

# Estimation of starvation and diel variation of the RNA/DNA ratios in field-caught *Sardina pilchardus* larvae off the north of Spain

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**ABSTRACT:** The aim of this study was to analyse the general larval condition, to determine the incidence of starvation and to investigate the effect of time of day on RNA/DNA ratios among field-caught *Sardina pilchardus* (L.) larvae. The larvae were collected during 4 research cruises off northern Spain, during March, April, May and June 1992. A highly sensitive fluorometric method for nucleic acid quantification was applied to larvae of *S. pilchardus*. The means of the RNA/DNA ratio were relatively high, so the larvae collected off northern Spain were generally in good condition. Low percentages of starving larvae (RNA/DNA ratio less than 1.3), ranging from 0 to 3.23%, were found over the 4 mo. The RNA/DNA ratios were significantly correlated with zooplankton biomass. Larvae collected at night revealed higher RNA/DNA ratios compared to larvae caught during the day. This seems to indicate that there is some endogenous rhythm in the production of RNA. It would then follow that, if there are diel changes in RNA concentrations, average RNA indices can be unrepresentative if there is any day/night bias in sampling.

**KEY WORDS:** Fish larvae · RNA/DNA · *Sardina pilchardus* · Starvation · Diel variation

## INTRODUCTION

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

Many studies have confirmed that food availability is a limiting factor for survival during these early phases (Setzler-Hamilton et al. 1987). Cushing (1995) noted that the relationship between food level and survival is equivocal. Leggett & Deblois (1994) have shown recent

evidence which suggests that failure to distinguish between zooplankton abundance and food availability for fish larvae may have compromised the evaluation of the importance of starvation on wild populations.

Several hypotheses—e.g. Hjort's (1914) 'critical period' hypothesis, Cushing's (1975, 1990) 'mismatch' hypothesis, and Lasker's (1975) 'stable ocean' hypothesis—link low fish larvae survival (and future recruitment) with starvation during the larval stage. Such relationships may be relatively simple to test in laboratory feeding experiments, but until recently they were difficult to demonstrate for sea-caught larvae. This situation has changed due to the introduction of condition indices, such as the RNA/DNA ratio, that make it possible to assess starvation in the ocean.

Accurate assessment of larvae condition provides a means to study the significance of the biotic and abiotic factors affecting fish populations during the

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critical planktonic stage (Suthers et al. 1996). RNA/DNA ratios have been used to assess the nutritional condition of a wide range of marine organisms, predominantly fish larvae (Buckley 1984, Robinson & Ware 1988, Suthers 1992, Bailey et al. 1995). This index is based on the assumption that the amount of deoxyribonucleic acid (DNA), the primary carrier of genetic information, is stable under changing environmental situations, while the amount of ribonucleic acid (RNA), which is directly involved in protein synthesis, is affected by the nutritional condition of the organism. The RNA/DNA ratio is therefore susceptible to changes in the environment which affect the physiology of the organism, e.g. low prey availability (McGurk et al. 1992, Chícharo & Chícharo 1995). Larvae in good condition tend to have a higher RNA/DNA ratio than those in poorer condition (e.g. Robinson & Ware 1988, Clemmesen 1994, Chícharo 1996). It has been reported that this ratio can respond quickly to changes in environmental conditions (Martin & Wright 1987), and this ratio has been used to give a measure of instantaneous growth in the field, in order to avoid periodic measurements (Buckley 1984, Clarke et al. 1989).

To assess starvation it is necessary to determine the RNA/DNA ratio below which larvae will be classified as starving. The idea of 'critical ratio' was originally discussed by Robinson & Ware (1988) and is based on a model of the general relationship between the RNA/DNA ratio, temperature and protein growth rate as determined and reported by Buckley (1984). Robinson & Ware (1988) defined the 'critical ratio' as the RNA/DNA ratio of a marine animal when the larval protein growth rate is zero. They calculated the 'critical ratio' for Pacific herring using Buckley's general model by setting the protein growth rate to zero and solving for RNA/DNA ratio at the temperature at which they captured the larvae. Rooker & Holt (1996) suggested that caution be exercised when applying this ratio to new species in field studies due to the inherent developmental variation seen in RNA/DNA ratios of many species of marine teleost larvae.

A solution to this problem is to calculate for each species, under controlled conditions, the mean RNA/DNA ratios of larvae deprived of food. In previous studies, this kind of calibration was done only with fed and starved laboratory-reared larvae (Buckley 1984, Clemmesen 1987, Robinson & Ware 1988, Pittman 1991, Chícharo 1993). The results of such studies should be regarded with caution as laboratory conditions hardly simulate natural conditions (Blaxter 1976, Theilacker 1980a, Mackenzie et al. 1990, Folkvord & Mokness 1995). Chícharo's studies (1996, 1997) made it possible to assess from a field experiment the RNA/DNA ratio indicative of starvation in *Sardina pilchardus* (RNA/DNA ratios less than 1.3).

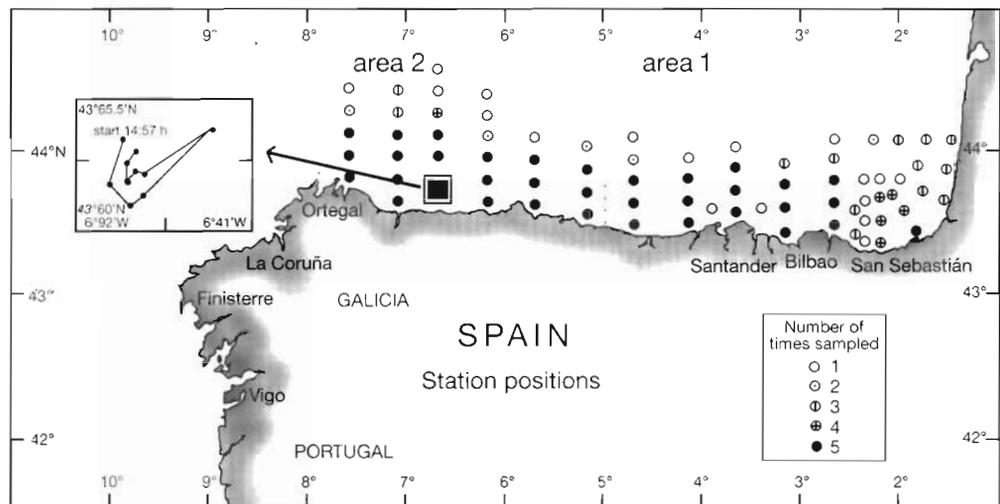
Recent studies have advocated caution in the use of RNA/DNA ratios because the techniques used to determine them have either been faulty (McGurk & Kusser 1992, Grémare & Vétion 1994, Canino & Calderone 1995, Suthers et al. 1996) or have lacked sufficient sensitivity (Bergeron et al. 1991, Richard et al. 1991, Mathers et al. 1994). Other workers have advocated caution in the use of ratios to remove the allometric effect of size and they have advised the use of a residual-based index from RNA content and an independently determined variable such as standard length or dry weight (Suthers et al. 1996). In addition, Houlihan (1991) and Mathers et al. (1994) have questioned the use of this index for calculation of instantaneous growth, since they found no correlation between RNA concentration and protein growth rate. Nevertheless, strong correlations between RNA/DNA and growth have been observed in a variety of species, such as *Ciuepa harengus*, *Ammodytes* sp., *Theragra chalcogramma*, *Paralichthys dentatus*, *Pseudopleuronectes americanus*, *Gadus morhua*, *Scomber scombrus* and *Morone saxatilis* (Buckley 1984). One aspect which has not been subject to a more detailed study is the diel variation of this index, which if such a fluctuation can be demonstrated, can also constitute an important limitation on the interpretation of results.

The first aim of this study was to determine the variation of the RNA/DNA ratios and the incidence of starvation among field-caught *Sardina pilchardus* larvae, in 2 hydrographic areas of the Cantabrian Sea (Bay of Biscay) over the period March to June 1992. In addition, we compared the zooplankton biomass with the RNA/DNA ratios in order to analyse the influence of prey availability on larval condition. Finally, variation in the nucleic acid indices at different hours of the day among *S. pilchardus* larvae caught throughout the spawning season was investigated.

## MATERIALS AND METHODS

**Field study.** During 1992 four research cruises were carried out covering the main spawning season of sardine off the north coast of Spain, which occurs from March to June. Thus, the northern Spanish continental shelf (45° to 42° N, 10° to 2° W) was sampled aboard the B/O 'Cornide de Saavedra' (6 to 20 March and 2 to 14 April 1992), the RV 'Valdivia' (4 to 24 May 1992) and the RRS 'Challenger' (23 May to 10 June 1992). The survey grid was divided into 2 areas: Area 1, from the boundary with French territorial waters to near Gijón; and Area 2, from the boundary with Area 1 to Cape Ortegal (Fig. 1). *In situ* temperature determinations were carried out by a CTD (conductivity/temperature/depth profiler) cast at selected stations.

Fig. 1. Location of sampling areas, stations and cruise tracks for the 40 h sampling (■). The number of cruises sampling stations from March to June 1992 shown



Mesozooplankton tows (including sardine larvae) were double oblique, with a bongo 200  $\mu\text{m}$  mesh net (diameter 50 cm), while zooplankton tows were double oblique, with a 53  $\mu\text{m}$  mesh net (diameter 10 cm). Bongo net hauls were designed to sample to about 100 m depth or to about 10 m from the bottom in the more shallow areas. On completion of the mesozooplankton haul, coarse mesh samples were immediately sorted for the bulk of sardine larvae and stored in liquid nitrogen ( $-196^{\circ}\text{C}$ ) for later RNA/DNA analysis. At each station a zooplankton sample was preserved in 4% buffered formaldehyde solution for taxonomic counts and another was frozen ( $-20^{\circ}\text{C}$ ) for biomass estimation. In Area 2, where the highest concentration of sardine larvae had been found, a special drift sampling to investigate the day-night condition of the larvae was carried out for 40 h (Fig. 1). During this 40 h sampling, 11 bongo net hauls were made with a minimum interval of 2 h and a maximum interval of 7 h.

**Laboratory procedures.** To determine the zooplankton biomass, samples were rinsed with an isotonic ammonium formate solution and heat dried to a constant weight in an electric oven at  $60^{\circ}\text{C}$ . The results were expressed as dry weight ( $\text{mg DW m}^{-3}$ ).

Before the nucleic acids were determined, standard lengths (SL) of thawed sardine larvae were measured under a dissecting microscope using an ocular micrometer. These lengths were corrected for shrinkage in the net according to Theilacker (1980b) and in the liquid nitrogen, using the following relationship:

$$\text{FrozenSL} = 0.422 + 0.92 \times \text{FreshSL} \quad (R = 0.87, n = 60)$$

This relationship was derived from an experiment conducted off southern Portugal during 1992 in which fresh length and frozen length were measured on the same fish larvae.

In this study, nucleic acids were extracted from whole body *Sardina pilchardus* by homogenising the larvae for 1 min in 600  $\mu\text{l}$  ice-cold tris buffer containing sodium dodecyl sulphate (SDS, final concentration 1%), using a sonicator. A highly sensitive fluorometric method for RNA/DNA quantification in individual organisms was applied.

Our analytical procedure was adapted from the methodology presented by Clemmesen (1988, 1990) for fish larvae, which allows individual larval analysis. It involves purification of tissue homogenates and subsequent fluorescence-photometric measurements using ethidium bromide (EB), a specific nucleic acids fluorochrome 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. The fluorescence was determined by exciting at 365 nm and reading at 590 nm with a spectrofluorometer (Hitachi model 650-10). Concentrations of nucleic acids were determined by running standard curves of DNA and RNA with EB every day, with known concentrations of calf thymus DNA and yeast RNA, in the appropriate range of values. Average recovery of added calf thymus DNA to larval samples (DNA spike) was  $92.1 \pm 4.6\%$  and average recovery of added yeast RNA (RNA-spike) was  $95.3 \pm 3.4\%$ . Total amounts of DNA and RNA in the post-larvae were corrected based on these average recovery efficiencies. The limit of detection (the analyte concentration giving a signal equal to the blank signal plus 2 standard deviations of the blank) was  $0.1 \mu\text{g ml}^{-1}$  for DNA and  $0.4 \mu\text{g ml}^{-1}$  for RNA. The coefficient of variability (sample standard deviation as percentage of the mean) was 4% for DNA and 10% for RNA when 10 aliquots of tissue homogenate were measured.

**Starvation percentages.** Larvae collected during the cruises with a RNA/DNA ratio equal to or below 1.3 were classified as starving. This value is the mean RNA/DNA ratio of sardine larvae obtained from an *in situ* experiment conducted off the coast of southern Portugal during 1992 (Chícharo 1996, 1997) in which larvae were deprived of food, inside net containers of 10 m mesh size, for 2 to 6 d. In this experiment *Sardina pilchardus* larvae ranged from 5.5 to 12.3 mm, and during the experiment daily measurements of water temperature were made (mean water temperature 15.3°C).

**Analysis.** The effects of time of year (cruises), areas and night and day (07:00 to 20:00 h, day; 20:00 to 07:00 h, night) on RNA/DNA ratios were examined by 3-way ANOVA. These effects on standard larval length were examined by a Kruskal-Wallis test (non-parametric ANOVA), due to the absence of normality. An index of condition was derived from the residuals of an overall, simple regression of  $\ln(\text{RNA}+1)$  on  $\ln(\text{SL})$ , according to Suther et al. (1996).

Using pooled samples (several larvae for each hour), the effect of time of day (hour) on RNA/larvae, DNA/larvae, SL, RNA/DNA and on the residuals of the regression of RNA on SL was examined by a conventional 1-way ANOVA with time of day as a categorical variate. As more than 2 comparisons were made, this analysis was followed by Tukey's test for comparison of means to compare means between hours, and the results were displayed on a graph. The relationships between RNA/DNA and zooplankton biomass were analysed by Pearson's correlation (Snedecor & Cochran 1989).

## RESULTS

### Oceanographic conditions

Between March and June 1992 ('Cornide', 'Valdivia' and 'Challenger' cruises) surface (5 m) water temperature ranged from 11.7 to 18.7°C. In March and April ('Cornide' cruises) a slight temperature gradient was present from east to west, with cooler (<12.5°C) water in the east (Area 1) and warmer water (>13°C) in the west (Area 2). In May ('Valdivia' cruise) surface water temperature was higher than 14°C over most of the areas except the coastal zone, halfway between Gijon and Ortegal, where surface temperatures were near 13°C (Lopez-Jamar et al. 1995). The lower surface temperature in this area is an indication of upwelling of deep cooler water, which starts at about this time of year and usually continues until the end of summer (Robles et al. 1992). From the end of May ('Challenger' cruise), a marked warming of surface water took place; the highest surface temperatures were recorded to the west of Bilbao (>17.5°C) (Lopez-Jamar et al. 1995).

Surface water salinity (5 m) in the study area generally ranged between 34.8 and 36.2 PSU, with lower values in the more eastern area (Area 1). Stratification values ( $\Delta\sigma_t$ , 0–50 m) were weak in March and April, indicating a well-mixed water column in the entire area, at least down to a depth of 50 m. By May stratification was relatively pronounced in the eastern area, mainly in the more inshore region. The remaining area showed moderate stratification except off north-west Galicia, where a well-mixed water column was present, indicative of upwelling. From late May and June a similar stratification pattern was maintained, but the area of highly stratified water in the eastern area became more extensive (Lopez-Jamar et al. 1995).

### Zooplankton biomass (potential prey)

The mean zooplankton biomass measured at the stations where sardine larvae were analysed biochemically was compared among time of the year (cruises) and areas. The results revealed higher values during March and April ('Cornide' cruises), but the values were not significantly different from those found in the other months (May and June  $p < 0.544$ ). When this parameter was compared between areas, the results indicated higher values for Area 1, but again the values were not significantly different from the other area ( $p < 0.769$ ).

Maximum biomass of zooplankton was recorded especially during the night (Area 2, 'Cornide' cruises; Area 1, 'Challenger' cruise) (Table 1). During the diurnal cycle, the maximum biomass of zooplankton was also recorded during the dark hours, with lower values occurring during the day (Table 1).

### Distribution and length of sardine larvae

*Sardina pilchardus* were caught throughout most of the area under investigation. During March and May the highest concentrations (ranging to 100 to 150 larvae  $\text{m}^{-2}$ ) were distributed along the northern coast of Spain to the east of Cape Ortegal. In April, the highest larval abundance was recorded off Santander. Abundance of sardine larvae decreased drastically along the west coast of Spain, where especially the outer shelf stations frequently yielded zero larvae (Lopez-Jamar et al. 1995).

The mean SL of field-caught larvae biochemically analysed ranged between 8 and 18 mm. When the total larval lengths were analysed, significant differences were observed between nighttime and daytime using the non-parametric Kruskal-Wallis test  $H$  ( $df = 1$ ,  $\chi^2_{0.05,1} = 4.414$ ,  $p < 0.036$ ). During the 40 h sampling as well the

Table 1. Mean and standard deviation of the RNA/DNA ratios, standard length, zooplankton biomass by time of year (cruise) and area, during daytime and nighttime periods (n: number of larvae analysed or number of stations for zooplankton biomass measurements). Data for the 40 h sample are also included in the 'Valdivia' data

Cruise		n	RNA/DNA Area 1	n	RNA/DNA Area 2	n	Length Area 1	n	Length Area 2	n	Zooplankton biomass Area 1	n	Zooplankton biomass Area 2
'Cornide'	Day	30	2.77 ± 0.51	20	4.15 ± 1.13	30	10.6 ± 2.0	20	15.4 ± 3.4	2	5.04 ± 0.2	2	5.18 ± 2.63
Mar/Apr	Night	29	2.75 ± 1.03	11	5.48 ± 1.01	29	12.9 ± 4.1	11	14.8 ± 2.1	3	4.68 ± 1.94	1	15.69
'Valdivia'	Day	42	3.89 ± 1.65	160	3.40 ± 1.28	42	19 ± 3.6	160	11.4 ± 2.9	3	2.77 ± 0.52	15	4.76 ± 2.48
May	Night	26	3.76 ± 1.59	107	3.81 ± 1.43	26	11.1 ± 3.6	107	12 ± 3.6	5	7.38 ± 2.49	10	5.2 ± 4.24
'Challenger'	Day	16	3.53 ± 1.24	14	2.73 ± 0.87	16	11.6 ± 2.8	14	13.1 ± 3.6	3	3.96 ± 2.71	3	3.93 ± 1.96
Jun	Night	5	4.28 ± 1.69	14	3.17 ± 1.09	5	12.6 ± 1.1	14	13.2 ± 2.1	1	12.75	1	4.61
40 h sample	Day			81	3.71 ± 1.47			81	11.6 ± 2			6	6.45 ± 3.02
	Night			62	4.09 ± 1.39			62	12.7 ± 2.5			5	7.05 ± 5.55
Total	Day	88	3.44 ± 1.38	194	3.43 ± 1.27	88	10.5 ± 3.1	194	11.9 ± 3.2	8	3.78 ± 1.76	20	4.68 ± 2.33
	Night	60	3.32 ± 1.45	132	3.87 ± 1.43	60	12.1 ± 3.8	136	12.4 ± 3.4	9	7.08 ± 3.21	12	6.02 ± 4.9

larger larvae were captured mainly at night (Fig. 2). Significant differences in length were also observed among areas,  $H$  ( $df = 1$ ,  $n=474 = 11.446$ ,  $p < 0.003$ ), and among times of year,  $H$  ( $df = 2$ ,  $n=474 = 8.414$ ,  $p < 0.015$ ).

### Starvation percentages

The starvation percentages were generally low. The highest value, 3.23%, was observed during the earliest cruises in March and April ('Cornide' cruises) in Area 2

(Table 2). Values near zero were found for the cruises in May and June ('Valdivia' and 'Challenger' cruises).

### Nutritional condition

This study measured the nucleic acid content of 474 sardine larvae, 90 during the 'Cornide' cruises (March and April), 335 during the 'Valdivia' cruise (May) and 49 during the 'Challenger' cruise (May–June). Among other things, a 40 h sampling was performed and 143

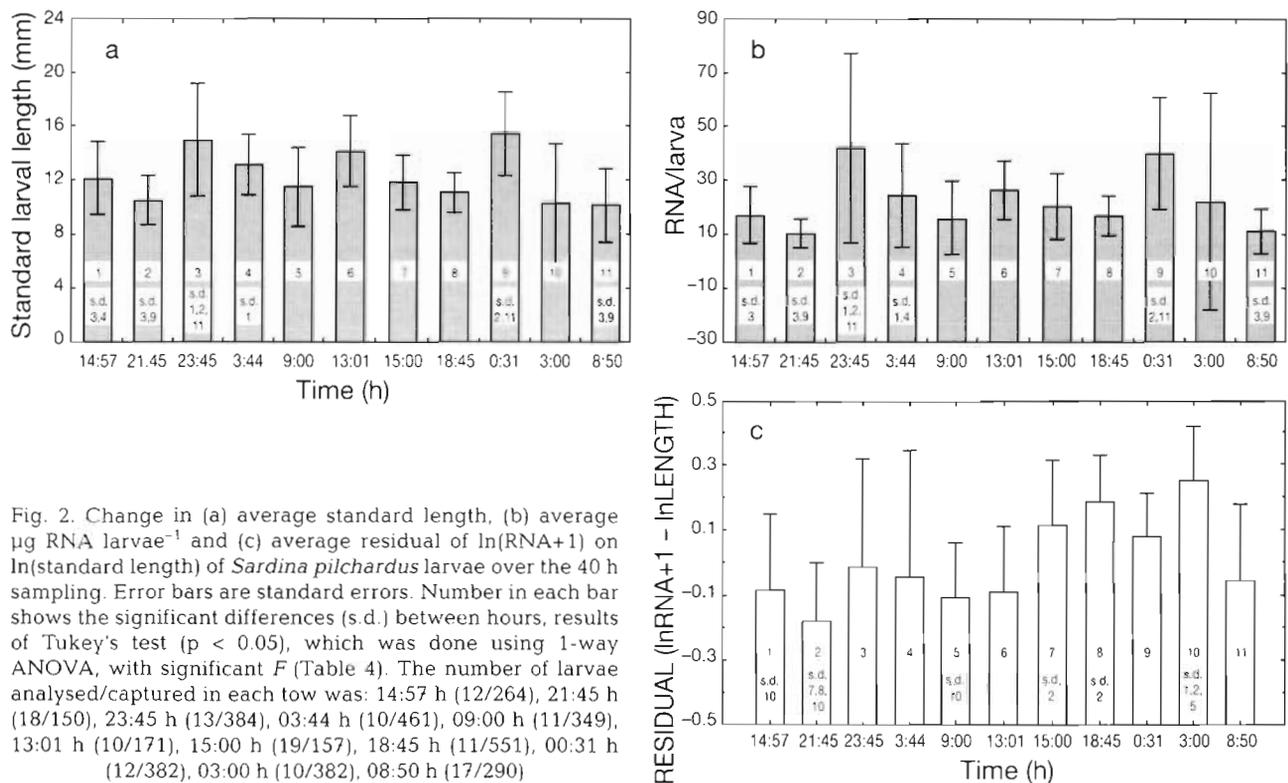


Fig. 2. Change in (a) average standard length, (b) average  $\mu\text{g RNA larvae}^{-1}$  and (c) average residual of  $\ln(\text{RNA}+1)$  on  $\ln(\text{standard length})$  of *Sardina pilchardus* larvae over the 40 h sampling. Error bars are standard errors. Number in each bar shows the significant differences (s.d.) between hours, results of Tukey's test ( $p < 0.05$ ), which was done using 1-way ANOVA, with significant  $F$  (Table 4). The number of larvae analysed/captured in each tow was: 14:57 h (12/264), 21:45 h (18/150), 23:45 h (13/384), 03:44 h (10/461), 09:00 h (11/349), 13:01 h (10/171), 15:00 h (19/157), 18:45 h (11/551), 00:31 h (12/382), 03:00 h (10/382), 08:50 h (17/290)

Table 2. Starvation percentages by time of year (cruises in March–April, May and June) and area (1, 2). Percentages below are number of larvae with RNA/DNA ratio <1.3 and total number of larvae analysed

Area	Mar/Apr	May	Jun	Total
1	1.7% (1/59)	0%	0%	2.03% (3/148)
2	3.23% (1/31)	0.75% (2/267)	0%	0.3% (1/326)
Total	0.6% (2/90)	0.6% (2/335)	0%	1.07% (4/474)

larvae were analysed during the 'Valdivia' cruise. The number of sardine larvae analysed was dependent on the quantity immediately frozen after capture in each cruise and zone.

The average values of RNA/DNA ratio were relatively high, but during March–April ('Cornide' cruises) and in Area 2 the mean values of these indices were the highest (Table 1).

Average RNA/DNA ratio values were higher during the night than during the day, especially in Area 2 (Table 1). The effects of day and night hours, time of year (cruises) and areas on RNA/DNA ratios examined by a 3-way ANOVA indicated that the effect of day and night hours was the only one with a significant  $F$  ( $p < 0.011$ ). Significant values were also found for the interaction between cruise and area ( $p < 0.001$ ) (Table 3). During the drift investigation, significant differences were found between the average RNA/DNA ratios of some hours ( $p < 0.0001$ ) (Table 4). The 1-way ANOVA followed by the Tukey test ( $p < 0.05$ ) (Fig. 3), revealed that at times such as at 18:45 h (twilight) or 03:00 h (night) the means of RNA/DNA ratios were significantly different from the means at other times of day (Fig. 3). The RNA concentration also was shown to have higher values at night (23:45 and 00:31 h) (Fig. 2).

Table 3. Summary of a 3-way ANOVA for combined data, fixed effect of RNA/DNA: 1 = cruise, 2 = area, 3 = day/night (07:00 to 20:00 h, day; 20:00 to 07:00 h, night). Ind. var.: independent variable; df: degrees of freedom; MS: mean square. \* $p < 0.05$

Ind. var.	df effect	MS effect	df error	MS error	$F$	p-level
1	2	1.82	462	1.694	1.074	0.342
2	1	4.454	462	1.694	2.629	0.106
3	1	11.081	462	1.694	6.541	0.011*
1,2	2	44.367	462	1.694	26.19	0.001*
1,3	2	2.297	462	1.694	1.356	0.259
2,3	1	3.554	462	1.694	2.098	0.148
1,2,3	2	2.344	462	1.694	1.384	0.252

Table 4. Summary of a 1-way ANOVA, fixed effect for 40 h sampling, with hours as independent variable. Dep. var.: dependent variable; df: degrees of freedom; MS: mean square

Dep. var.	df effect	MS effect	df error	MS error	$F$	p-level
Length	10	46.258	132	7.792	5.937	0.0001
DNA/larvae	10	88.307	132	14.911	5.922	0.0001
RNA/larvae	10	1480.4	132	342.726	4.32	0.0001
Residual RNA	10	0.227	132	0.050	4.502	0.0001
RNA/DNA	10	8.695	132	1.576	5.519	0.0001

To prevent length from influencing RNA/DNA ratios, the mean residual condition (residuals of RNA on SL) was used, but it showed a trend similar to the RNA/DNA (Fig. 2c).

The general correlation between the RNA/DNA ratio and larval length was not significant ( $r^2 = 0.052$ ;  $p < 0.093$ ;  $n = 474$ ), and similar results were found for drift sampling ( $r^2 = 0.023$ ,  $p < 0.665$ ;  $n = 143$ ). Zooplankton biomass was related to the nutritional condition of fish larvae. During normal cruises zooplankton biomass explained 17% of the observed variability ( $r^2 = 0.17$ ;  $p < 0.002$ ;  $n = 49$ ) (Fig. 4a). Moreover, when drift sampling data was considered on its own, a significant correlation between RNA/DNA ratios and zooplankton biomass was also evident ( $r^2 = 0.442$ ;  $p < 0.026$ ;  $n = 11$ ) (Fig. 4b), but in this case variation was explained to a high degree (44%).

To discern the influence of zooplankton prey on RNA/DNA ratios of sardine larvae, the residuals of the relationship between zooplankton biomass and RNA/DNA ratios were analysed by time of the drift sampling and the results also show high values during the night hours (Fig. 5).

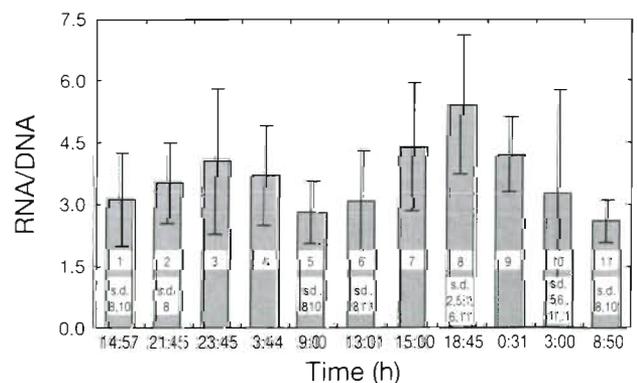


Fig. 3. Change in average RNA/DNA ratios of *Sardina pilchardus* larvae during the 40 h sampling. Error bars are standard error. Number in each bar shows the significant differences (s.d.) between hours, results of Tukey's test ( $p < 0.05$ ), which was done using 1-way ANOVA, with significant  $F$  (Table 4)

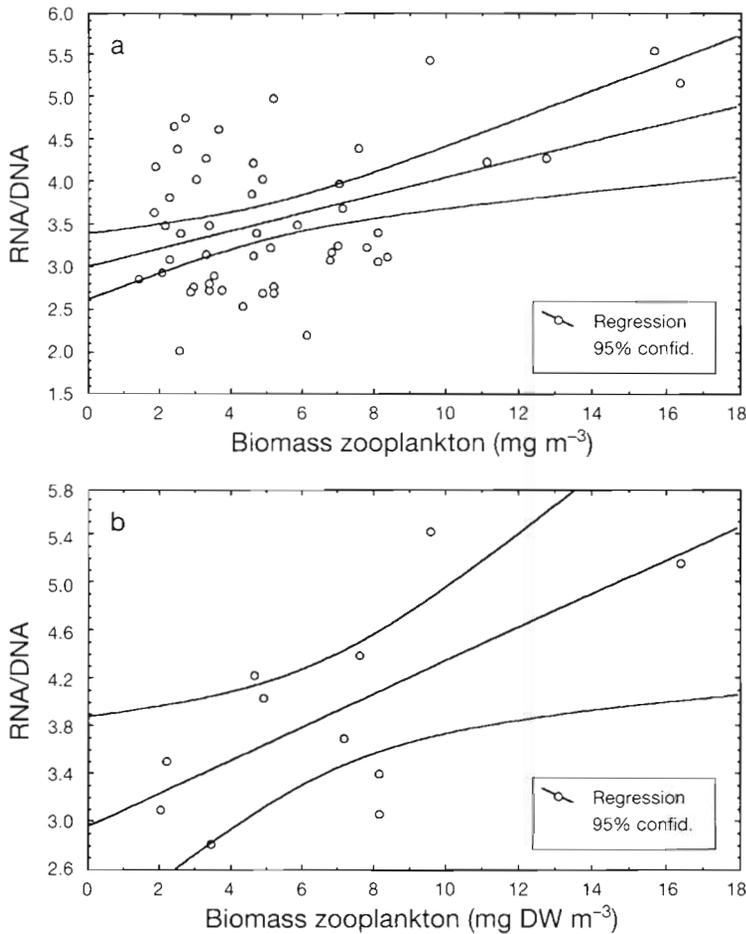


Fig. 4. Relationship of RNA/DNA on zooplankton biomass: (a) total cruises; (b) 40 h sampling data. DW: dry weight

## DISCUSSION

### Starvation and nutritional condition

To assess the importance of starvation for field-caught larvae it is necessary to determine the RNA/DNA ratio below which larvae are classified as starving. Until now, this kind of calibration has been done only in laboratory conditions (Buckley 1984, Clemmesen 1987, Robinson & Ware 1988, Pittman 1991, Chícharo 1993), which hardly simulate natural conditions (Blaxter 1976, Theilacker 1980, Mackenzie et al. 1990, Folkvord & Mokness 1995). Chícharo's studies (1996, 1997) made it possible to assess from a field experiment the RNA/DNA ratio indicative of starvation in *Sardina pilchardus* (RNA/DNA ratios less than 1.3).

The utilisation of the mean RNA/DNA ratio obtained from starved fish larvae to determine starvation percentage has some problems. In fact, the RNA/DNA ratio is temperature dependent and also seems to

change with larval size. These are important limitations because when we work with field samples the variability of temperatures and larval size is usually high. However, despite the wide range of water temperatures measured during this study (11.7 to 18.7°C), the great majority of samples were taken during May, when water temperature was close to the water temperature during the field experiment (15.3°C).

The range of larval length studied in the field experiment was 5.5 to 12.3 mm (Chícharo 1997) and bigger larvae (8 to 18 mm) were found in the sea. Thus, if this ratio increases with age (length) (Clemmesen 1994, Rooker & Holt 1996), we could be stating that some starved larvae are in good condition. Nevertheless, according to Clemmesen (1994) the increase in the RNA/DNA ratio with length was less pronounced in starving larvae compared to fed larvae. Also, if we increase our ratio (1.3; Chícharo 1997) to 1.5 [the ratio Clemmesen (1994) gave for a starved herring larvae 18 mm in length], the same starvation percentages are obtained.

Only 0.84% (4/474) of sardine larvae collected during this study were classified as starving. This result is lower than that found for sardine larvae (4.8%) collected on the Algarve continental shelf (southern Portugal) during May 1992 (Chícharo 1997). In addition, there are few reports of finding larvae in poor condition at sea.

Since the initial studies of larval condition conducted by Ehrlich et al. (1976) and O'Connell (1976, 1980), who showed evidence that only 8% of wild *Clupea harengus* larvae were in condition of starvation, many other studies have confirmed those results. Buckley

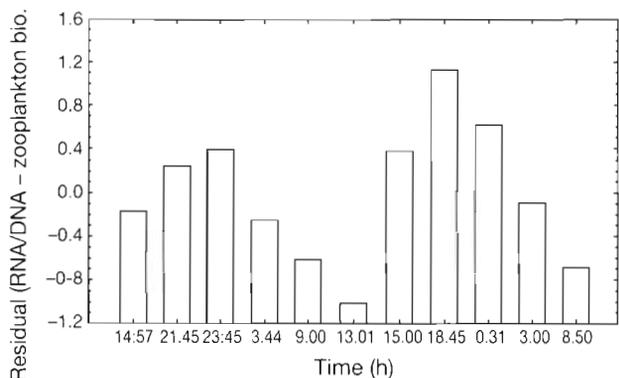


Fig. 5. Change in average residual of RNA/DNA ratios on zooplankton biomass ( $\text{mg m}^{-3}$ ) during the 40 h sampling

(1984) found 2 and 7% of starved larvae of *Melanogrammus aeglefinus* and *Ammodytes* sp., respectively. Clemmesen (1994) found only 1% of *C. harengus* larvae with RNA/DNA ratios below the critical level. Meanwhile, McGurk et al. (1992) detected 8% of starved larvae of the same species, but in a different area. Thus, the percentage of larvae in poor condition may vary between taxa, study area and over time. In fact, the results of the present study indicate some variability of starving larvae between areas and months (cruises).

The highest starvation percentages were found in Area 2 and during the months of March and April. Surprisingly, the mean value of zooplankton biomass was highest during this period and in this area. This result was probably related to the well-mixed column in the area. However, the highest starvation percentages determined in such conditions may be related to the existence of some abnormal larvae. According to McGurk et al. (1992), there are larvae which may not have successfully developed their feeding abilities and are therefore not able to take advantage of food availability.

The general condition of sardine larvae, measured by the RNA/DNA ratio during the sampling period, was very good. Complementary findings are summarised by McFadzen et al. (1997) for measurements of larval condition (histological methods) and by Conway et al. (1994) for measurements of sardine larvae feeding which were made on the same cruises. The first study showed that most specimens were in medium or good condition and the second suggested, based on the quantity of food in the guts of larvae, that in all months there was generally sufficient food available to the larvae. Moreover, in the present study, zooplankton biomass seems to explain part of the variation in RNA/DNA ratios which was revealed by the significant correlation between this index and the potential prey of sardine larvae. This seems to agree with several other recent studies, namely, Canino et al. (1991), Canino (1994), Bailey et al. (1995), Suthers (1996), and Suthers et al. (1996). However, results of sardine larval condition measured by histological methods (McFadzen et al. 1997), on the same cruises, indicated the lack of any overall correlation between food availability and the RNA/DNA index. It should be recognised that the measures of food availability in McFadzen et al.'s study were derived from estimates of food concentrations (number l<sup>-1</sup>) and in our study they were derived from biomass determinations.

Relating starvation and larval condition to food availability in the plankton is complex due to small-scale temporal and spatial patchiness in the distribution of fish larvae and prey (Mackenzie & Kiørboe 1995). Zooplankton concentration or biomass estimates may under-

estimate prey availability when zooplankton production is being consumed as quickly as it is being produced (Hunter 1981). Sometimes it is difficult to distinguish between the influences of prey availability, which contribute to an enhanced condition, and the effects of predation, which may result in loss of weak larvae in the samples taken and also to an enhanced general condition of collected larvae (Bailey & Houde 1989).

### Diel variation of nucleic acids

Our results seem to indicate a significant diel effect for RNA/DNA, RNA residuals and RNA/larvae. In the nucleic acids indices, this kind of diel effect has not been reported very often in previous studies with fish larvae. In fact, Bailey et al. (1995) found no significant diel effect on RNA content using SL as covariate with *Theragra chalcogramma* larvae. However, with *Sciaenops ocellatus* larvae, Rooker & Holt (1996) found diel changes in RNA/DNA ratios, with higher values during daytime periods and markedly reduced ratios at night. The results of a 3-way ANOVA (cruise, area and day/night) on the general RNA/DNA ratios in the present study also suggested a significant diel effect, but higher values were found during the night in the present study. Also, when we analysed the variation during a diurnal cycle, the results suggested that at twilight and during early hours of the night RNA/DNA values were significantly higher. Thus, this difference may be a specific characteristic of this particular species.

Because larger individuals were found during the nighttime in our study and because some authors (Suthers 1992, Clemmesen 1994, Rooker & Holt 1996, Suthers et al. 1996) have argued that the RNA/DNA ratio increases with age, we tried to eliminate any vestiges of a length influence using the RNA residual indices. In fact, the absence of normally distributed length data showed that we were selecting size class. In any event, our study found no significant relationship between RNA/DNA ratios and length. When we analysed the variations of residual RNA on standard length during the 40 h sampling, similar RNA/DNA ratios were found, with somewhat higher values being noted during dark hours. This could be explained by the following hypothesis: an endogenous rhythm exists that raises the concentration of RNA in *Sardina pilchardus* larvae during certain hours. It is probably the case that circadian periodicities in endocrine activity are responsible for observed patterns. Circadian periodicities in cell division rate or in growth-regulating hormones has been reported for several eukaryotic organisms: algae (Makarov et al. 1995, Costas et al. 1996), insects (Zeng et al. 1996), rats and sheep (Coon

et al. 1995) and marine fishes (Bates et al. 1989). In a study by Makarov et al. (1995), in marine macroalgae the majority of nuclear divisions took place during the dark period. In mammals the night-to-day ratio of melatonin, the hormone that co-ordinates daily and seasonal physiology, is less than 2 in sheep and exceeds 150 in rats (Coon et al. 1995). The light/dark regimen seems to induce a circadian protein synthesis rhythm in these organisms which is probably responsible for the diel variations in RNA/DNA ratios.

We excluded the hypothesis that a sudden increase in RNA concentration takes place in response to an increase in food availability because, firstly, according to Clemmesen (1994), the RNA/DNA ratios do not reflect increase in prey availability over a period of hours. In fact, a sudden increase in food availability at first leads to an increase in the activity of the ribosome followed later by an increase in their numbers. The methodology used measures only the ribosome content. Secondly, despite the increase in food availability at night most fish larvae do not feed at night (Suthers & Sundby 1996). According to Conway et al. (1991), the diel variation in feeding incidence in *Sardina pilchardus* reveals lowest feeding incidence during the dark, between 20:00 and 04:00 h GMT. This observation applies equally well to most other species of fish (Last 1980, Suthers et al. 1996) and is related to the fact that fish larvae are visual feeders. However, the results of Conway et al. (1991) also suggest that, surprisingly, smaller larvae (<15 mm) show the least diurnal effect, maintaining almost the same feeding incidence throughout the day and the night. This contrasts with other observations (Arthur 1976) that clupeid larvae do not feed at night.

In the light of the foregoing observations, only one other hypothesis may be developed to try to explain the fact that RNA indices are higher during the night. This hypothesis assumes that the higher values result from the RNA of the prey, which remain in the sardine guts after the day's feeding activity. In fact, there were no differences in the mean number of food items per feeding larvae during daytime or nighttime hours, except during the period 20:00 to 24:00 h, when the highest value was recorded (Conway et al. 1991). However, the rapid digestion rate of larval prey (Arthur 1974) and the loss of gut contents through defecation that occurs in response to net capture and preservation (Colton et al. 1980, Blaxter & Hunter 1982, Conway et al. 1991) make this hypothesis difficult to prove. Moreover, when we removed the influence of zooplankton biomass on RNA/DNA ratios, the residuals of RNA/DNA ratios still revealed a periodic fluctuation.

Although it is still necessary to confirm under controlled conditions (constant light, temperature, etc.) the hypothesis that an endogenous rhythm exists, it can be

concluded from the present study that, because of these diel changes in RNA concentrations, average RNA indices may be unrepresentative when there is a day/night bias in sampling.

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