



## Comparison of RNA/DNA ratios obtained with two methods for nucleic acid quantification in gobiid larvae

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### Abstract

In this paper, RNA and DNA contents and RNA/DNA ratios obtained with two fluorometric methods for the extraction and quantification of nucleic acids in individual estuarine gobiid larvae are compared. A total of 141 gobiid larvae, collected in the Mira and Guadiana estuaries (Portugal), were analysed. This study indicated that the nucleic acids content and RNA/DNA ratios of individual larvae varied with the procedure used. The two methods yielded similar log DNA values but different log RNA and log (RNA/DNA). The differences could be due to a number of factors related to specific steps of tested protocols. Comparison of nucleic acids contents and calibration of the two methods by regression analysis provided a reasonable basis for comparison although differences between estimates were evident. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Fluorimetric methods; Gobiid larvae; RNA/DNA

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### 1. Introduction

The management of marine fish populations is dependent on understanding the nature and causes of variability in recruitment and year-class strength. Variability in annual recruitment is thought to be largely controlled by starvation, predation, and unfavourable advection which determines survival during the early life-history stages (Houde, 1987).

Any means to assess the nutritional condition of field-caught larvae would help in estimating larval survival and year-class fluctuations (Richard et al., 1991). The nutritional condition of fish larvae has been characterised morphometrically (Blaxter, 1971; Ehrlich et al., 1976), histologically (e.g. Strussmann and Takashima, 1990), by

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measuring proteolytic enzymes (Ueberschar, 1988) or by the relative amounts of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) (Bergeron and Boulhic, 1994; Mathers et al., 1994; Bailey et al., 1995; Rooker and Holt, 1996; Bergeron and Person-Le Ruyet, 1997; Canino, 1997; Chícharo, 1997, 1998; Chícharo et al., 1998a,b). Despite the current debate about the use of nucleic acids-based indices (see Bergeron, 1997 for a review) they are ubiquitous in the literature.

Several methods have been developed for determining the concentration of nucleic acids in fish larvae. Initial methods, based upon UV light absorption (e.g. Buckley, 1979), were limited by sample size, thus requiring pooled samples of fish larvae (typically > 800 µg). Recent development of highly sensitive fluorometric techniques for direct measurement of nucleic acid content has permitted the analysis of individual larvae (Clemmesen, 1988, 1993; Caldarone and Buckley, 1991; Mathers et al., 1994; Canino and Caldarone, 1995). These protocols are based upon the fluorescence of specific dyes (e.g. ethidium bromide, Hoechst H33258 or bisbenzimidazole) when bound to nucleic acids. The fluorescence of the nucleic acid–fluorophore is measured sequentially, either by specific fluorophores or by digestion of RNA by ribonuclease (Robinson and Ware, 1988; Clemmesen, 1988, 1993). Total fluorescence is then partitioned into DNA and RNA components.

In this paper, RNA and DNA contents and RNA/DNA ratios of individual estuarine gobiid larvae obtained with two sample preparation procedures [Caldarone and Buckley (1991) and Clemmesen (1988, 1993)] are compared. Both ease and applicability of protocols are discussed in the light of the results.

## 2. Material and methods

### 2.1. Sampling

Gobiid larvae were collected monthly from estuaries of the Mira ( $n = 86$ ) and Guadiana rivers ( $n = 70$ ) between May and September 1997 (Fig. 1). Zooplankton tows at a constant speed of 2 knots were made at 1-m depth with a conical net (1.60 × 0.37 m, 0.5 mm mesh-size). Fish larvae were sorted in a black glass tray, immediately frozen in liquid nitrogen (−197°C) and later stored at −80°C. Fish larvae were identified using the keys provided in Newell and Newell (1963) and Russell (1976).

### 2.2. Extraction of nucleic acids

In the laboratory, larvae were thawed and measured (to the nearest 0.1 mm) under a dissecting microscope equipped with a ocular micrometer. The procedures outlined by Caldarone and Buckley (1991) [conventional fluorimetric analysis (CFA)] and developed by Clemmesen (1988, 1993) and further modified by Chícharo (1996) [modified fluorimetric analysis (MFA)] were used to quantify nucleic acids in individual fish larvae.

Fish larvae were extracted in 0.15 ml of 1% sarcosine (sodium *N*-lauroylsarcosine) in

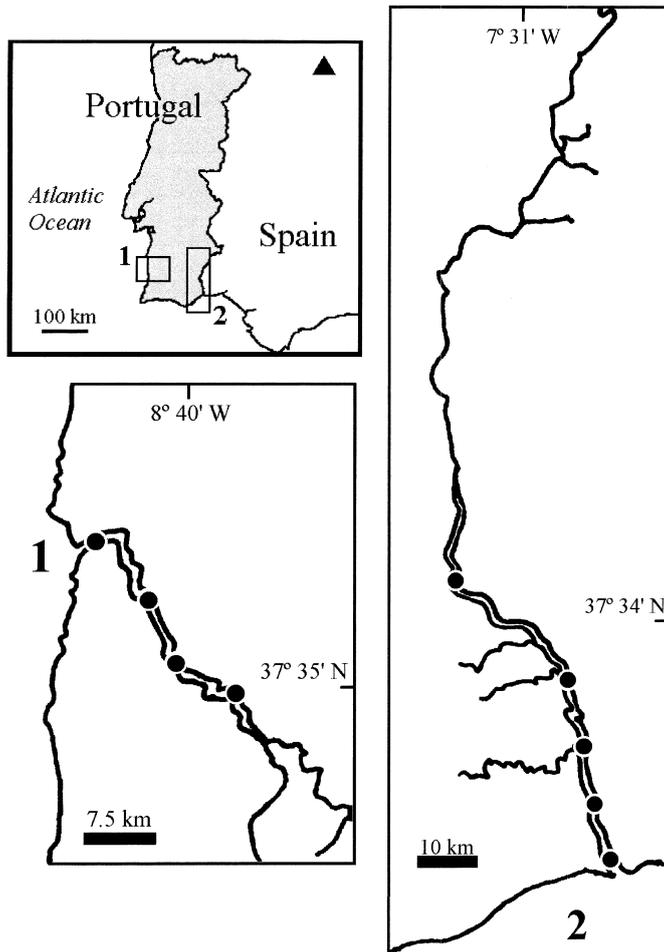


Fig. 1. Location of sampling areas (rectangles) and stations (●). (1) Mira river and (2) Guadiana river.

Tris–EDTA buffer (Trizma, pH 8.0) to give a final concentration of 0.1%. After centrifugation, aliquots of the supernatant were used for further analyses (Fig. 2).

### 2.2.1. CFA

A 0.2-ml aliquot of extracted sample was combined with 0.4 ml of Tris–NaCl (Trizma, pH 7.5) and 0.05 ml of ethidium bromide (EB) ( $0.1 \text{ mg} \cdot \text{ml}^{-1}$ ). Another 0.2-ml aliquot of the same extracted sample was combined with 0.35 ml of Tris–NaCl and 0.05 ml of ribonuclease A (Type-II A,  $0.12 \text{ } \mu\text{g} \cdot \text{ml}^{-1}$ ). This mixture was incubated at  $37^\circ\text{C}$  for 30 min, allowed to reach room temperature for at least 15 min, and stained with 0.05 ml of EB (Fig. 3).

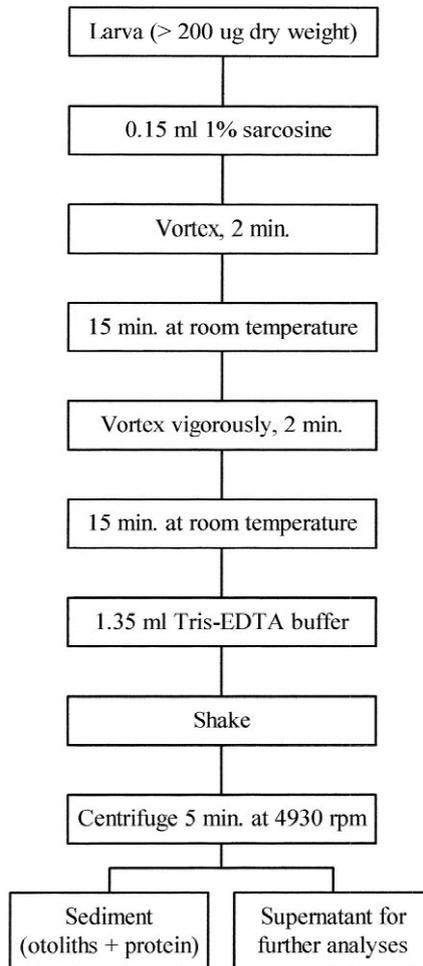


Fig. 2. Flow-chart of sarcosine extraction procedure according to Caldarone and Buckley (1991).

### 2.2.2. MFA

For purification of nucleic acids, a third 0.6 ml aliquot of the extracted sample was washed first with 0.6 ml of phenol–chloroform–isoamyl alcohol (49.5:49.5:1, v/v) and then with 0.3 ml of chloroform–isoamyl alcohol (24:1, v/v) as shown in Fig. 4. After these purification steps, 0.2-ml aliquots of the supernatant were treated as above for CFA.

### 2.3. Fluorescence assays

Calculations of nucleic acids concentration were identical for both procedures. Endogenous sample fluorescence (blank) was subtracted from total sample-EB dye

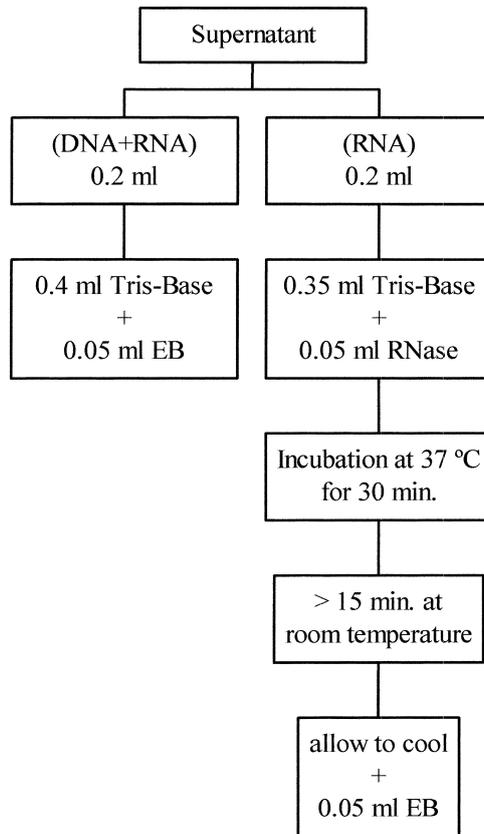


Fig. 3. Flow-chart of the quantification methodology described by Caldarone and Buckley (1991).

fluorescence. The fluorescence due to total RNA, mainly ribosomal, was calculated as the difference between total fluorescence (RNA and DNA) and the fluorescence measured after ribonuclease treatment, which is assumed to be due to DNA. Fluorescence was determined by exciting at 365 nm and reading at 590 nm with a spectrofluorometer (Hitachi Model 650-10). Concentrations were determined by running standard curves of DNA–EB and RNA–EB every day with known concentrations of  $\lambda$ -DNA ( $0.25 \mu\text{g} \cdot \mu\text{l}^{-1}$ ) and 16s–23s RNA ( $4 \mu\text{g} \cdot \mu\text{l}^{-1}$ ), in the appropriate range of values. All chemicals used in the procedures described above were analytical grade.

The limit of detection, i.e. the analyte concentration giving a signal equal to the blank signal plus 2 standard deviations of the blank (Miller and Miller, 1988), was  $0.16 \mu\text{g} \cdot \text{ml}^{-1}$  for DNA and  $0.46 \mu\text{g} \cdot \text{ml}^{-1}$  for RNA. Percent recovery of added  $\lambda$ -DNA to eight larvae homogenates (DNA spike) was 95.3% for CFA and 88.8% for MFA, and the recovery of added 16s + 23s RNA (RNA spike) was 105.6% for CFA and 62.8% for MFA. Total amounts of nucleic acids were corrected based on these values. The coefficient of variation (Zar, 1996) calculated for estimates from eight homogenate

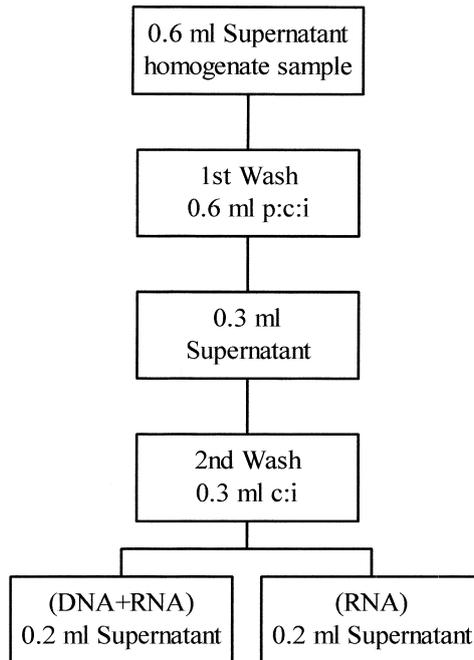


Fig. 4. Flow chart of the purification and quantification of nucleic acids adapted from Clemmesen (1993) by Chicharo (1996). p, phenol; c, chloroform; i, isoamylalcohol.

samples was: (1) 1.5% for DNA and 3.5% for RNA when using CFA, and (2) 14.8% for DNA and 17.8% for RNA when using MFA.

#### 2.4. Data analysis

Predicted nucleic acids contents were log-transformed to correct for non-normality. Normality was then evaluated using the Kolmogorov–Smirnov  $k$  test (Zar, 1996). Differences between rivers and methods were compared using Student's  $t$ -tests (Zar, 1996). The relationships between log RNA, log DNA, and log (RNA/DNA) predicted by the two methods were studied using correlation coefficients (Zar, 1996). Functional relationships between variables were derived using geometric mean regression analysis (Sokal and Rohlf, 1981).

### 3. Results

Larvae ranged in length from 3.3 to 23.0 mm (mean SL of  $10.55 \pm 3.63$  mm for river Mira and  $10.07 \pm 4.13$  mm for river Guadiana). Mean contents of nucleic acids were higher in larvae from the Guadiana river except for RNA content obtained from MFA. In

Table 1

Summary statistics for nucleic acids contents and RNA/DNA ratios obtained in this study; values are presented as mean  $\pm$  standard deviation (S.D.) and range

	<i>n</i>	SL (mm)	Caldarone and Buckley (1991)		Clemmesen (1993)	
			Mean $\pm$ S.D.	Range	Mean $\pm$ S.D.	Range
Mira		3.34–18.25				
$\mu\text{g DNA}$	86		10.78 $\pm$ 9.22	0.17–64.91	11.42 $\pm$ 10.41	2.18–66.87
$\mu\text{g RNA}$	86		30.49 $\pm$ 23.70	2.22–138.12	66.68 $\pm$ 68.16	3.24–320.94
RNA/DNA	86		6.46 $\pm$ 14.28	0.14–114.32	6.96 $\pm$ 6.27	0.25–34.65
Guadiana		4.13–23.03				
$\mu\text{g DNA}$	70		15.01 $\pm$ 17.85	2.17–76.39	14.28 $\pm$ 19.87	0.73–114.40
$\mu\text{g RNA}$	70		35.63 $\pm$ 60.38	1.06–315.49	65.08 $\pm$ 120.73	2.12–619.96
RNA/DNA	70		2.42 $\pm$ 1.26	0.19–6.23	4.12 $\pm$ 3.84	0.23–27.02

contrast, mean RNA/DNA ratios and standard deviation were lower for the Guadiana river (2.42  $\pm$  1.26 with CFA and 4.12  $\pm$  3.84 with MFA) than for the Mira river (6.46  $\pm$  14.28 and 6.96  $\pm$  6.27, respectively) (Table 1).

Resulting distributions of log-transformed data were normal (Kolmogorov–Smirnov test,  $P > 0.05$ ). Log-transformed data were different between rivers (Student *t*-test,  $***P < 0.001$ ) except for log DNA (Student *t*-test,  $P > 0.276$ ) (Table 2). Pooled differences between methods were non-significant (Student *t*-test,  $P > 0.826$ ) for log DNA but highly significant (Student *t*-test,  $***P < 0.001$ ) for log RNA and log (RNA/DNA) (Table 2). When comparing methods within each estuary, only log (RNA/DNA) was significantly different in the Mira estuary (Student *t*-test,  $***P < 0.001$ ). In contrast, the two methods produced similar values of log DNA in the Guadiana estuary (Table 3). Log DNA was more strongly correlated between the two methods than log RNA or log (RNA/DNA), with similar functional regression equations between estuaries (Fig. 5).

Table 2

Comparison of log RNA, log DNA and log(RNA/DNA) of gobiid larvae caught in the estuaries of the Mira and Guadiana rivers (Student *t*-test results)<sup>a</sup>

Depend. Var.	d.f.	<i>t</i>	<i>P</i>
River (Guadiana vs. Mira)	155		
log DNA		1.093	0.276 <sup>NS</sup>
log RNA		– 2.793	0.006 <sup>***</sup>
log (RNA/DNA)		– 4.782	0.000 <sup>***</sup>
Method (CFA vs. MFA)	155		
log DNA		– 2.220	0.826 <sup>NS</sup>
log RNA		– 6.266	0.000 <sup>***</sup>
log (RNA/DNA)		– 5.585	0.000 <sup>***</sup>

<sup>a</sup> Methods were Clemmesen (1988, 1993) (MFA) and Caldarone and Buckley (1991) (CFA). d.f., degrees of freedom; NS, non-significant;  $***P < 0.001$ .

Table 3

Comparison of log DNA, log RNA and log (RNA/DNA) obtained with the two methods [MFA — Clemmesen (1988, 1993) and CFA — Caldarone and Buckley (1991)] for larvae collected in the Mira and Guadiana rivers (Student *t*-test)<sup>a</sup>

River	d.f.	<i>t</i>	<i>P</i>
Depend. Var.			
Mira	148		
log DNA		− 0.145	0.885 <sup>NS</sup>
log RNA		− 4.339	0.000***
log (RNA/DNA)		− 4.375	0.000***
Guadiana	120		
log DNA		0.723	0.471 <sup>NS</sup>
log RNA		− 1.256	0.211 <sup>NS</sup>
log (RNA/DNA)		− 2.974	0.004***

<sup>a</sup> d.f., degrees of freedom; NS, non-significant; \*\*\**P* < 0.001.

#### 4. Discussion

The two rivers studied herein are the most important watercourses in southern Portugal. They have contrasting hydrological and biological characteristics, namely their extension, discharge, estuarine fauna and anthropogenic impact. Moreover, gobiids are among the most common and representative families of temperate and sub-tropical estuaries, e.g. Mira and Guadiana in Portugal (Costa et al., 1987; Chícharo, 1988). In fact, gobiids are described as resident species in several Portuguese estuaries (Ré et al., 1983; Ré, 1984; Costa et al., 1987; Antunes et al., 1988; Chícharo, 1988). Hence, they can be considered as good indicators of environmental changes occurring throughout their life-cycle.

This study indicated that the RNA/DNA ratios of individual larvae varied with the method of quantification. Without any means of determining which method is the most accurate, inter-calibration of methodologies is important in order to allow gross comparison of results between laboratories or estimates.

Percent recovery of nucleic acids after purification was lower than published results (Clemmesen, 1988; Canino and Caldarone, 1995; Chícharo, 1998; Chícharo et al., 1998a). The lower RNA recovery rates might indicate some RNA loss during the procedure (Caldarone and Buckley, 1991; Canino and Caldarone, 1995), probably due to its smaller molecular size (Clemmesen, 1993), or to variable activity of RNase. In addition, purification of homogenates might have contaminated the samples (Grémare and Vétiou, 1994). On the other hand, the percent recovery of nucleic acids as well as the coefficients of variation obtained with CFA were within the range mentioned by Canino and Caldarone (1995) and Wagner et al. (1998). On the contrary, the variability of determinations when using the MFA was higher than those referred to by Clemmesen (1988, 1993); Chícharo (1997, 1998) and Chícharo et al. (1998a,b). Room temperature did not affect the results and values of CV obtained in this study because the assays were run in an environment with acceptable temperature range ( $\pm 2^\circ\text{C}$ ) as referred to by Caldarone and Buckley (1991). Detection limits were within published values (Clem-

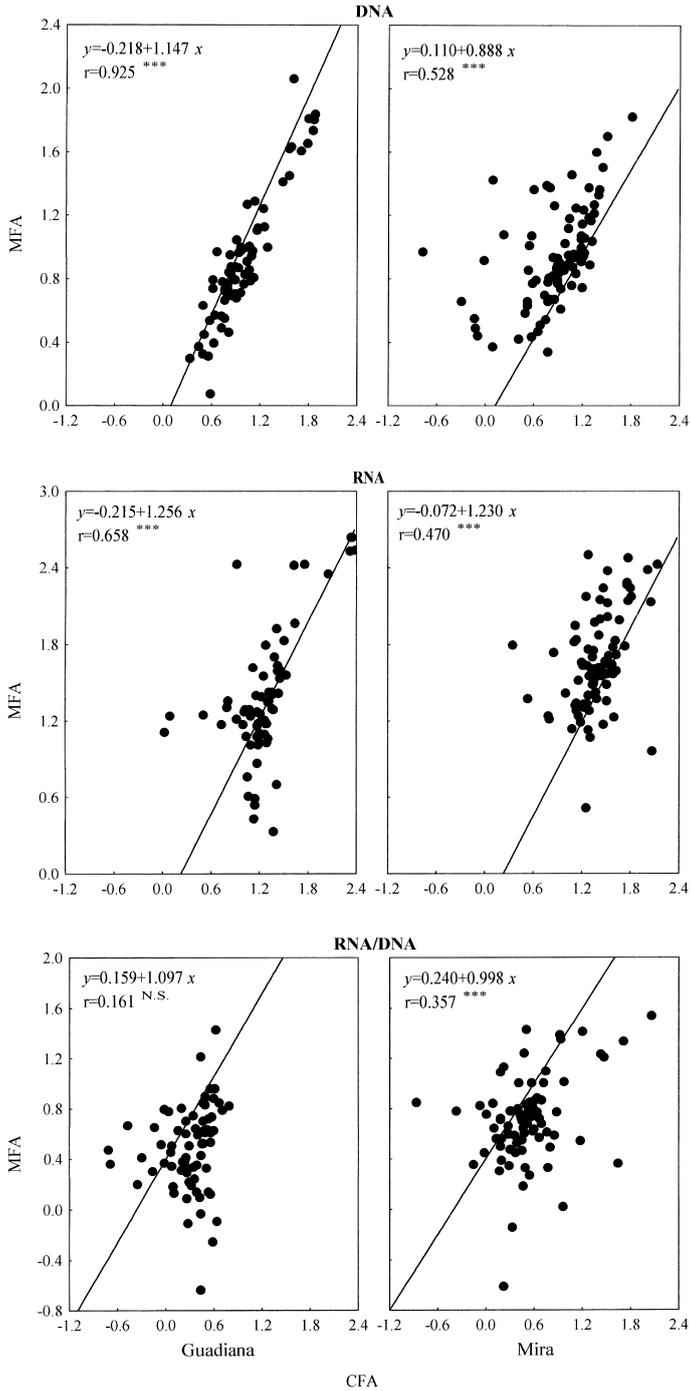


Fig. 5. Scatterplots of paired comparisons between log (nucleic acids concentration) and log (RNA/DNA) values and respective functional relationships and correlation coefficients.

mesen, 1987, 1988; Caldarone and Buckley, 1991; Canino and Caldarone, 1995; Chícharo et al., 1998a).

The two methods yielded similar log DNA values but different log RNA and log (RNA/DNA). The activity of RNase could be partially responsible for the differences observed when comparing the two methods. Clemmesen (1993) and Caldarone and Buckley (1991) reported residual quantities of RNA remaining in the homogenates after RNase digestion,  $\approx 3\%$  and  $< 1\%$ , respectively. These trace quantities seem reasonably low and, according to those authors, do not affect the estimates. Moreover, the purification of homogenates with organic solvents could lead to their contamination, thus increasing the fluorescence of the samples and contributing to an overestimation of nucleic acids contents. On the other hand, larvae were relatively bigger in river Guadiana, perhaps in a more advanced ontogenic stage. This could account for the differences observed in log RNA and log (RNA/DNA). McGurk et al. (1992) analysed differences in nucleic acids contents and RNA/DNA ratios obtained with three different methods. They also found significant differences for  $\ln$  RNA and  $\ln$  (RNA/DNA) which they attributed to the interference of other compounds. These substances could have either absorbed the fluorescence, or contributed to the residual fluorescence or inhibited the kinetics of RNase. Mathers et al. (1994) compared the methods of Munro and Fleck (1966) and Clemmesen (1988) and reported differences for the RNA content in larvae  $< 200 \mu\text{g}$  dry weight. Those authors proposed modifications of the procedure for larvae  $200\text{--}800 \mu\text{g}$  dry weight that overcame the difficulties mentioned by Buckley (1984) for larvae  $< 800 \mu\text{g}$  dry weight. Grémare and Vétion (1994) compared seven fluorimetric methods and stressed the importance of: detergent concentration in the extraction buffer, centrifugation of homogenates, and interference from using organic solvents (chloroform, phenol and isoamylalcohol), namely their effect on the linearity of the fluorophore response. Those authors proposed an alternative protocol, which incorporates these recommendations. Recently, Chícharo (1996) compared the condition of *Sardina pilchardus* larvae using the spectrophotometric method of Schmidt and Tannhauser (1945) and the fluorimetric procedure of Clemmesen (1988, 1993), and found no significant differences in both RNA and DNA contents and RNA/DNA ratios.

Comparison of nucleic acids contents and calibration of the two methods by geometric mean regression analysis provided a reasonable basis for comparison although differences between estimates were evident. The equations obtained in this study can be used to relate the results although they explain only partially the variability of data. Moreover, the differences in the slopes of the equations calculated might indicate that the procedures apply differently to distinct larval populations. In fact, *Pomatoschistus* spp. dominated among the gobiid larvae collected in the Guadiana river (Chícharo, 1988; Esteves, unpublished results), whereas in the Mira river, *Gobius* spp. and *Pomatoschistus* spp. are equally important in ichthyoplankton samples and much less common than in other Portuguese estuarine systems (Costa et al., 1987). It would be interesting to investigate whether these observations are the result of zoological differences or due to a methodological artefact. Canino and Caldarone (1995) compared FIA and CFA by functional regression and found higher correlation coefficients. Unexpectedly, they obtained better fits for RNA, considering that RNA content is calculated indirectly. McGurk et al. (1992); Grémare and Vétion (1994) and Mathers et al. (1994) reported

differences between methods but did not provide parameters for inter-calibration of results.

Although results showed that a correction factor must be applied to compare data derived from the two procedures, both protocols seem to produce biologically reasonable estimates. More sensitive fluorimetric methods assays (e.g. Clemmesen, 1993) require an extensive amount of operator attention and lengthy extraction procedures, thus restricting the number of samples that can be processed in a day (Canino and Caldaroni, 1995). Nevertheless, the protocol of Clemmesen (1988, 1993) is used in many laboratories. On the other hand, relatively simpler methods (e.g. Caldaroni and Buckley, 1991) provide a quick assay of RNA and DNA contents in fish larvae with a very reasonable degree of sensitivity and eliminating the purification step, as recommended by Grémare and Vétion (1994) and Bergeron (1997). Hence, they are appropriate for the analysis of large numbers of field-caught larvae (up to 100 larvae/day).

Different methodologies can lead to different results making direct comparisons of data impossible. Standardisation and inter-calibration of analytical procedures has been encouraged (Clemmesen, 1993). Several authors have compared methodologies for extraction and quantification of nucleic acids (McGurk et al. 1992; Grémare and Vétion, 1994; Canino and Caldaroni, 1995; Chícharo, 1996) and obtained significant differences. Nevertheless, the majority does not provide any means of correcting the results. In this study, equations are derived for the inter-calibration of results between the two fluorimetric methods. The comparison of methodologies at a specific level will limit the range of nucleic acids concentrations and could confirm the results reported herein for gobiids from rivers Mira and Guadiana.

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