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The DNA/RNA ratio as a useful indicator of the nutritional condition in juveniles of *Ruditapes decussatus**

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SUMMARY: Condition indices have been widely used to assess how healthy individuals are under certain circumstances. It has been shown that the RNA/DNA ratio is a good indicator of the nutritional condition of several marine organisms. A very sensitive fluorometric method for RNA/DNA quantification (Clemmesen, 1990) was applied to fed and starved post-larvae laboratory-reared clam, *Ruditapes decussatus*. The presented study shows that the RNA/DNA ratio is a useful indicator of nutritional stress for bivalve spat. The decrease in RNA/DNA ratio recorded here, under starved conditions was lower than in similar experiments on fish. A lower metabolic rate in clams may result in a slower response with respect to RNA/DNA ratios than that observed in vertebrates.

Key words: Bivalves, nutritional condition, RNA/DNA, *Ruditapes decussatus*

RESUMEN: LA RELACIÓN RNA/DNA COMO UN INDICADOR DEL ESTADO NUTRICIONAL DE JUVENILES DE *RUDITAPES DECUSSATUS*. – Ha sido demostrado que la relación RNA/DNA es un buen indicador del estado nutricional de varios organismos marinos. Se ha aplicado una técnica fluorimétrica muy sensible para la quantificación de la relación RNA/DNA (Clemmesen, 1990) en juveniles de almeja *Ruditapes decussatus*, cultivados en laboratorio, con y sin alimento En el presente trabajo se muestra que esta relación también es muy útil para indicar la condición nutricional de bivalvos juveniles. La disminución de la relación RNA/DNA que ha ocorrido, en condiciones de inanición, fué menor que la encontrada en experiencias similares con peces y bivalvos adultos. La menor tasa metabólica de las almejas pudiera ser la explicación.

Palabras clave: Bivalvos, estado nutricional, RNA/DNA, *Ruditapes decussatus*.

INTRODUCTION

Determination of the nutritional condition using the RNA/DNA ratio has been conducted on a wide range of marine organisms, but mainly on fish (Bullow, 1970; Buckley, 1984 and Robinson and Ware, 1988) and crustaceans (Anger and Hirche, 1990), Application of the method to bivalves (Holland and Gabbott, 1971 and Wright and Hetzel, 1985) has been limited, especially concerning post-larvae. The use of 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) is directly involved in protein synthesis and by inference, with nutritional condition. Consequently the RNA/DNA ratio should be a good indicator of the condition of the individuals from wild populations. Nevertheless a previous laboratory calibration is necessary, which is the aim of this work.

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An attempt was made to study how nutritional stress the RNA/DNA ratio. Is very important to know whether a juvenile *Ruditapes decussatus*, captured in the field, is in nutritional stress, and therefore more susceptible to negative influences of the environment, e.g. pollution or other factors. The RNA/DNA ratio is an ecophysiological condition index, which summarises the physiological activity of animals (growth, reproduction, secretion, etc.) under given environmental conditions (Lucas and Beninger, 1985).

MATERIALS AND METHODS

The juveniles used in this work were obtained from a hatchery. Individuals were kept in seawater, previously filtered through a 1 µm filter system and sterilised with UV light. Seawater temperature was maintained at 20-23 °C. Initially, bivalves were split into two groups by their dimensions: i) 300-600 µm and ii) 600-3200 µm. Each group was split again into another two, one held in starved conditions and the other fed with the algae *Tetraselmis suecica*. After 23 days, a high mortality occurred among the starved groups and the experiment ended. Twelve individuals of each group were taken every day and then measured and weighed on a microbalance. After that, the bivalves were placed in liquid nitrogen. Nucleic acids were extracted from whole juveniles of *Ruditapes decussatus* by homogenising the frozen tissue for 1 min. in 400 µl of ice-cold tris buffer containing sodium dodecly sulphate (SDS) and using a sonicator. A very sensitive fluorometric method for RNA/DNA quantification in individual organisms was applied to fed and starved laboratory reared bivalves. A more conventional technique, such as the spectrophotometric method, (based on Schmidt and Thannhauser (1945) and modified by Munro and Fleck (1966)), required a minimum of 800 mg dry weight per sample (Buckley, 1984), so pooled samples had to be analysed and thus estimates of individual variability were impossible.

The analytical procedure (Fig. 1) was adapted from the methodology presented by Clemmesen (1990) for fish larvae, which involves purification of tissue homogenates and subsequent fluorescence-photometric measurements using ethidium bromide, a specific nucleic acid dye. The homogenate was shaken for 15 min. on a mixer. After 15 min. of centrifugation (6000 rpm) the supernatant was transferred to a new vial and 300 µl of tris-NaCl buffer saturated phenol (80%) and 300 µl of chloroform/isoamyalcohol (24:1 v/v) were added. After shaking for 10 min. and centrifuge for the same time, the resulting aqueous nucleic acid phase (top layer) was taken and transferred to a new vial and the phenol-chloroform/isoamyalcohol phase discarded. To eliminate the phenol traces in the aqueous nucleic acid solution the samples were washed with 300 µl of chloroform/isoamyalcohol then shaken for 5 min. and the aqueous phase was harvested by 5 min. of centrifugation. Fluorophor ethidium bromide (EB) was used to determine both RNA and DNA fluorescence of the sample. The ribonuclease A was used to distinguish between DNA and RNA fluorescence. After adding 20 µl of the EB stock solution (0.1 mg/ml in distilled water) to an aliquot of the sample the fluorescence was measured. Another aliquot of the sample was treated with ribonuclease A incubated at 37 °C for 30 min., cooled to room temperature and the fluorescence measured after the addition of EB. The fluorescence due to total RNA

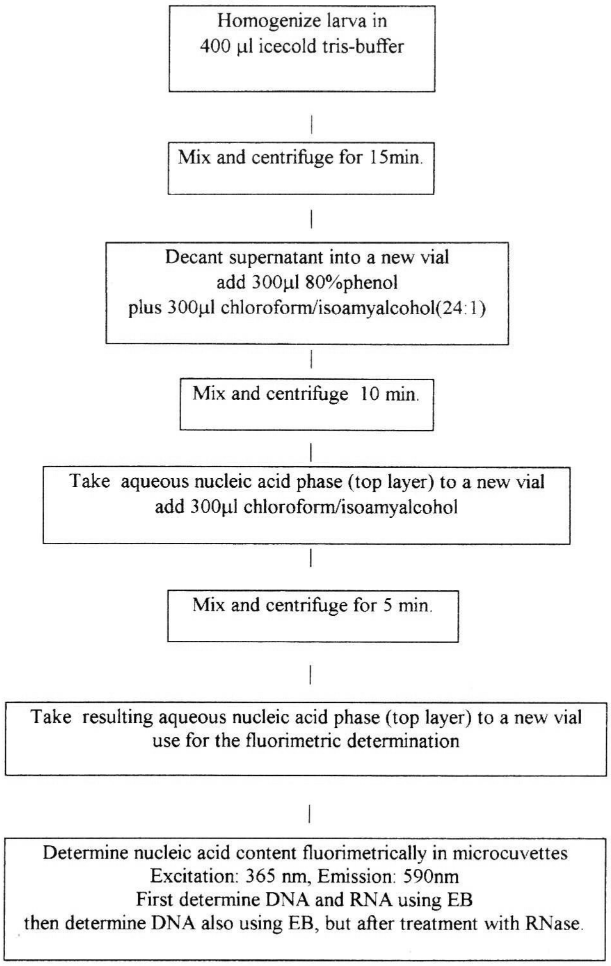

FIG. 1. – Flowchart of the analytical procedure.

(ribosomal and messenger) was calculated as the difference between total fluorescence (RNA and DNA) and the fluorescence after ribonuclease treatment, which is assumed to be due to DNA, after subtraction of self-fluorescence of the enzyme. Fluorescence was determined by exciting at 365 nm and reading at 590 nm on a Hitachi Spectrofluorometer. Concentrations of nucleic acids were determined by using standard curves of DNA and RNA with EB built up with known concentrations of calf DNA and yeast RNA, in the appropriate range of value.

A small modification of Clemmesen's methodology was made in order to be able to work with the small amounts of tissue presented by the bivalve post-larvae. The concentration of SDS in the Tris-buffer was reduced from 2% to 0.5% due to the interference in the assays when higher concentrations were used. The final volumes were also reduced from 600 µl to 220 µl and no dilutions were done in the final aqueous nucleic acid phase in order to allow the readings of such small amounts of nucleic acids. Average recovery efficiency was 74.7% (SD=2.1%) for RNA and 65.5% (SD=2.9%) for DNA. Total amounts of RNA and DNA 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 two standard deviations of the blank) was 0.123 µg for RNA and 0.032 µg for DNA. The coefficient of variability (sample standard deviation as percentage of the mean) was 4.6% for RNA and 3.5% for DNA when 10 aliquots of tissue homogenate were measured.

RESULTS

Results indicate that clams kept in starved conditions showed a decrease in the ratio of RNA to DNA compared with fed clams (Fig. 2). This decrease was more obvious in the group of 600-3200 µm than in the group of 300-600 µm. Nevertheless only 11% of all the starving bivalves showed RNA/DNA ratios higher than 1.5, and only 10% of the fed post-larvae showed ratios lower than 1, which seems to indicate that the "critical level" of the RNA/DNA ratios in *Ruditapes decussatus* post-larvae will be around 1-1.5, but a more intensive study is necessary in this field. The exponential relation between the amount of RNA per bivalve and its length on fed post- larvae is described by the equation (Fig. 3):

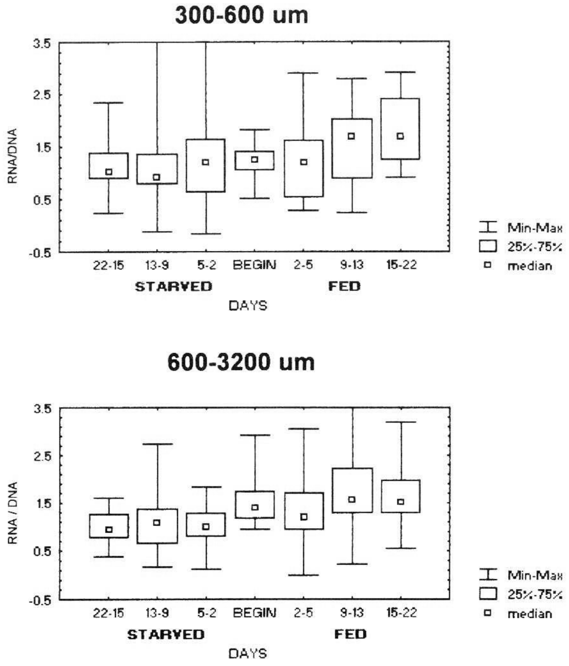

FIG. 2. – Evolution of the RNA/DNA ratios during the experiment. Increasing days are counted from the beginning of the experiment (day 0) to the left (starved)and to the right (fed).

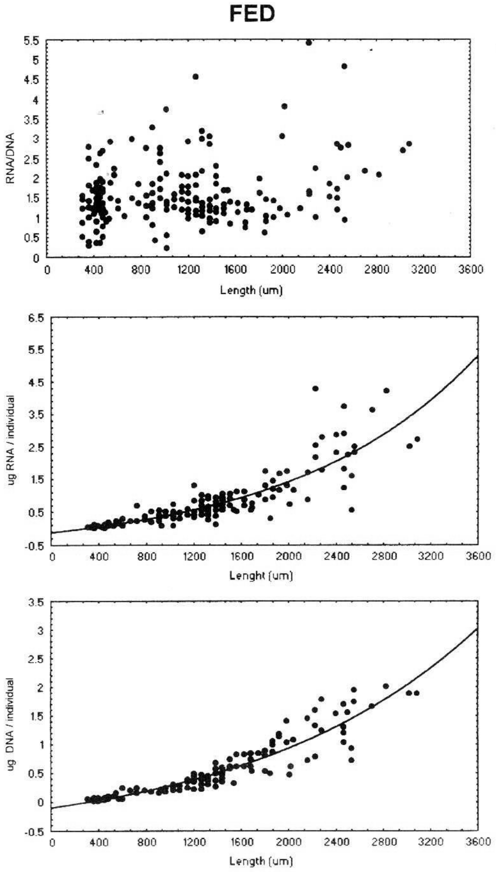

FIG. 3. – Relations between the RNA/DNA ratios vs. length (top graph), µg RNA/individual vs. length (middlegraph) and µg DNA/individual vs. length (bottom graph) of fed *Ruditapes decussatus* post-larvae.

$$y=-0.70208+\exp(-0.534+(0.000647)*x)$$
(n=214, r=0.88 and variance explained:77.28%)

and for starved post-larvae (Fig.3):

$$y=-0.57883+\exp(-0.6389+(0.000510)*x)$$
(n=271,r=0.89and variance explained:79.01%)

The amount of DNA versus individual length for fed bivalves was described by the equation (Fig. 3):

$$y=-0.64128+\exp(-0.598+(0.000527)*x)$$
(n=214, r=0.94,and variance explained:89.18%)

and for starved post-larvae (Fig. 4):

$$y=-0.55665+\exp(-0.70401+0.000541)*x)$$
(n=271,r=0.98,and variance explained:94.44%)

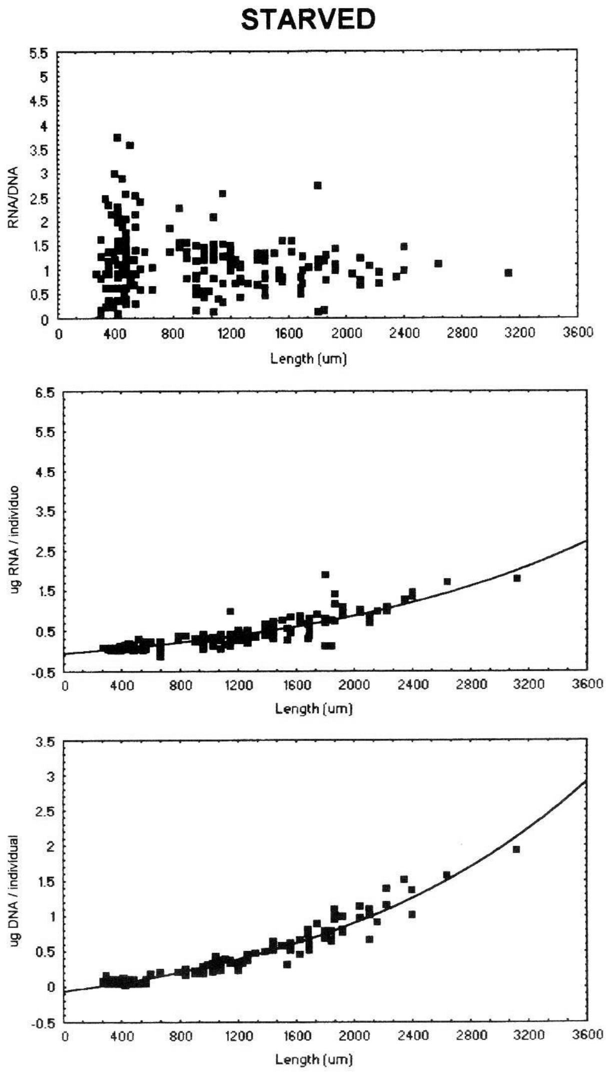

FIG. 4. – Relations between the RNA/DNA ratios vs. length (top graph), µg RNA/individual vs. length (middle graph) and µg DNA/individual vs. length (bottom graph) of starved *Ruditapes decussatus* post-larvae.

The Mann-Whitney non-parametric test showed that there was a significant difference ($p<0.05$) between the RNA/DNA ratios of starved and fed groups. The differences in the amounts of RNA were significantly different only for the 600-3200 µm group, and no significant differences were found when the amount of DNA was compared among these 2 groups (Fig. 5 and 6).

DISCUSSION

The exponential relation between the amount of RNA per bivalve and its length was more obvious in the fed group, while the amount of DNA versus individual length was not different between the two groups. These results are in agreement with the characteristics of these two macromolecules. In fact the amount of DNA per individual, as the carrier of genetic information, showed a similar pattern when related to the length in fed and starved conditions. The amount of RNA per individual, depending on the physiological condition of individual post-larvae, showed a faster increase in length in fed conditions than in starved conditions. To be independent of the length of the organisms, the RNA/DNA ratio has been recommended (Buckley,1984) to analyse and compare the nutritional state of individuals with different dimensions.

The decrease in RNA/DNA ratio recorded here was lower than that previously observed in similar experiments on fish and adult bivalves. In this experiment the magnitude of the decrease was 28.88 % in the group of 300-600 µm and 32.26% in the group of 600-3200 µm, after 22 days of starvation. Bullow (1970) in his study of golden shiners, found a 33-35% decrease in RNA/DNA ratio after 14 days, and 56% after 45 days of starvation. According to Wright and Hetzel (1985), starvation of oysters for 55 days resulted in a 36.5% decrease in RNA/DNA ratios. A slower response with respect to RNA/DNA ratios by bivalves may probably be related to a lower metabolic rate. In this experiment there was a smoother decrease in RNA/DNA of the smaller bivalves (300-600 µm) which may be related to the fact that these are newly settled and may have some energetic reserves from the larval stages still available. Although the 1 µm filtration was rigorously monitored it is likely that the clams in this medium derived nutrients through absorption

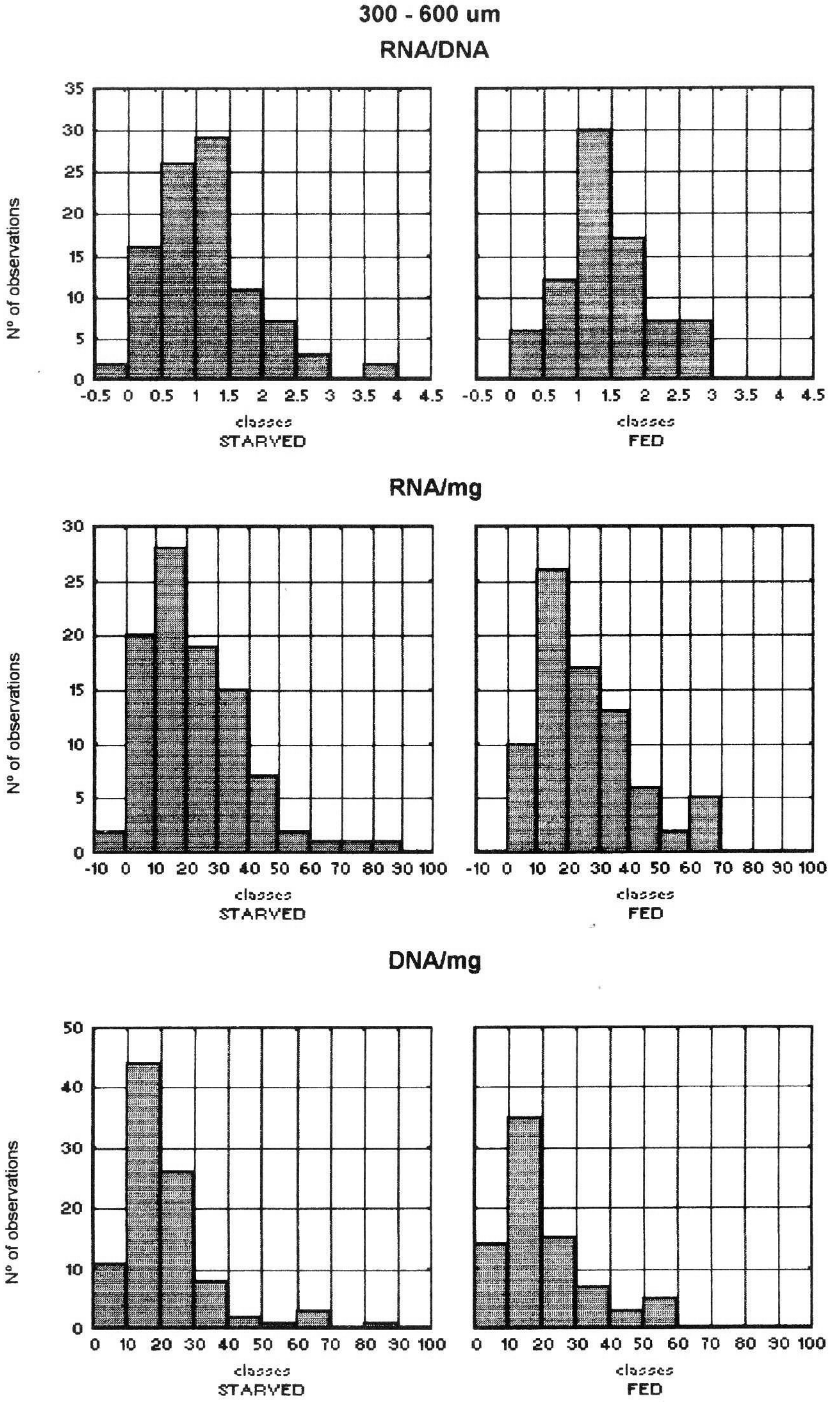

FIG. 5. – Distribution of the RNA/DNA ratios (top graph, Mann-Whitney test U=2834, p=0.0041), µg RNA/individual (middle graph, Mann-Whitney test U=3165, p=0.063) and µg DNA/individual (bottom graph, Mann-Whitney test U=3649, p=0.6692) in classes for starved and fed *Ruditapes decussatus* post-larvae (300-600 µm).

of dissolved organics and filtration of particulates less than 1 µm in size. The RNA/DNA ratio, besides providing information about the nutritional condition of an organism, has also shown potential for assessing instantaneous growth rate, particularly in studies of larvae and juveniles (Buckley, 1984) and for the detection of pollution stress on organisms (Barron and Adelman, 1984).

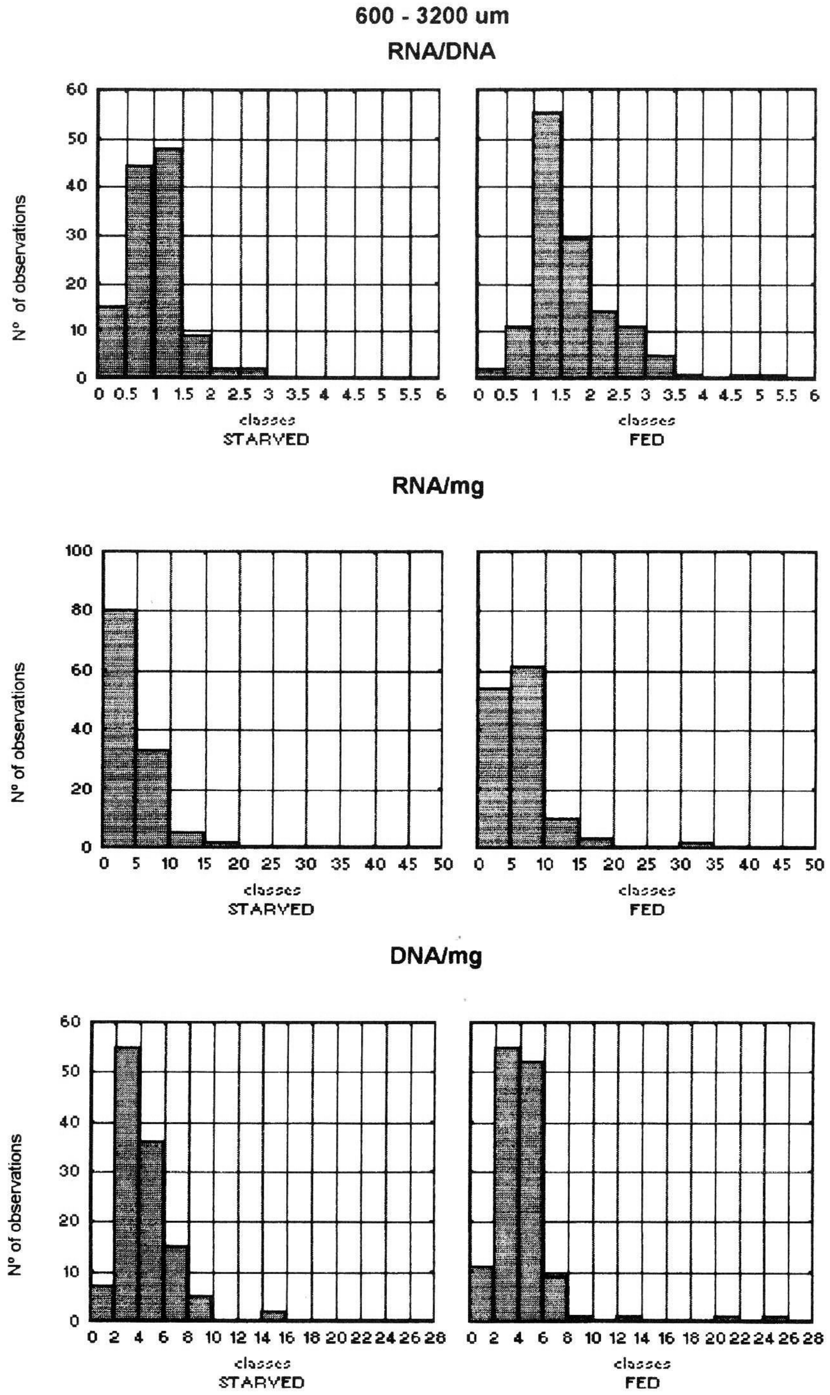

FIG. 6. – Distribution of the RNA/DNA ratios (top graph, Mann-Whitney test U=3507, p=0.0000), μg RNA/individual (middle graph, Mann-Whitney test U=4974, p=0.000) and μg DNA/individual (bottom graph, Mann-Whitney test U=7636, p=0.6973) in classes for starved and fed, *Ruditapes decussatus*, post-larvae (600-3200 μm).

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REFERENCES

Anger, K. and H.-J. Hirche. – 1990. Nucleic acids and growth of larvae and juvenile spidercrab, *Hyas araneus*. *Marine Biology*, 705: 403-411.

Barron, G. and I. Adelman. – 1984. Nucleic acid, protein content, and growth of larval fish sublethally exposed to various toxicants. *Can. J. Fish. Aquat. Sci.* , 41: 141-190.

Buckley, L. – 1984 . RNA/DNA ratio: an index of larval fish growth in the sea. *Mar. Biol.* , 80: 291-298.

Bullow, J. – 1970. RNA/DNA ratios as indicators of recent growth rates of fish. *J. Fish. Res. Board Can.*, 27: 2343-2349

Clemmesen, C. – 1990. Improvements in the determinations of RNA and DNA content in individual marine fish larvae. *ICES, CM Biol. Oceanogr. Committee*, 98, 14 p.

Holland, D. and P. Gabbott. – 1971. A micro-analytical scheme for determination of protein, carbohydrate, lipid and RNA levels in marine invertebrate larvae. *J. mar. biol.Ass. U. K.*, 51: 659-668.

Lucas, A. and P. Beninger. – 1985. The use of physiological condition indices in marine bivalve aquaculture. *Aquaculture*, 44: 187-200.

Munro, H. N. and A. Fleck. – 1966. The detection of nucleic acid. In: *Glick*, D. (ed) *Methods of biochemical analysis*. Vol. 17. Interscience Publishers, N. Y., pp. 113-176.

Robinson, S. M. and D. Ware. – 1988. Ontogenetic development of growth rates in larval Pacific herring, Clupea harengus pallasi, measured with RNA/DNA ratios in the Strait of Georgia, British Columbia. *Can. J. Fish. Aquat. Sci.*, 45: 1422-1429.

Schmidt, G. and S. J. Thannauser. – 1945. A method for the determination of deoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissue. *J. Biol. Chem.*, 161: 83-89.

Wright, D. and E. Hetzel. – 1985. Use of RNA:DNA ratios as an indicator of nutritional stress in the American oyster Crassostrea virginica. *Mar. Ecol. Prog. Ser.* ,25: 199-206.