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Starvation percentages in field caught *Sardina pilchardus* larvae off southern Portugal*

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ABSTRACT: Starvation has been proposed as a major event only in small larvae that are learning to feed on planktonic prey. However, there are few comparisons of percentages of starving larvae either among locations, times or phases of larval development. The percentage of starvation of *Sardina pilchardus* larvae of different lengths was analysed in four distinct areas off southern Portugal. A highly sensitive fluorometric method for RNA/DNA quantification was applied to field caught S. pilchardus larvae during April and May 1992. Using in situ determination of critical RNA/DNA ratio (1.3), we classified as starving only a small percentage (2.22%) of first-feeding (<8 mm) sardine larvae, but bigger larvae appeared to be more vulnerable to starvation. We concluded that sardine larvae analysed were generally in good condition (total percentage of starving larvae - 4.64%), but with some variation within each region.

Key words: Fish larvae, *Sardina pilchardus*, RNA/DNA, Starvation.

RESUMEN: VARIACIÓN DEL PORCENTAGE DE INANICIÓN EN LARVAS DE *SARDINA PILCHARDUS*.– La inanición en larvas de peces ha sido considerada muy importante después de la reabsorción del vitelo. Poco se conoce de la importancia relativa de la inanición en distintas fases del desarrollo larvario. Se ha calculado el porcentage de inanición de las larvas de sardina por classe de comprimento en cuatro áreas distintas a lo largo del sur de Portugal. Las larvas se recogieron en una campaña de investigación en los meses de Abril y Mayo de 1992. Se ha aplicado una técnica fluorimétrica muy sensible para la quantificación de la relación RNA/DNA en larvas de peces de *Sardina pilchardus*. El porcentage total de larvas en inanición - con índices RNA/DNA abajo del nível crítico (1.3) - ha sido 4.64 %, pero para las larvas cerca de su primera alimentación, intervalo de comprimento < 8 mm, los porcentages de inanición fue inferior (2.22%). En el presente trabajo se demuestra que la inanición no será muy frecuente entre las larvas de *S. pilchardus* recogidas, pero en determinadas zonas su importancia puede aumentar significativamente.

Palabras clave: Larvas de peces, inanición, RNA/DNA, *Sardina pilchardus*.

INTRODUCTION

Condition indices have been widely used to assess the importance of starvation in field caught fish larvae (O'Connel, 1980, Buckley, 1984, Theilacker, 1986, Robinson and Ware, 1988 and Clemmesen, 1994). Recent studies have suggested that the RNA/DNA ratio is one of the best indicators of the nutritional condition of several marine organisms (Clemmesen, 1994 and Bailey *et al*., 1995). This index is based on the assumption that the amount of ribonucleic acid (RNA) is directly involved in protein synthesis which is affected by the nutritional condition of the organism, while the amount of deoxyribonucleic acid (DNA), the primary carrier of genetic information, is stable under changing environmental situations. The RNA/DNA ratio is an ecophysiological condition index, which summarises the physiological activity of differently

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sized animals. It is therefore, susceptible to changes in the environment, which affect the physiology of the organisms e.g. low prey availability (Mcgurk *et al*., 1992 and Chícharo and Chícharo, 1995).

Starvation has been proposed as one of the major sources of fish larval mortality (Suthers, 1992). However, the relative importance of this event seems to vary according to species. Houde (1987) indicated that there are species 'insensitive to prey' and species 'prey sensitive', in which latter category clupeiformes are included.

A clupeiform species, the Iberian sardine (*Sardine pilchardus*) is the traditional target of an important pelagic fishery on the Atlantic coast of the Iberian Peninsula (Pestana, 1989). The annual recruitment to these stocks shows a high variability that may be reflected in overall stock abundance, thus affecting the fishery (Porteiro *et al*., 1986 and Robles *et al*., 1992). It is commonly assumed that to understand the recruitment variability, it is necessary to study the factors which determine the rate of survival during the early life history stages.

Several hypotheses - e. g. Hjort's (1914) 'critical period' hypothesis, Cushing's (1975, 1990) 'match-mismatch' hypothesis, and Lasker's (1975) 'stable ocean' hypothesis - link high starvation percentages at the time of first feeding with fish larvae survival and future recruitment. Other studies, however, provide some evidence that starvation occurs during all larval stages (Leggett and Deblois, 1994).

Knowledge about the early life history of the sardine, especially that which is related to the importance of starvation is scarce. To assess the importance of starvation on field caught larvae it is necessary to determine the RNA/DNA critical ratio, below which larvae will be classified as starving. Until now, this kind of calibration has been done only with fed and starved laboratory reared larvae (Buckley, 1984; Clemmesen, 1987; Robinson and Ware, 1988; Pittman, 1991 and Chícharo, 1993). The results of such studies should be regarded with caution as laboratory conditions hardly simulate natural conditions (Blaxter, 1975; Theilacker, 1980; Mackenzie *et al*,. 1990 and Folkvord and Mokness, 1995). The first aim of this study was to access from a field experiment the level of RNA/DNA ratio indicative of starvation in *Sardina pilchardus* larvae. The second aim was to determine the incidence of starvation through critical RNA/DNA ratio analysis among distinct length classes of sardines collected in specific areas off the southern coast of Portugal.

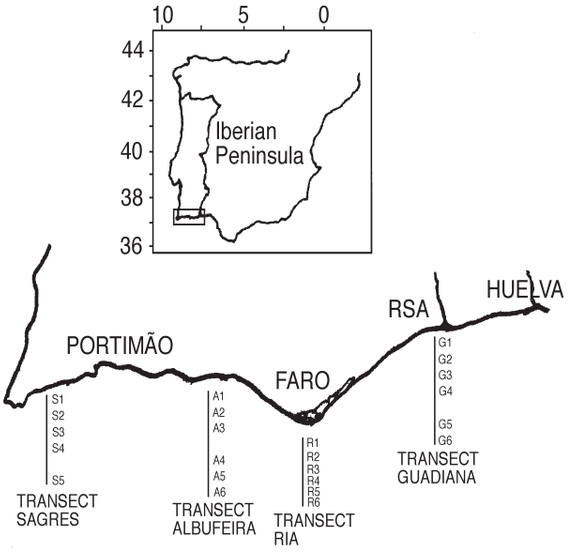

Fig. 1. – Locations of sampling stations on Algarve's continental shelf, southern Portugal. Major cities are indicated - V.R.S.A, Fa<ro, Portimão. The Guadiana is the major river of this coast, Ria is a coastal lagoon, Algufeira and Sagres are coastal villages.

MATERIAL AND METHODS

Field study

Hydrographic and plankton sampling were carried out during a research cruise on Portugal's southern continental shelf (37-36ºN, 9-7ºW) aboard the German vessel 'Poseidon' (29 April-7 May 1992). Sardine larvae were captured along four transects, (Guadiana, Ria, Albufeira and Sagres) between V. R. S. António and Sagres, out to the 300 m isobathimetric (Fig. 1). The Guadiana transect commenced close to the Guadiana estuary, Ria transect commenced off the Ria Formosa, a coastal lagoon without any significant freshwater inflow; and transects Albufeira and Sagres commenced off villages with the same name. In each, transect 5-6 stations were sampled. In situ temperature determinations were carried out by CTD cast at selected stations. Mesozooplankton tows were double oblique, with a 80 cm diameter bongo net of 505 μm mesh. All the tows were made during day time. On completion of the mesozooplankton haul, coarse mesh samples were immediately sorted for the bulk of sardine larvae and stored in liquid nitrogen (-196 ºC) for later RNA and DNA analysis.

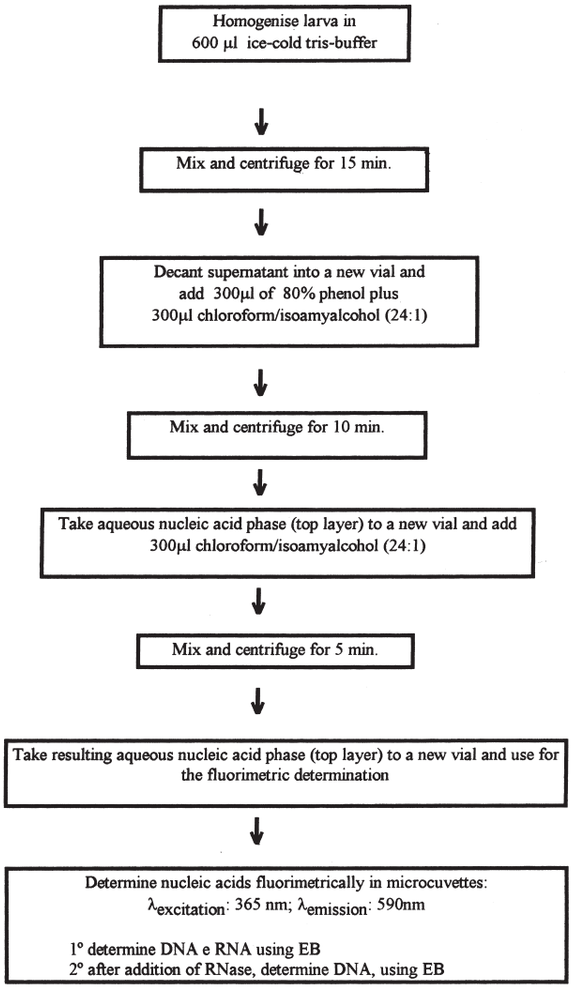

Fig. 2. – Flowchart of the analytical procedure for nucleic acids determinations.

Laboratory procedures

Standard lengths of thawed sardine larvae were measured under a dissecting microscope using an ocular micrometer before nucleic acid determinations. A highly sensitive fluorometric method for RNA and DNA quantification in individual organisms was applied to caught larvae. The analytical procedure (Fig. 2) was adapted from the methodology presented by Clemmesen (1988,1990) for fish larvae, which involves purification of tissue homogenates and subsequent fluorescence-photometric measurements using ethidium bromide (EB), a specific nucleic acid flurochrome dye. The fluorescence due to total RNA (mainly ribosomal) can then be calculated as the difference between total fluorescence (RNA and DNA) and the fluorescence after ribonuclease A (type II-A) treatment, which is assumed to be due to DNA, after the subtraction of the self-fluorescence of the enzyme. The fluorescence was determined by exciting at 365 nm and reading at 590 nm in an Hitachi Espectrofluorometer. Concentrations of nucleic acids were determined by using standard curves of DNA and RNA with EB built up daily, with known concentrations of calf DNA and yeast RNA.

Starvation percentages

For assessment of starvation rates in field caught larvae it is necessary to firstly determine the critical level of RNA/DNA. This calibration was carried out in a field experiment, during the months of January and February of 1991. Short and slow tows were made during the major spawning period of *S. pilchardus* to collect eggs for this experiment. These eggs were placed in special cylindrical net containers measuring 25 cm in diameter and 50 cm in height (Fig.3). Two kinds of net containers were used which had different mesh sizes. The one measuring 10 µm was filled with sea water, which had previously been filtered through a 1 µm filter system. This procedure did not allow the entry of relevant food organisms. The other had mesh size of 500 µm and was filled with in situ sea water, allowing the prey of sardine larvae to flow freely through the containers. Three mesh containers for each treatment were used and ca 200 eggs were placed in each container. The containers were kept submerged for 10 days, and cleaned every two days to avoid clogging. The sea water temperature during the experiment averaged 15.3 ºC. Once the collected eggs were in an advanced stage of development, hatching occurred approximately 1-2 days later, based on Blaxter (1969) and Miranda and Iglesias (1990). The absorption of the yolk sac occurred approximately 3-6 days after hatching. It was observed that the duration of the starvation period for larvae inside the 10 µm net containers was approximately 2-6 days. Ten days after, at the end of the experiment all the remaining larvae were stored in liquid nitrogen for subsequent nucleic acid analysis. The critical ratio of RNA/DNA was considered to be the mean RNA/DNA ratio of the starving larvae from the 10 µm mesh net containers.

Larvae collected during the cruise with RNA/DNA ratios equal to or below the critical ratio were classified as starving. In order to analyse the

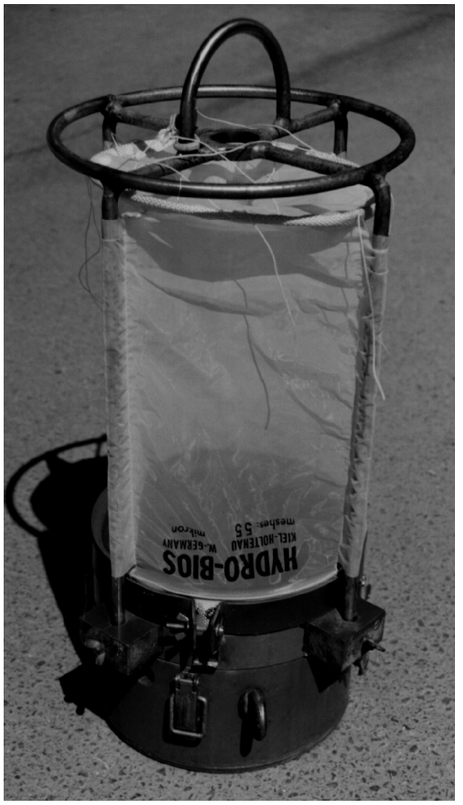

Fig. 3 – Cylindrical net container with 25 cm diameter and 50 cm high, used for calibration of RNA/DNA ratio of sardine larvae. There were two types of containers: one with mesh size of 10 mm which did not allow the entrance of sardine prey and the other with mesh size of 500 mm through which food organisms could pass.

percentage of starvation by length classes, the results were divided into three length classes: <8 mm, 8-16 mm and 16-22 mm. The larval length class of less than 8 mm was considered as belonging to first-feeding larvae, after Silva and Miranda (1992), who pointed out that at 14.5 ºC sardine larvae exhaust endogenous reserves and start feeding between 4-7 days, which corresponds to a larval length of 5-6 mm.

Analysis

After the application of the Kolmorogof-Smirnov test to investigate the data normality, a t-student test was applied to compare the RNA/DNA ratios of larvae between starvation treatments. A conventional one-way ANOVA followed by a means comparisons Tukey test was applied to compare parameters between the transects. The relationship between the parameters was analysed using Spearman's rank correlation.

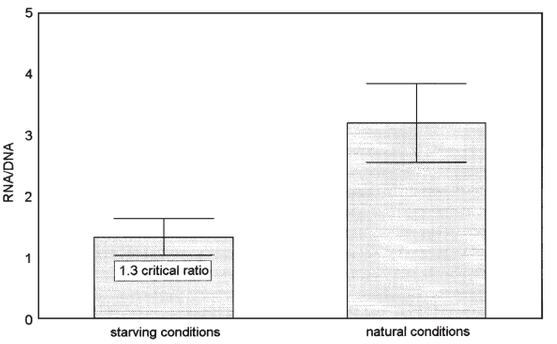

Fig. 4. – RND/DNA mean of sardine larvae in different feeding conditions. Starving conditions were simulated inside net containers of 10 mm mesh size, and natural conditions inside net containers of 500 mm mesh size containers (see containers on Fig. 3) Error bars represent one standard derivation.

RESULTS

Starvation experiment

Survival during the validation experiment was not very high: 5% (10 larvae/200 eggs) among the starved larvae and 9 % (18 larvae/200 eggs) among the larvae with food available. Therefore, only a small number of larvae of lengths between 4.5 mm and 7 mm, was available for the nucleic acid measurements (10 from each container). Nevertheless, the mean RNA/DNA ratio of the starved larvae (net container with 10 μm mesh size), 1.3 (±0.35), was significantly different ($p<0.05$) from the mean RNA/DNA of fed larvae (net container with 500 μm mesh size), 3.2(±0.64) (Fig. 4). Thus a field caught sardine larvae with an RNA/DNA ratio less than 1.3 was classified as starving (assuming that the critical ratio is approximately the same for all size of larvae). Accordingly, the critical value of RNA/DNA ratio was 1.3.

Field study

Sea surface temperature ranged between 14.4 and 17.4 ºC (Fig. 5). Significant differences in temperature were found between transects ($p<0.001$): it was higher at the Guadiana transect and lower at the Sagres transect. The Tukey test revealed the latter area as being significantly different from all others ($p<0.05$). It was found that there is a trend for the water temperature to increase from Sagres to the Guadiana (west to east coast) (Fig. 5). Colder water was found adjacent to the southwest coast of Portu-

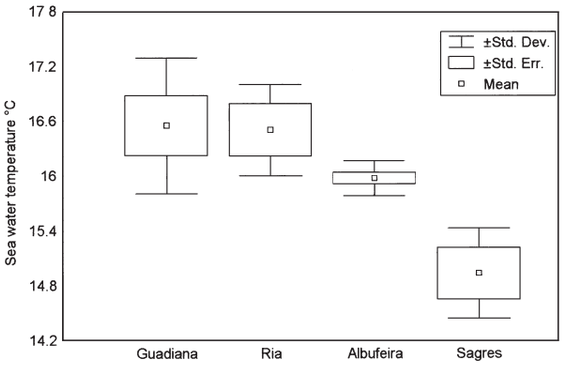

Fig. 5. – Variation of the surface teperature between transects. Std Dev. represent one standard deviation and Std. Err. represent one standard error of the mean.

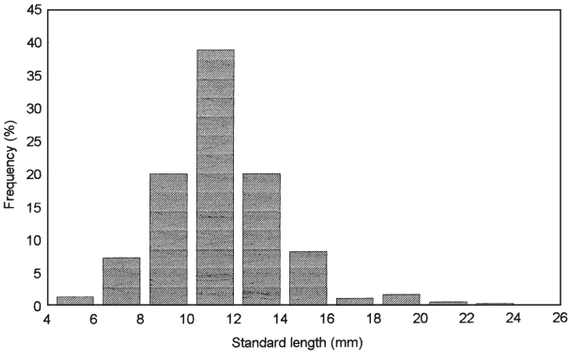

Fig. 6. – Length frequency distribution of *Sardina pilchardus* larvae of data pool from field captured individuals off southern Portugal.

gal. Near Sagres the transect was indicative of upwelling on the western coast. Off the central south coast relatively higher temperatures were observed. Stratification of water column was not significant, with the exception of the Albufeira transect, where a thermocline was detected, between 31 and 39 m.

S. pilchardus were the predominant fish larvae at all stations. The sardine larvae were more abundant in the Albufeira transect, particularly near the coast. When the larval lengths were analysed, no significant differences were registered among the transects ($p<0.46$). The standard length of analysed field caught larvae ranged between 4-22 mm, but the intermediate classes, 8-16 mm, were the most common (Fig.6), except for some stations in the Albufeira transect (Fig. 7).

The RNA/DNA ratio measured individually among 302 sardine larvae varied between 0.42 and 12.98. The RNA/DNA ratio was not correlated with length (Fig. 8) ($r=0.186$; $p=0.562$) or temperature ($r=-0.310$; $p=0.211$) There was considerable variability on the RNA/DNA ratios of sardine larvae, but in general the values were above the critical ratio. The total percentage of field caught sardine larvae with RNA/DNA ratios of less than 1.3, was 4.64% (Fig. 9). However, there

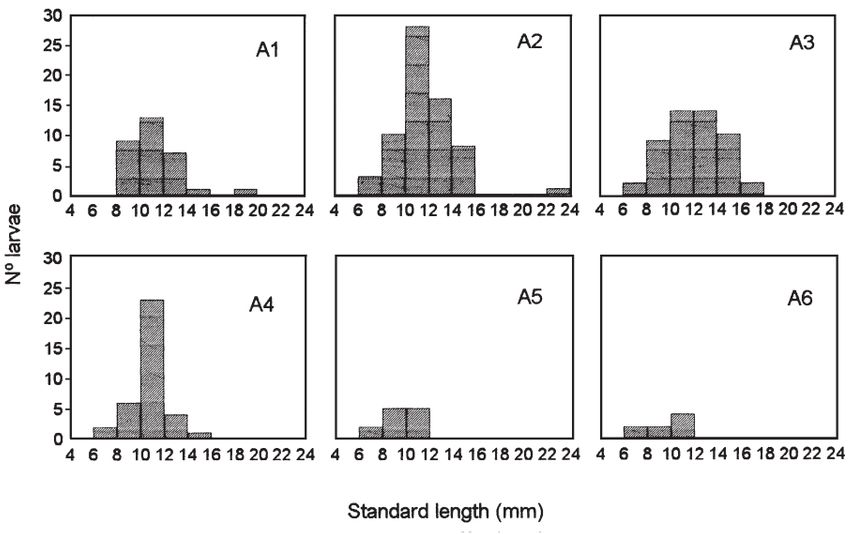

Fig. 7. – Length frequency distribution of *Sardina pilchardus* larvae captured at 6 stations (A1..A6) along the Albufeira transect, where larvae were more abundant.

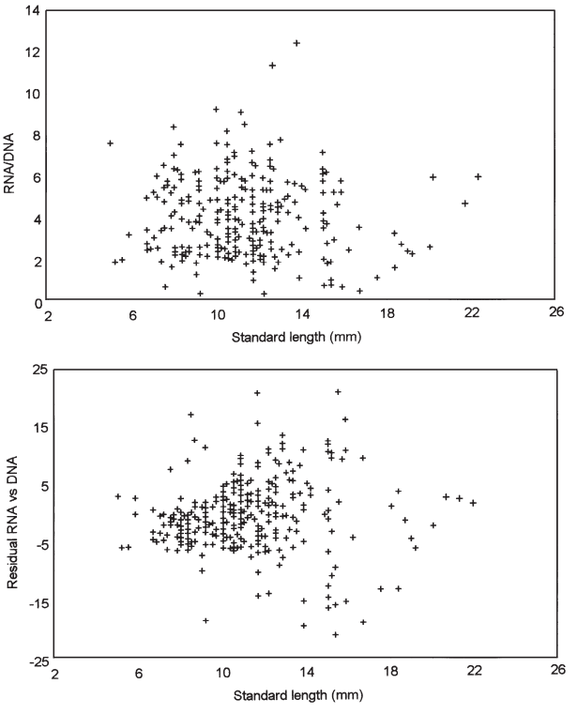

Fig. 8. – Plot of RNA/DNA ratios and its residuals against sardine larval length.

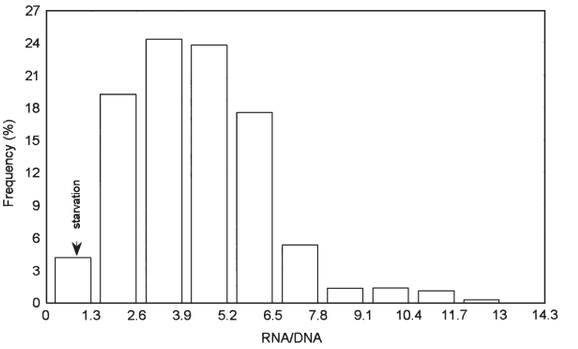

Fig. 9. – RNA/DNA ratio frequency distribution of *Sardina pilchardus* larvae of all field captured indiduals off southern Portugal.

was some variability among transects as to the percentage of starvation detected (range 0-66.67%) (Table 1). Variability was noted also especially as to the nutritional condition of larvae between stations of the Albufeira transect (Fig. 10), but no significant differences on RNA/DNA ratio were registered among stations of this transect ($p<0.191$).

When the total percentage of starvation by length classes was analysed, the results showed that the most vulnerable length class to starvation was between 8-24 mm, with 3.61% classified as starving for 8-16 mm interval, and especially for the 16-24 mm class with 37.5% starving, and lastly the larval length class less than 8 mm which represented 2.22% starving (Table 1).

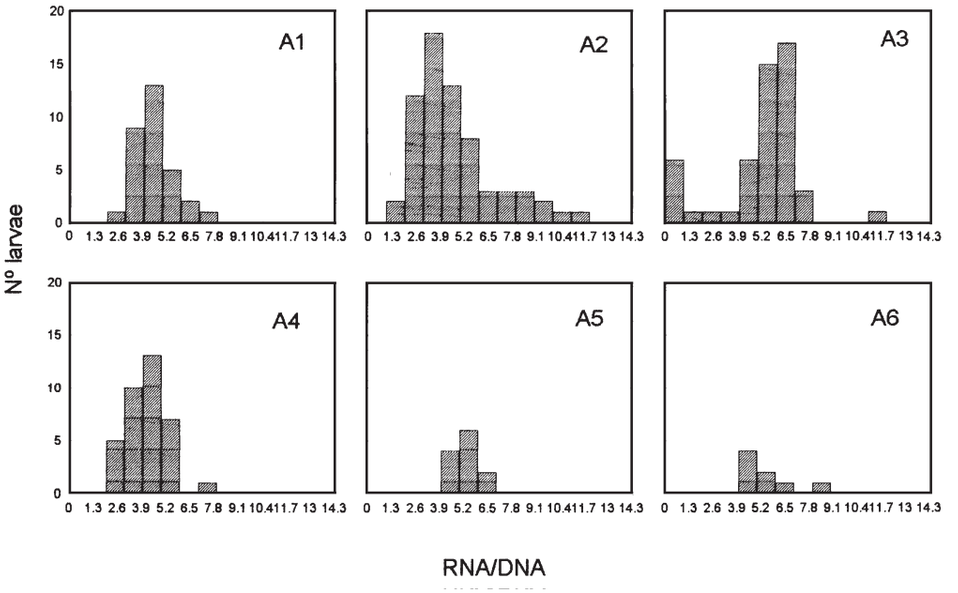

Fig. 10. – RNA/DNA ratios frequency distribution of *Sardina pilchardus* larvae captured at 6 stations (A1..A6) along the Albufeira transect. These results are shown in more detail because it was the place where larvae were more abundant.

Table 1. – Starvation percentage by length class and by trasect (below percentage withun parenthesis is given number of starved larvae/total number of larvae analysed on that length class or transect; - means no larvae were captured).

Larval length	Guadiana	Ria	Albufeira	Sagres	Total
<8 mm	0% (0/2)	0% (0/14)	0% (0/13)	12.5% (2/16)	2.22% (2/45)
8-16 mm	0% (0/35)	10% (1/10)	3.57% (7/196)	12.5% (1/8)	3.61% (9/249)
16-24 mm	20 (1/5)	-	66.67% (2/3)	-	37.5% (3/8)
Total	2.38% (1/42)	4.17% (1/24)	4.25% (9/212)	12.5% (3/24)	4.64% (14/302)

DISCUSSION

Starvation experiment

Results from the validation experiment revealed that the RNA/DNA ratio as an index was capable of identifying *S. pilchardus* larvae according to their nutritional history. This has already been demonstrated in other species, namely *Clupea harengus* (Clemmesen, 1987, 1994), *Pseudopleuronectes americanus* (Buckley, 1980), *Gadus morhua* (Buckley, 1979), *Scophthalmus maximus* (Clemmesen, 1987) and *Hippoglossus hippoglossus* (Pittman, 1991).

To assess the importance of starvation on field caught larvae it is necessary to determine the RNA/DNA critical ratio. The starvation experiment with *S. pilchardus* first-feeding larvae revealed that the critical RNA/DNA ratio is 1.3. Besides the likely variability with length, it was assumed that the critical ratio was approximately the same for all size of larvae captured in the field. However, it is likely that larger larvae can persist with a lower ratio. According to Suthers (1996) larger larvae do have lower proportions of RNA. Nevertheless Mcgurk *et al*. (1992) and Clemmesen (1994) demonstrated that RNA/DNA increases with length, but that this increase is less pronounced in starving larvae when compared with fed larvae, and that it is the starving conditions which should be considered when establishing the critical ratio. It is well accepted that there is some variability in the critical RNA/DNA ratio among fish species, and the duration of starvation periods. However, according to Clemmesen (1994) there are indications that the RNA/DNA ratio reached after starvation periods of days to weeks results in a value ca. 1 near the 'point of no return', in the majority of fish larvae species. Also, it seems that there is a certain similarity between distinct taxonomic groups. In fact, an RNA/DNA critical ratio of 1.1 was registered on bivalve post-larvae (Chícharo and Chícharo, 1995).

Comparison of starvation percentages by transect

Comparison of starvation percentages among transects revealed higher values in Sagres. This can be related to lower temperature, which increases the duration of larval life and which in turn increases the starvation probability (Cushing, 1990). However our study revealed that temperature had a low and nonsignificant negative correlation coefficient with RNA/DNA ratio. Nevertheless, the ratio of major organic components including nucleic acids may be altered in response to temperature (Buckley *et al*., 1990). The chemical composition of fish larvae revealed, as pointed out by Buckley *et al*. (1990, 1991), a complex relation with temperature during not only incubation periods but also during the final stages of gamete maturation.

Otherwise, results showed relative low incidence of starvation in other transects. This can be associated with the proximity of an estuary system (Guadiana) or a coastal lagoon (Ria), features which have been considered as potential contributors to coastal ocean productivity (Pilkey *et al*., 1989), and especially to zooplankton production, which may be consumed as quickly as it is produced (Hunter, 1981), and is in the appropriate range of dimensions for fish larvae food. However, on the Albufeira transect, which was not close to any of these productive systems, starvation percentage was also low. The highest densities of sardine larvae were found in this transect. Thus it could be argued that plankton patchiness (Steele, 1978) could be responsible for a high abundance of larvae, and also, probably for the level of their potential prey in this area.

Less than 5% of sardine larvae collected during this study were in a poor condition. At most stations there were no larvae below the critical ratio, suggesting that starving larvae have either been lost to predation or starvation, or that their feeding abilities are such that they can meet their basic nutritional requirements (Robinson and Ware, 1988). However, larvae in a poor condition may be underestimated as they can be rapidly consumed by predators. There

are few reports of finding high percentages of larvae in a poor condition at sea (Margulies, 1993 - 62-63%). Most of the studies reveal low starvation percentages (range:1-8%) (Ehrlich *et al*., 1976; O'Connell, 1976, 1980; Buckley, 1984, Clemmesen, 1994).

There has been some debate about the relative significance of predation and starvation in determining larval survival (Cushing, 1990 and Gotceitas *et al*.,1996). There are persistent suggestions by those who consider predation as the main cause of mortality that starvation may be a minor component of larval mortality (Bailey and Houde, 1989; Pepin, 1989; Litvak and Leggett, 1992 and Pepin and Shears, 1995). However, Suthers (1992) cites a solid body of evidence showing declining condition with a declining of prey availability. Many studies have confirmed that food availability is a limiting factor for survival (Setzler-Hamilton *et al*., 1987).

Cushing (1995) notes that the demonstration of a relationship between food level and survival is equivocal. Leggett and Deblois (1994) have also showed recent evidence, which suggests that failure to distinguish between zooplankton abundance and food availability for fish larvae may compromise the evaluation of the importance of starvation on wild populations in certain studies.

Comparison of starvation percentages by length classes

When percentages of starvation were analysed by length classes, a small number of first-feeding sardine larvae was classified as starving (2.22%). Mcgurk *et al*. (1992) also using RNA/DNA ratios, classified as starving 11 to 23 % of first-feeding (<13 mm long) herring larvae. By contrast, Blaxter (1969), concluded that the sardine is more susceptible to starvation than the herring at the transition from endogenous to exogenous food.

In fact my data on sardine larvae may be biased as losses of small larvae through the meshes may have occurred due to either escapement or extrusion (Smith and Richardson, 1977). However, according to the these authors, extrusion is unlikely to occur at a 2 knot towing speed, being the speed at which the net was towed in our study. Escapement may have occurred, however, based on the skull width of the sardine larvae, which according to Colton *et al*. (1980) is the more meaningful measurement in studies of mesh retention of larval fishes, therefore only larvae smaller than 5 mm (skull width ca 500 μm, Chicharo, 1996) should have passed through the meshes. Despite that, on stations A5 and A6, where the same mesh net was used, first-feeding sardine larvae, with 4 to 8 mm, seemed to predominate and no starving individuals were found. So that the low incidence of starvation of small larvae could be accounted for by factors other than escapement of larvae through the meshes. Besides, it is necessary to consider that if the most probable phase in poor condition is the first-feeding larvae stage which is the stage most susceptible to predation (Shepherd and Cushing, 1980; Bailey and Houde, 1989 and Houde, 1989), then these small larvae will be more rapidly eliminated by the action of predation, and will not be strongly represented in the samples. Nevertheless, results from recent studies, investigating factors affecting predation risk to larval fishes, call into question whether a larger body size-at-age does necessarily increase larval survival (Litvak and Leggett, 1992).

Moreover the 'critical period' is not the only phase susceptible of starvation as the post-first feeding larval stage is also vulnerable to starvation. The length class, which had the highest incidence of starvation, was 16-24 mm, and according to Silva and Miranda (1992) such larvae are post-first feeding. This agrees with the general conclusions of Leggett and Deblois (1994) in relation to fish larvae, in general, e.g. that starvation should not be restricted to small larvae.

Furthermore, Peterman *et al*. (1988), who tested Hjort's hypotheses in *Engraulis mordax,* concluded that the 'critical period' (if one exists) is more likely to occur at a later stage than during a few days of the yolk-sac absorption. The early larval stages of sardine average only a small portion of the total larval duration. The first feeding occurs between 3-6 days from hatching, and the whole planktonic life takes, depending on temperature, between 20-25 days (Blaxter, 1969 and Miranda *et al*., 1990).

Regardless of the larval stages considered whether, it be first feeding or post-first feeding, it can be concluded from the research carried out on the cruise, that, in general, the percentage of starvation in field caught *S. pilchardus* larvae was not very high. In further studies, it will be necessary to carry out more intensive studies to determine the total mortality rates and to examine in detail the interplay between starvation and predation. Such studies will help us to understand further the complexity of the circumstances that determine larval survival.

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