An attempt to determine variability of RNA/DNA ratios during *Dicentrarchus labrax* larval development

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RNA and DNA content of seabass (*Dicentrarchus labrax*) larvae were determined from day 10 to day 60 after hatching in an aquaculture facility. Our results show a two-phase modulation of the RNA/DNA ratio throughout seabass larvae development. From day 10 after hatching there is an increase in the RNA/DNA ratio reaching maximal values at day 30 of age. Then, the RNA/DNA ratio decreased reaching minimal values at day 60 after hatching. These results are consistent with a high rate of protein synthesis during the first 30 days of seabass development compared with the rest of the developmental period studied. The results are compared with that of otolith growth carried out for the same species larvae.

Keywords: *marine fish, larvae, DNA/RNA ratio, Dicentrarchus labrax.*

APROXIMACIÓ A LA DETERMINACIÓ DE LA VARIABILITAT DE LA RELACIÓ DNA/RNA DURANT EL DESENVOLUPAMENT LARVAL DEL LLOBARRO *Dicentrarchus labrax.* S'ha determinat el contingut de DNA (àcid desoxiribonucleic) i de RNA (àcid ribonucleic) en larves de llobarro (*Dicentrarchus labrax*) d'edats compreses entre 10 i 60 dies de vida, procedents d'una planta de cultiu. Els resultats obtinguts mostren dues fases diferenciades de desenvolupament d'aquestes larves, basades en la relació RNA/DNA avaluada. S'observa un augment de la proporció de RNA/DNA des del dia 10 fins el dia 30 de vida, per a decréixer a continuació, trobant els valors mínims en les larves de 60 dies de vida.

Paraules clau: *peixos marins, larves, relació DNA/RNA, Dicentrarchus labrax.*

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Introduction

Determination of larval condition has widespread applications in the field of fish ecology, as an indicator of sub-lethal effects. A methodology used to assess larval condition based on the quantification of larval nucleic acids (RNA/DNA) concentration allows to infer growth and nutritional status accurately (Buckley, 1984; McLaughlin *et al.*, 1995). This method is based on the assumption that the concentration of RNA changes as a function of the amount of protein synthesis occurring in the cell whereas DNA concentration indicates the number of cells in an organism (Buckley, 1980; 1984). Therefore, the RNA/DNA ratio is considered a measure of protein synthetic activity per cell (Buckley, 1984; Bulow, 1987; Robinson and Ware, 1988; Hovenkamp, 1990; Hovenkamp and Witte, 1991). In this contribution we applied this technique to asses larval condition during development of a temperate water species reared at controlled conditions (*D. labrax*, L. 1758).

Material and methods

a) Rearing conditions

Larvae were obtained from naturally spawning adult sea bass larvae, hatched from the same spawning and reared at the Es Murterar Aquaculture facility (Mallorca, Spain). The experimental conditions were as follows: sea bass were kept indoors in a 1200-liter tank filled with freely circulating seawater. Initial specimen density was 39 larvae/l. Natural light cycles were maintained. Food (*Artemia* spp) was supplied *ad libitum* and according to the nutritional requirements of the sea bass during the experimental period. Water temperature (measured daily at noon) was kept at 19-20°C and the salinity at 39 psu. Specimens were hatched in February 1996 and were killed at regular ten days intervals. Larvae were measured to the nearest 0.1 mm under a dissecting microscope, dried on blotting paper, and weighed to the nearest 1 µg. The larvae were subsequently frozen in liquid nitrogen.

b) Nucleic acid extraction

Total RNA and DNA were extracted from larvae samples by repeated washing of the homogenised tissue with organic solvents (Sambrook, 1989) with modifications. Briefly, each larvae was homogenised in 1 ml saturated phenol (pH 7.5) plus 100 µl of distilled water, during 30 s using an Ultra-Turrax T25 homogeneizer (IKA, Staufen, Germany) and then incubated for 5 min. at room temperature. Two hundred µl of chloroform were then added to homogenates, vortexed for 30 s, and then incubated at room temperature for 5 min. The samples were centrifuged at 10,000 g for 10 min at 4°C. The aqueous phases were separated and mixed with 500 µl of 100% isopropyl alcohol. The mixtures were briefly shacked, incubated for 10 min at room temperature and then centrifuged at 10,000 g for 10 min at 4°C. The nucleic acid precipitate (DNA plus RNA) was washed twice with 1 ml of 75% ethanol and dissolved in DNAse-RNAse-free water (nucleic acid solution).

c) Enzimatic determination of the relative content of DNA and RNA

This technique is based on the method described by Clemmesen (1988). Two aliquots (termed RNA and DNA aliquots) of the nucleic acid solution were processed in parallel. To the RNA aliquot (5 µl) 5 µl of DNAse solution (Dnase I, Rnase free, 10U/µl Boehringer Mannheim, Germany) and 40 µl of DNAse buffer (40 mM Tris-HCl. PH 7.9, 19 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$) were added. To the DNA aliquot (10 µl), 2 µl of RNAse buffer (Boehringer Mannheim, Germany) 5 µl of RNAse (RNase, Dnase free, 0.5 (g/µl; Boehringer Mannheim, Germany) and 33 µl of nuclease-free water were added. Both samples were incubated during one hour at 37°C. The reactions were terminated by adding 900 µl of nuclease-free water and placing the tubes at room temperature.

d) Quantification of DNA and RNA by fluorescence analysis

DNA and RNA concentration were quantified by a method based on that of Higuchi and Dollinger (1992) originally described for quanti-

fication of amplified DNA sequences. Briefly DNA and RNA aliquots (50 µl) were mixed with water to a final volume of 900 µl. Then, 50 µl or SYBR Green II (FMC BioProducts, Rockland, ME, USA) solution at 1/100 dilution was added. Fluorescence was measured in a fluorescence spectro-fluorometer (model RF-1501; Shimadzu, Japan). For SYBR Green II fluorescence determination excitation was at 490 nm and emission at 520 nm. The amount of fluorescence of each sample was measured and the fluorescence units were evaluated using standard curves which consisted of 5 points of DNA (herring sperm DNA, Sigma Chemical CO. Saint Louis, MO, USA) or RNA (*E. coli* ribosomal RNA, Sigma Chemical CO. Saint Louis, MO, USA) content (1-5 µg, resulting in a linear relationship). The standard curves were performed prior each set of samples fluorescence determinations.

Results

In the aquaculture facility sea bass larvae size increased from 4.5 mm total length (TL) at 10 days of age to 19 mm TL at 60 days following a linear relationship (r = 0.77) (Table 1, Fig.1A). In the other hand, larvae weight increased from 0.48 mg at 10 days of age to 30.67 mg at 60 days reaching a plateau at 50 days (Table 1, Fig. 1B).

The DNA content increase from 2.50 µg at age 10 to 42 µg at age 60, showing a big increase between 40 and 50 days (Table 1; Fig. 2 A). The RNA content increase from 2.6 µg at age 10 to 32.68 µg at age 50, showing a dcrease at age 60 (21.7 µg) (Table 1; Fig. 2 B).

The RNA/DNA ratio was 1.04 initially, increasing to near 2 at day 30. Then the RNA/DNA ratio decreased reaching minimum values (0.5) at day 60 (Table 1, Fig. 2 C). Focusing in these results we consider two age groups, 10 to 40 days and older. The non-parametric Mann-Whitney test showed significant differences on the RNA, DNA and RNA/DNA content between those groups (Table 2, Fig. 2 C).

Discussion

RNA/DNA ratios are considered as useful independent growth measure of individual fish larvae (Buckley, 1984). DNA of individual cells is fairly constant, so the DNA content of whole animals increases proportional to increases in cell number (growth). However, RNA content of cells is variable, and reflects active protein synthesis. RNA/DNA ratios have been found to be accurate indicators of recent feeding of larvae, higher ratios indicate better feeding condition (Canino, 1994).

First our results show an increase of the RNA/DNA ratio from 1.04 at 10 days of development to 1.86 (0.29 at day 30 of development (Table 1; Fig. 2 C). Clemmesen (1989) proposed that the RNA/DNA ratio of 1 is an indicator of a critical level of feeding conditions. In the acua-

Age	Mean length (mm)	Mean weight (mg)	Mean DNA (µg)	Mean RNA (µg)	Mean RNA/DNA	n
10	4.50	0.48	2.50	2.6	1.04	1
20	9.06 (0.70)	2.11 (0.22)	9.53 (6.96)	12.62 (8.27)	1.67 (1.02)	5
30	10.80 (0.84)	5.70 (1.07)	14.18 (5.09)	25.56 (7.63)	1.86 (0.29)	5
40	14.33 (1.15)	9.67 (5.04)	12.76 (7.96)	19.53 (15.45)	1.44 (0.30)	3
50	17.50 (0.58)	32.93 (3.72)	38.84 (12.17)	32.68 (11.77)	0.90 (0.34)	4
60	19.00 (1.00)	30.67 (8.27)	42.00 (17.17)	21.70 (7.68)	0.53 (0.04)	3

Table 1. Summary of the data analysed on *Dicentrarchus labrax* reared larvae in relation to age (days from eclosion).s.d. in brackets. n : number of fish analysed.

Taula 1. *Resum de les dades analitzades de les larves cultivades de* Dicentrarchus labrax *en relació a l'edat (dies després de l'eclosió), desviació estàndard entre parèntesi, n : nombre d'exemplars analitzats.*

Table 2. Results of the Mann-Whitney test on the *Dicentrarchus labrax* larvae. n : number of fish. s.d. : standard deviation. p :

Taula 2. *Resultats del test de Mann-Whitney sobre les larves de* Dicentrarchus labrax. *n: nombre de peixos. s.d.: desviació estàndard. p: probabilitat.*

	Age (days)	n	mean	s.d.	p
DNA (µg)	10-40	14	11.38	6.59	0.0001
	>40	7	40.20	13.23	
RNA (µg)	10-40	14	18.00	11.29	0.07
	>40	7	27.98	11.1	
RNA/DNA	10-40	14	1.64	0.64	0.003
	>40	7	0.74	0.31	

culture facility seabass larvae started exogenous feeding on age 15-16 days (*Artemia* spp.). Our RNA/DNA ratio data suggest that seabass larvae in captivity are at the level of critical feeding during yolk utilisation, but improving rapidly once exogenous feeding started and follows a continuous improvement in feeding condition reaching its maximum at day 30 of development. The critical condition demonstrated by larvae before the exogenous feeding started may be explained by the fact of its high rate of RNA synthesis (indicating protein synthesis) as its shown in Figure 2 B. Protein synthesis is a high energy-requiring process that rapidly exhaust yolk energetic reserves. Once the abundant and more energetic exogenous feeding started, the high ratio of protein synthesis can be maintained without energy deficit and the larvae rapidly improve its condition and size (Table 1; Fig. 1 A). An increase of RNA/DNA with larval size has been also reported previously on herrings (Clemmesen, 1994).

In the other hand, our results clearly show that beyond day 30 of development, the RNA/DNA ratio diminish steadily reaching low values (lower than the critical ratio of 1) at day 50 (0.90 ± 0.34) and even lower at day 60 (0.53 ± 0.04) (Table 1; Fig. 2 C). Seabass initiate metamorphosis precisely between 50-60 days of development. This, correlates with the observed slow down of larvae weight (Table 1; Fig. 1B) and

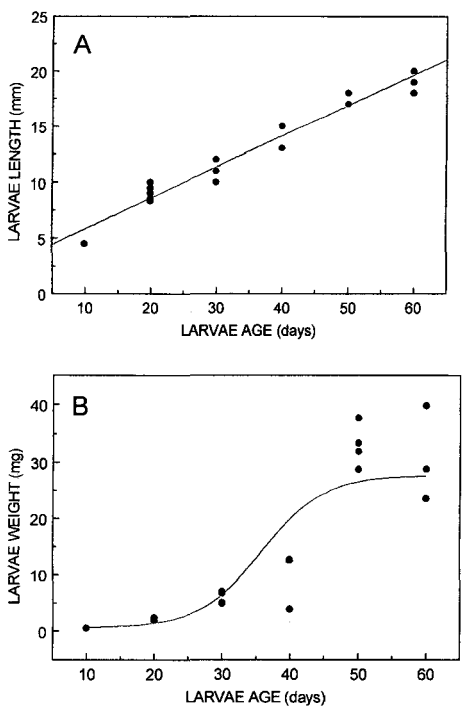

Fig. 1. A- Linear relationship (r = 0.77) between total length (mm) and age (days after hatching) of sea bass (*D. labrax*) larvae from aquaculture facility. B- Relationship between total weight (mg) and age of sea bass larvae from aquaculture facility, reaching a plateau at day 50.

Fig. 1. *A- Relació lineal (r = 0.77) entre longitud total (mm) i edat (dies després d'incubació) de llobarro (*D. labrax*) larves d'aquicultura. B- Relació entre pes total (mg) i edat de larves de llobarro d'aquicultura, atanyent una pendent horitzontal el dia 50.*

active protein synthesis (RNA/DNA ratio). Stage-dependent RNA/DNA ratios have been reported in sole (Richard *et al.*, 1991). Thus, our larvae were undertaking a critical stage corresponding to the end of metamorphosis with low RNA/DNA ratios despite the increase in larval size. This indicate that metamorphosis is a critical period in seabass development in terms of feeding conditions (as indicated by RNA/DNA ratios) probably by the increased metabolic expenses due to increased mobility and body changes (formation of scales, etc).

Daily growth increments of the otolith is another method of assessing larval growth and condition (e. g. Govoni *et al.*, 1985, Suthers *et al.*, 1996). Our results of RNA/DNA ratio as individual seabass larvae growth measure are in concordance with available data on otolith growth (Gutiérrez and Morales-Nin, 1986), that demonstrated in seabass larvae reared in an aquaculture facility a similar biphasic pattern in the thickness of the daily growth increments.

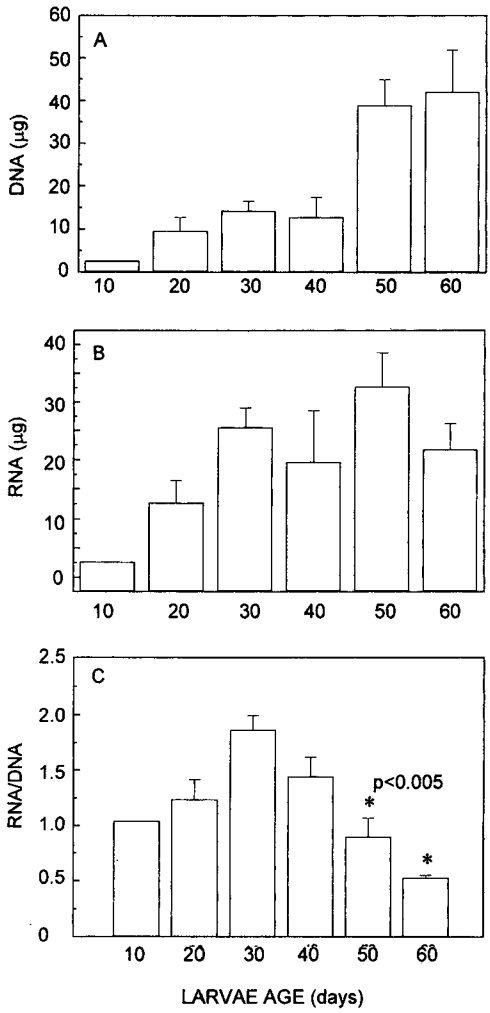

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Fig. 2. A.- DNA content (µg) of fed sea bass (*D. labrax*) larvae in relation to larval age (days after hatching). B.- RNA content (µg) of fed sea bass larvae in relation to larval age. C.- RNA/DNA ratio of fed seabass larvae in relation to larval age. Asterisks refers to significant differences between age groups (Mann-Whitney U-test, p<0.005). ***Fig. 2.*** *A. Contingut d'ADN (µg) de larves de llobarro (*D. labrax*) engreixades en relació a l'edat larval (dies després d'incubació). B.-contingut d'ARN (µg) de larves alimentades en relació a l'edat larval. C.-RNA/DNA proporció de larves de llobarro alimentades en relació amb l'edat larval. Els asteriscs indiquen diferències significatives entre categories d'edat (Mann - Whitney U-test, p < 0.005).*

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