistré aux stations d'échantillonnage où l'abondance des œufs et des larves a été maximale chaque mois et chaque marée (LT-marée basse, HT-marée haute). Les données concernant la localisation des larves de sardine récoltées avec le chalut à panneaux sont également données.

A) Eggs		Tide	Station	Temperature (ºC)	Salinity	Chlorophyll a (mg.L^{-1})	Seston (mg.L^{-1})	Organic Matter (mg.L^{-1})
Plankton net	Mar-02	LT	1	17.6	35.4	3.4	5.3	4.1
		HT	4	18.5	35.3	0.5	21.5	0.7
	Apr-02	LT	1	15.8	36.0	0.8	6.8	5.1
		HT	3	16.2	35.1	0.9	16.0	5.0
	May-02	LT	2	16.5	34.8	1.2	10.7	4.0
		HT	4	16.9	35.7	2.9	10.0	6.7
	Jun-02	LT	2	18.3	34.9	0.8	10.3	4.0
		HT	1	17.5	36.9	1.1	6.3	4.0
	Oct-02	HT	2	22.1	33.8	1.1	4.0	4.0
	Nov-02	HT	3	18.9	35.9	0.4	7.7	5.0
	Dec-02	LT	1	15.1	35.6	0.6	4.7	2.3
		HT	1	15.7	34.9	0.6	3.7	2.0
	Jan-03	LT	1	15.7	35	0.8	16.3	4.7
		avg. ± st.dev		.17.3 ± 1.9	35.3 ± 0.7	1.2 ± 0.9	9.5 ± 5.5	4.0 ± 1.5

B) Larvae		Tide	Station	Temperature (ºC)	Salinity	Chlorophyll a (mg.L^{-1})	Seston (mg.L^{-1})	Organic Matter (mg.L^{-1})
Plankton net	Mar-02	LT	4	18.0	25.6	1.5	10.1	4.1
		HT	4	18.5	35.3	0.5	21.5	5.7
	Apr-02	LT	4	16.2	32.4	1.0	15.8	6.2
	Jun-02	LT	4	19.6	26.1	0.8	16.0	6.4
		HT	4	18.2	35.8	2.2	12.0	7.6
	Oct-02	HT	2	22.1	33.8	1.1	4.0	4.0
	Nov-02	LT	1	18.3	36.0	0.3	13.3	6.0
		HT	4	18.8	35.9	0.5	4.3	3.0
	Dec-02	LT	1	15.1	35.6	0.6	4.7	2.3
	Feb-03	LT	1	13.0	35.9	1.6	6.7	2.0
		avg. ± st.dev.		17.8 ± 2.5	33.2 ± 4.1	1.0 ± 0.6	10.8 ± 5.9	4.7 ± 1.9
Otter trawl	Feb-03	LT	5	11.5 ± 0.1	4.2 ± 2.3	2.8	36.0	5.3

Table 3. *Sardina pilchardus.* Summary of the t-tests and U-tests performed to investigate if there is a significant difference between the average values of each parameter registered in the sampling stations where the abundance of sardine eggs and larvae was maximum.

Tableau 3. *Sardina pilchardus.* Résumé des tests t et U réalisés afin d'étudier s'il existe une différence significative entre la moyenne des valeurs de chaque paramètre enregistré dans les stations d'échantillonnage où l'abondance des œufs de sardines et des larves a été maximale.

During winter, the absence of offshore transport and maximum water column stability might facilitate the retention of sardine larval stages near coastal spawning grounds (Coombs et al., 2006), eventually allowing early larval stages to enter estuaries through tidal transport. The

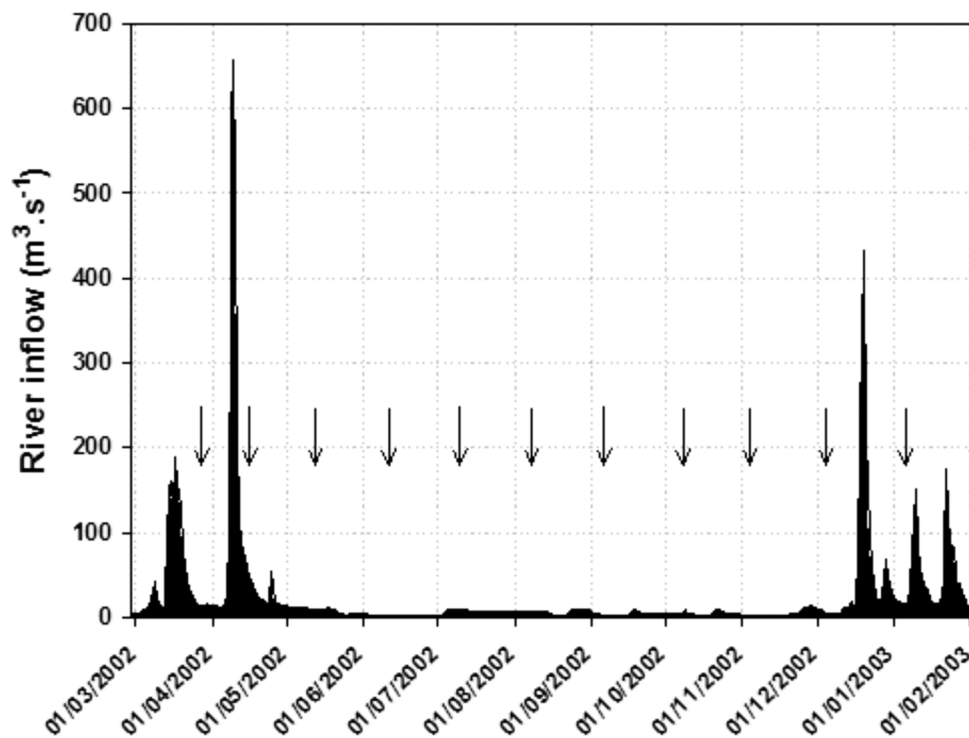| Parameter	test result
Temperature	$t = -0.534$, $P = 0.599$, $d.f. = 21$
Salinity	$U = 113.000$, $P = 0.687$
Chlorophyll a	$U = 118.000$, $P = 0.926$
Seston	$t = -0.565$, $P = 0.578$, $d.f. = 21$
Organic Matter	$t = -1.086$, $P = 0.299$, $d.f. = 21$

Figure 6. *Sardina pilchardus.* Guadiana river inflow measured at Pulo do Lobo hydrometric station, between March 2002 and February 2003. The arrows on the graph correspond to the days when zooplankton samples were collected.

Figure 6. *Sardina pilchardus.* Débits du Guadiana mesurés à la station du Pulo do Lobo, entre mars 2002 et février 2003. Les flèches sur le graphique correspondent à l'époque où des échantillons de zooplancton ont été recueillis.

sardine larvae collected with the zooplankton net were mainly limited to the low Guadiana estuary and had an average length of 5.0 mm. In the Tagus estuary, sardine larvae were also restricted to the mouth of the estuary, but the average total length was 20.5 mm (Ré, 1984). However, the late sardine larvae collected with the otter trawl, in the present study, had an average length of 32.8 mm, corresponding to an age of 60 days (Dulčić, 1995). In the 60 days prior to the capture of these larvae, the average river inflow was 53.7 ± 76.0 m^3.s^{-1} (maximum daily average 431.7 m^3.s^{-1}) (see Fig. 6). The residence time of the low estuary is 6 days for a constant river inflow of 10 m^3.s^{-1} (Oliveira et al., 2006). Given this, the presence of these late sardine larvae in the Guadiana estuary, 14 km upstream from the river mouth, is surely not accidental. It is possible that the presence of these late sardine larvae results from active and passive retention mechanisms (Hare et al., 2005). Moreover, these larvae were collected during the ebb of one of the biggest tides in early February (max. 3.06 m; min. 0.90 m) (IH, 2008). Examples of active retention mechanisms are *a*) selective tidal stream transport and residual bottom inflow, which might be synchronized with vertical migrations of larvae with formed gas bladder (Ré,

1984) and *b*) lateral migrations to the margins, where the river flow is lower or to take advantage of the residual up-estuary flow near the margins, which occurs in the low Guadiana estuary (Oliveira et al., 2006), and thus, counter-acting strong river inflows. Examples of passive retention mechanisms are tidal and wind advection, although wind has little effect in the advection of inert particles in narrow estuaries (Braunschweig et al., 2003), like the Guadiana. The relative importance of these mechanisms differs among species and changes with larval development (Hare et al., 2005). In Chesapeake Bay (USA), the net up-estuary migration of the Atlantic menhaden *Brevoortia tyrannus* (Latrobe, 1802) (Clupeidae) larvae was dominated by residual bottom inflow and wind forcing (Hare et al., 2005). An estuarine anchovy, *Anchoa mitchilli* (Valenciennes, 1848) (Clupeidae), performs up-estuary migration by tracking the diurnal migrations of their main preys (Copepoda: *Acartia tonsa* Dana) (North & Houde, 2004).

The distinct abiotic and biotic characteristics of the locations where maximum abundance of eggs and larvae where collected might help explaining the reason to capture late sardine larvae at 14 km from the river mouth. It is likely that water temperature was not the parameter that led
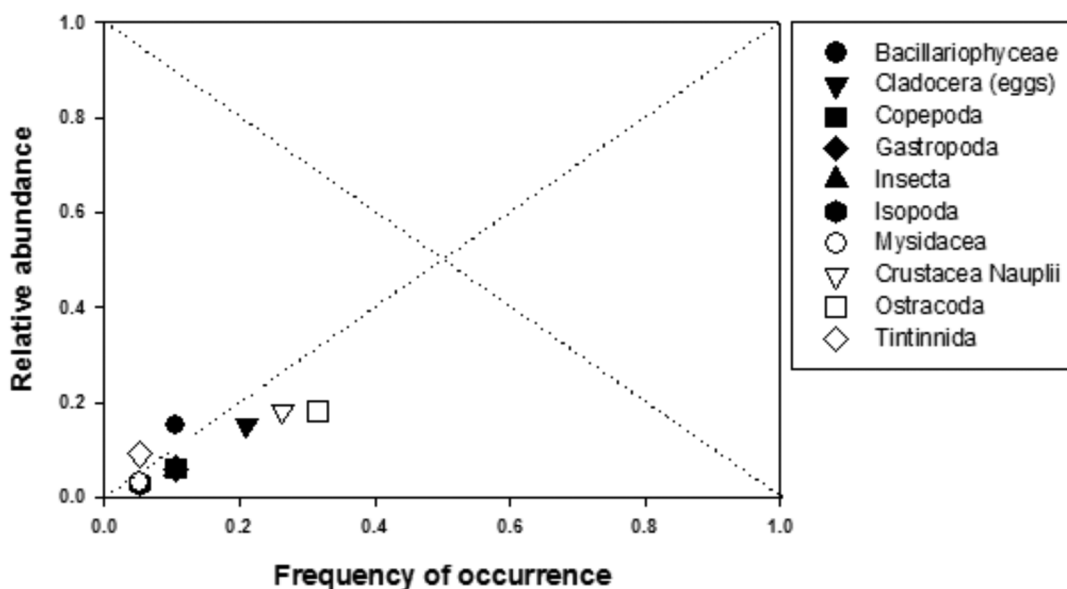
Figure 7. *Sardina pilchardus.* Frequency of occurrence *versus* relative abundance of each prey present in the digestive system of sardine larvae collected with the otter trawl.

Figure 7. *Sardina pilchardus.* Fréquence d'apparition par rapport à l'abondance relative de chaque proie présente dans le système digestif des larves de sardines recueillies avec le chalut à panneaux.

sardine larvae to undertake a migration into the estuary, since colder temperatures where registered inside the estuary than in the coast in February 2003 (Morais, 2007), which would prevent faster larval development. However, sardine larvae might have followed a low salinity cue to migrate upstream (maximum inflow registered in previous months December/January), where they can usually find areas of higher productivity (Morais, 2007). Indeed, station 5 had higher concentrations of chlorophyll *a*, seston and organic matter than the average concentrations determined in the stations where maximum abundances of eggs and early larvae were found. The exploitation of estuarine resources during the early development might be another strategy used by sardine larvae to enhance their survival, as usually done in upwelling areas (Regner et al., 1987; Mercado et al., 2007). Moreover, there is one paradigmatic example, described by Santos et al. (2007), which found that sardine larvae might achieve higher survival rates in the Western Iberia Buoyant Plume and in the convergence zones formed by the interaction between this hydrographic feature and the Iberian Poleward Current.

Relating the migration of sardine larvae with the migration of a particular prey or with the differential abundance of a prey along the Guadiana estuary is impossible, because no preys where found in the digestive system of the early larvae collected with the zooplankton net. This was due to the fact that the collecting device induces regurgitation and defecation of gut content

(Fernández & González-Quirós, 2006), despite they were collected during the day, the period when they predominantly eat (Schumann, 1963), and because some early larvae were still using the nutritional reserves of the yolk-sac. Moreover, it is possible that naked (e.g. ciliates) and non-naked protozoans constitute a relevant component of the diet of these sardine larvae (Rossi et al., 2006), which cannot be detected with gut content analysis. Nevertheless, it is relevant to point that the first and third most important prey for late sardine larvae, the ostracods and cladoceran eggs, are mainly deposited on the bottom while in their resting phase, suggesting that these larvae might have performed the migration to station 5 close to the bottom.

In conclusion, if only the collections done with the zooplankton net were considered, the findings drawn from this study would be equal to all the other studies made in other temperate estuaries; i.e. sardine larvae are restricted to the last few kilometres of an estuary, tidal transport explains their presence inside the estuary and they are mainly found in locations with high salinity (Ré, 1984; Chícharo, 1988; Ribeiro, 1991; Duarte, 1993; Drake et al., 2002; Ramos et al., 2006). The data from the otter trawl, however, clearly show that tidal transport does not explain the presence of late sardine larvae 14 km upstream from the mouth of the estuary and also that sardine larvae can be found in locations with reduced salinity. Finally, this work urges larval ichthyologists to think in the information that is being lost when only the traditional zooplankton gears

and sampling strategies are used in studies of estuarine ichthyoplankton. A solution may be achieved by assembling information from larvae collected with zooplankton nets during day and night samplings and with light traps specially designed to operate in estuaries to capture photopositive fish larvae.

Acknowledgments

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