View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by OceanRep

© 1988 Verlag Paul Parey, Hamburg und Berlin
ISSN 0341-6836

A RNA and DNA fluorescence technique to evaluate the nutritional condition of individual marine fish larvae

Catriona M. Clemmesen

Institut für Meereskunde, Kiel

Ms. received 28. 12. 1987
Ms. accepted 1. 9. 1988

Communicated by Walter Nellen

Abstract

It has been shown that the RNA/DNA ratio is a good indicator of the nutritional status of fish larvae. The presented analytical procedure involves purification of fish larvae tissue homogenates and subsequent fluorescence-photometric measurement using specific nucleic acid fluorescent dyes. Ethidium bromide is used to determine DNA and RNA together, bisbenzimidazole (Hoechst 33258) reacts specifically with DNA. Concentrations as low as 0.1 µg/ml (approximately the amount of nucleic acids in a 25 µg dry weight herring yolk sac larvae) can be measured with these fluorescent dyes.

Treatment of samples with ribonuclease A and subsequent fluorescence measurements using ethidium bromid was compared with DNA-determinations using bisbenzimidazole. The bisbenzimidazole procedure was given preference, since it resulted in better reproducability.

With the presented method the physiological condition of individual larvae and the amount of variability can be determined.

Kurzfassung

Beschreibung einer RNA- und DNA-Fluoreszenz-Methode zur Abschätzung des Ernährungszustandes von individuellen marinen Fischlarven

Das RNA/DNA-Verhältnis hat sich als eine geeignete Größe zur Bestimmung des Ernährungszustandes von Fischlarven erwiesen. Die hier vorgestellte Methodik zur RNA- und DNA-Analytik beinhaltet die Reinigung von Fischlarven-Gewebehomogenaten und die anschließende fluoreszenzphotometrische Messung mit nukleinsäurespezifischen Fluoreszenzfarbstoffen. Die gleichzeitige Bestimmung von RNA und DNA erfolgt mit Ethidiumbromid. Für den separaten Nachweis von DNA wird Bisbenzimidazol (Hoechst 33258) verwendet. Mit diesen beiden Fluoreszenzfarbstoffen können sehr geringe Nukleinsäurekonzentrationen im Bereich von 0,1 µg/ml gemessen werden. Diese Nukleinsäuremengen entsprechen etwa den Gehalten in Hering-Dottersacklarven bei einem Fischlarven-Trockengewicht von ca. 25 µg.

Ein Vergleich zwischen der Behandlung der Proben mit Ribonuklease A bei anschließender Fluoreszenzmessung mit Ethidiumbromid und der DNA-Bestimmung unter Verwendung von Bisbenzimidazol wurde durchgeführt. Wegen der besseren Reproduzierbarkeit wurde die DNA-Bisbenzimidazol-Messung bevorzugt.

Die hier vorgestellte Methode ist geeignet, den physiologischen Zustand von individuell gemessenen Fischlarven zu bestimmen. Damit ist es jetzt möglich, die individuelle Variabilität und die Streuung innerhalb einer Gruppe zu erfassen.

Resumen

Un método de fluorescencia para la determinación de RNA y DNA con el fin de evaluar el estado nutricional individual de larvas de peces marinos

Ha sido demostrado que la relación RNA/DNA es un buen indicador del estado nutricional de larvas de peces. En el presente trabajo es presentado un método de análisis que incluye la purificación de tejidos homogenizados de larvas de peces para efectuar posteriormente la medición de RNA y DNA a través de técnicas fotométricas-fluorescentes, usando colorantes fluorescentes específicos para ácidos nucleicos. Para determinar DNA y RNA en conjunto fue usado ethidiumbromid, mientras que

U.S. Copyright Clearance Center Code Statement: 0341-6836/88/3202/0134 $ 02.50/0

bisbenzimidazol (Hoechst 33258) reacciona específicamente con el DNA. Bajas concentraciones, de hasta 0.1 µg/ml (valor equivalente a la concentración de ácidos nucleicos encontrada en una larva con saco vitelino de arenque de 25 µg de peso seco), pueden ser medidas con estos colorantes fluorescentes.

Los resultados obtenidos con muestras tratadas con ribonucleasa A, en las que posteriormente se midió la fluorescencia usando bromuro de ethidio, fueron comparados con las estimativas de DNA usando bisbenzimidazol. Este último fue preferido ya que se logró una mejor reproductividad de los resultados.

El método aquí descrito permite estimar las condiciones fisiológicas individualmente, así como la variabilidad existente dentro de un determinado grupo de larvas.

Introduction

Understanding the variability in recruitment is essential to the evaluation of the dynamics of fish populations. It is assumed that predation and/or starvation may be major factors causing the high mortality during the larval stages (CUSHING 1975; HUNTER 1976; LASKER 1975; MAY 1974; NELLEN 1986). A means for diagnosing the nutritional status of fish larvae might therefore help in elucidating larval survival and possibly predicting subsequent year classes.

Nucleic acids play a major role in growth and development. The amount of desoxyribonucleic acid (DNA), the carrier of genetic information, is stable under changing environmental situations and has been used as an indicator of biomass (DORTCH et al. 1983) and cell number (REGNAULT and LUQUET 1974). Ribonucleic acid (RNA) is directly involved in protein synthesis. Consequently larval growth is dependent on the amount of available RNA. The relationship between RNA and DNA is an index of the cell's metabolic intensity. It has been shown that the RNA/DNA ratio is a useful indicator of the nutritional condition and has been used in several larval fish studies. Starved larvae had lower RNA/DNA ratios than fed larvae and the RNA/DNA ratio showed a linear decrease with the length of the starvation interval (BUCKLEY 1980, 1981, 1984; BUCKLEY and LOUGH 1987; CLEMMESEN 1987; MARTIN et al. 1985). The particular value of the RNA/DNA method lies in detecting early stages of starvation. This is important, since it is assumed that weak or moribund larvae may be rapidly eliminated by predation.

For the extraction and quantification of fish nucleic acids the SCHMIDT-THANNHAUSER method (1945) as modified by MUNRO and FLECK (1966) has been used. BUCKLEY (1979) adapted the procedure to measure microquantities of RNA and DNA present in larval fish and eggs. However this technique has its limitations, since larvae less than 800 µg dry weight must be pooled to provide sufficient tissue for analysis. Pooled samples only allow the estimation of the nutritional status of a population, individual variability and distribution cannot be determined. This might be essential for detecting starving individuals to determine whether starvation plays a major role in the recruitment process. Therefore a more sensitive fluorometric method for RNA and DNA quantification in individual fish larvae was developed.

Materials and methods

Chemicals

All chemicals used in the procedures were analytical grade. Ethidium bromide (EB) yeast RNA and ribonuclease A (from bovine pancreas 65 Kunitz units/mg) were obtained from Serva, Heidelberg, West Germany. Calf thymus DNA and bisbenzimidazole (Hoechst 33258) were purchased from Sigma chemicals, West Germany. Proteinase K and sodium dodecyl sulfate (SDS) were obtained from Merck, Darmstadt, West Germany. Ribonuclease A was dissolved in Tris-NaCl buffer (pH 7.5) at a final concentration of 0.02 mg/ml. The DNA and RNA solutions were spectrophotometrically assayed at 260 nm in Tris-EDTA buffer pH 9.0.

Extraction of nucleic acids

Based on an extraction and purification method described by MANIATIS et al. (1982) the following method was developed. Individual fish larvae were homogenized in a Potter Elvehjem microhomogeniser at 0 °C using 0.3 ml of 0.05 M Tris-HCl buffer containing 0.1 M NaCl, 0.01 M EDTA, pH 9.0 and 0.2 mg/ml proteinase K. The complete homogenate was transferred to a 1.5 ml capped vial, SDS at a final concentration of 2 % was added. The sample was shaken for 15 min on an Eppendorf mixer. After 15 min of centrifugation (6000 rpm) the supernatant was decanted into a new vial and 0.3 ml of 80 % phenol and 0.3 ml chloroform/isoamylalcohol (24 : 1) were added. After shaking for 10 min at 20 °C the aqueous phase was harvested by centrifugation and the phenol-chloroform/isoamylalcohol phase was discarded. This procedure was carried out twice. To eliminate the phenol traces in the aqueous nucleic acid solution the samples were washed twice with chloroform/isoamylalcohol (see flow chart of analytical procedure for further details Fig. 1). Finally 0.2 ml of Tris-buffer were added to the purified nucleic acid solution.

Fluorescence assay

The fluorometric assay of DNA and RNA depends on the use of specific fluorophors which react with nucleic acids and result in an increase in fluorescence intensity. The fluorophor ethidium bromid (EB) (KARSTEN and WOLLENBERGER 1972, 1977; LE PECQ and PAOLETTI 1966; PRASAD et al. 1972) was used to determine both RNA and DNA. It is an intercalating agent which specifically reacts with base-paired regions of DNA and RNA. Bisbenzimidazole is a DNA specific fluorescence dye, since it preferably reacts with adenine-thymine base areas, which only occur in DNA and has been used on DNA solutions, cell and tissue homogenates (BRUNK et al. 1979; CESARONE et al. 1979; DEFLAUN et al. 1986; LABARCA and PAIGEN 1980).

Two approaches to determine DNA and RNA separately were tested. The first trial dealt with the use of EB to determine DNA and RNA together followed by a second independent fluorescence measurement using a specific DNA fluorophor (bisbenzimidazole). The DNA concentration determined with bisbenzimidazole via a bisbenzimidazole calibration curve was used to calculate the EB – fluorescence due to DNA. The EB – fluorescence due to RNA was estimated by subtracting this value from the total fluorescence (DNA and RNA). The RNA concentration was then calculated from the RNA-EB calibration curve.

The second approach used ribonuclease A to distinguish between DNA and RNA fluorescence. The fluorescence of DNA and RNA in a sample is measured. An aliquot of the sample is treated with ribonuclease (0.02 mg/ml), incubated at 37 °C for one hour, cooled to room temperature and the fluorescence measured. The fluorescence due to RNA was then calculated as the difference between total fluorescence (RNA and DNA) and fluorescence after ribonuclease treatment, which is assumed to be due to DNA.

After adding 0.05 ml of the EB stock solution (0.1 mg/ml in aqua dest.) to 0.6 ml of sample the fluorescence was measured in microcuvettes. The full intensity of fluorescence is reached in less than one minute and remains constant for over one hour. The fluorescence was determined by exciting at 365 nm and reading the emission at 590 nm using a Kontron spectrofluorometer (model SFM 25). Hereafter, 0.05 ml of the bisbenzimidazole solution (0.02 mg/ml in aqua dest.) was added to 0.6 ml sample volume and measured at 352 nm excitation and 448 nm emission wavelength at 25 °C. Concentrations of nucleic acids were determined by using standard curves of DNA and RNA with EB and bisbenzimidazole.

Fish larvae samples used for developing the analytical procedure and for first trials had been stored in a frozen state at −74 °C and were thawed prior to analysis. The herring

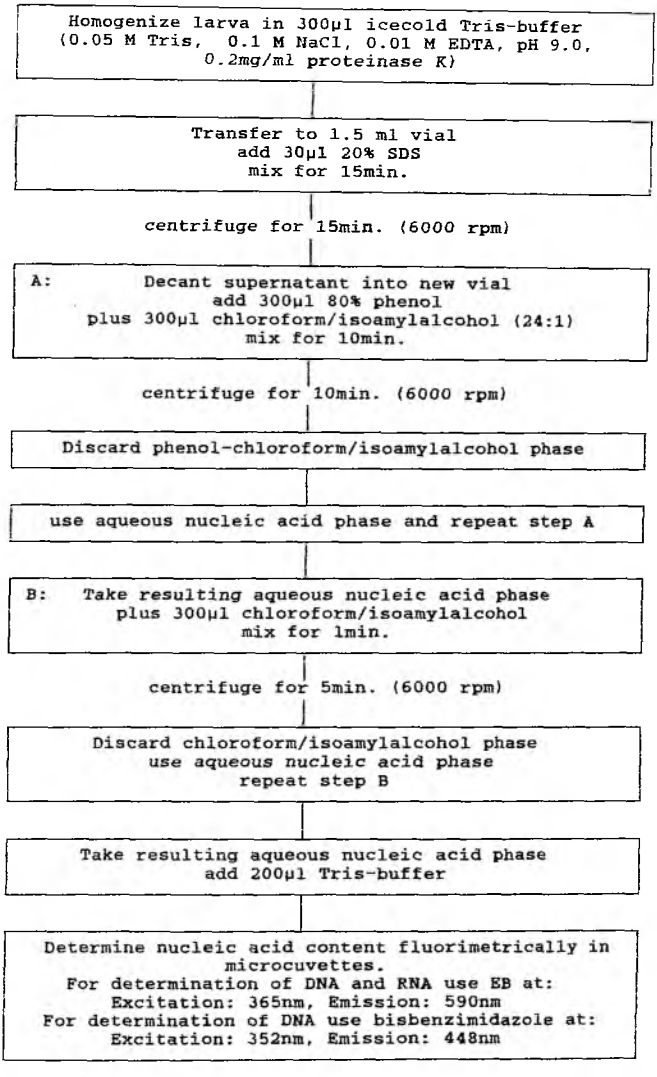

Fig. 1. Flowchart of the analytical procedure

(Clupea harengus) and turbot larvae *(Scophthalmus maximus)* had been reared in the laboratory at two different food densities using *Brachionus plicatilis* and *Artemia* nauplii or had been deprived of food organisms over varying time intervals.

Results

Fluorescence standard curve

When calf thymus DNA and yeast RNA concentrations were measured using the ethidium bromide technique a standard curve as shown in Fig. 2 was obtained. The DNA-bisbenzimidazole calibration curve is also presented in Fig. 2.

Testing of the analytical procedure

Best results concerning fluorescence intensity and reproducability were obtained when two washing steps with the phenol/chloroform/isoamylalcohol mixture and two washing steps with chloroform/isoamylalcohol were performed. A third washing step with phenol-chloroform-isoamylalcohol did not result in a higher fluorescence yield, indicating that most of the interfering substances had been removed (Table 1). Additional centrifugation after the SDS treatment resulted in a higher fluorescence intensity and better reproducability ($\bar{x}$ = 45.6 ± 2.35 n = 10, Vx = 5.2 %, RNA-DNA fluorescence in herring larvae compared to $\bar{x}$ = 38.0 ± 10.1, Vx = 26.6 % when the centrifugation step was omitted). It is assumed that cellular material, which has "quenching" effects can be removed by the centrifugation step. It was therefore generally included in the analytical procedure.

Table 1. **Influence of different numbers of washing steps in the analytical procedure on the fluorescence-measurements of DNA and RNA with ethidium bromid using herring larvae homogenates**

Analytical procedure	sample 1 % Fluorescence	sample 2 % Fluorescence
I. 2× phenol/chloroform/ isoamylalcohol washings 1× chloroform/isoamylalcohol washing	65.0	18.5
II. 2× phenol/chloroform/ isoamylalcohol washings 2× chloroform/isoamylalcohol washing	93.4	21.9
III. 3× phenol/chloroform/ isoamylalcohol washings 1× chloroform/isoamylalcohol washing	62.9	15.0
IV. 3× phenol/chloroform/ isoamylalcohol washings 2× chloroform/isoamylalcohol washings	92.0	19.7

Background fluorescence

The background fluorescence of the purified nucleic acid samples determined before the addition of EB or bisbenzimidazole was negligible. It was in the range of 1–4 % of the nucleic acid fluorophor fluorescence values.

Proportionality

Fig. 3. shows that ethidium bromid fluorescence due to DNA and RNA and bisbenzimidazole-DNA fluorescence give a linear increase with the amount of purified tissue homogenate.

The coefficient of variability (sample standard deviation as percentage of the mean) was 2.1 % for DNA and 2.3 % for RNA when 14 aliquots of herring larvae homogenates were measured.

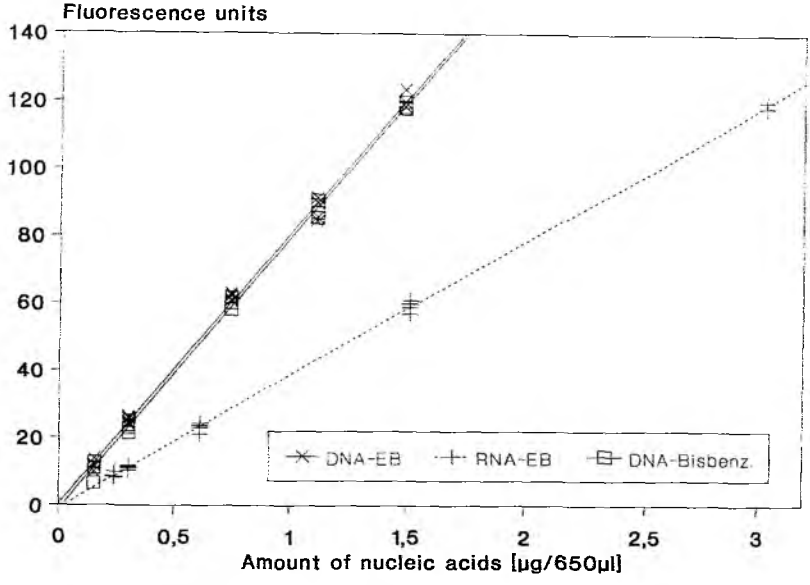

Fig. 2. DNA-EB, RNA-EB, and DNA-bisbenzimidazole standard curves using different amounts of calf thymus DNA and yeast RNA. The curves were fitted by linear regression:
DNA-EB: $Y = 0.453 + 80.587\ X$; $r = 0.997$
RNA-EB: $Y = -0.799 + 39.676\ X$; $r = 0.999$
excitation 365 nm, emission 590 nm
DNA-bisbenzimidazole: $Y = -1.203 + 81.023\ X$; $r = 0.998$
excitation 352 nm, emission 448 nm

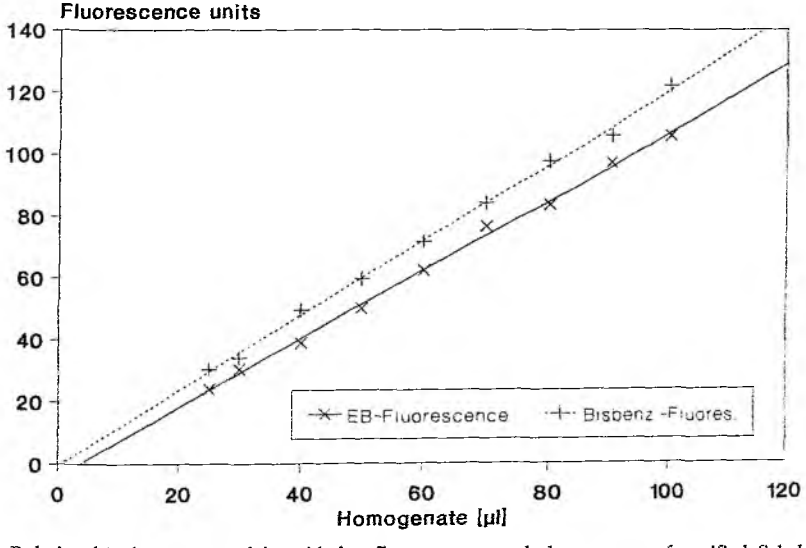

Fig. 3. Relationship between nucleic acid dye fluorescence and the amount of purified fish larvae nucleic acids (homogenate). The curves were fitted by linear regression:
EB-fluorescence in relation to the amount of homogenate:
$Y = -4.020 + 1.107\ X$; $r = 0.997$
Bisbenzimidazole-fluorescence in relation to the amount of homogenate:
$Y = -0.651 + 1.212\ X$; $r = 0.998$

Determination of nucleic acid contents of freeze-dried material compared to frozen samples thawed prior to analysis

To evaluate whether freeze-drying of the fish larvae samples results in different nucleic acid determinations as compared to measurements on larvae, which have been stored frozen and were thawed prior to analysis, a batch of frozen yolksac herring larvae stored at −80 °C for 18 month from the same experiment was split into two sample groups. One group was measured immediatly, the other was freeze-dried for 24 hours. The DNA- and RNA-contents of 10 individually measured freeze-dried (DNA: $\bar{x}$ = 0.22 µg ± 0.03 µg; RNA: $\bar{x}$ = 0.59 µg ± 0.10 µg) and 10 frozen larvae (DNA: $\bar{x}$ = 0.21 µg ± 0.03 µg, RNA: $\bar{x}$ = 0.68 µg ± 0.12 µg) were measured. The presented means and the standard deviations indicate that freeze-drying has no significant effect on the DNA or RNA concentrations. The higher variability observed within the RNA determinations is expected, since the RNA content is dependent on the physiological condition and might vary extremely between members of the same group.

Comparison of bisbenzimidazole and ribonuclease treatment to determine the DNA concentration

Comparison of the two different methods to determine DNA independent of RNA showed that treatment with ribonuclease A resulted in a higher DNA concentration (DNA: $\bar{x}$ = 0.21 µg ± 0.08 µg) compared to the samples treated with bisbenzimidazole (DNA: $\bar{x}$ = 0.19 µg ± 0.02 µg). It was also demonstrated that the reproducability of the determinations between the aliquots was better with the DNA specific dye (Vx = 8.4 %) compared to the ribonuclease treatment (Vx = 35.4 %).

Table 2. **RNA/DNA ratios of mackerel larvae caught in the Celtic Sea from one sample taken with the Longhurst-Hardy-plankton recorder.** Samples were frozen in liquid nitrogen, stored there for a few days, were subsequently freeze-dried and then kept on silica gel

No.	mg dryweight/ larva	RNA/DNA ratio
1	0.161	11.0
2	0.176	4.3
3	0.177	2.4
4	0.187	6.0
5	0.194	10.3
6	0.194	8.3
7	0.203	3.8
		$\bar{x}$ = 6.6

Application of the proposed analytical procedure on larvae reared in the laboratory under defined nutritional conditions gave the results shown in Fig. 4. Eighteen-day-old turbot larvae from three different feeding groups were compared. Obvious differences in the RNA/DNA ratios between feeding and starving larvae were observed. It was also demonstrated that the variation within the groups can vary remarkably. This is especially obvious in the group fed a medium food density.

Results on RNA/DNA ratios of mackerel larvae caught in the Celtic Sea (Clemmesen and Coombs in prep.) show the importance of individual analysis to determine "outsiders" (see e.g. larva no. 3 in Table 2). If these larvae had been measured as a pooled sample a RNA/DNA ratio of 6.6 would have resulted and would have camouflaged the extremes.

Discussion

The aim was to develop a highly sensitive, reproducible, and easy method for quantitative determinations of DNA and RNA contents in whole-body homogenates to determine the nutritional status of fish larvae. At first a very simple analytical procedure without the use of phenol-chloroform mixtures was tested based on methods by Beers and Wittliff

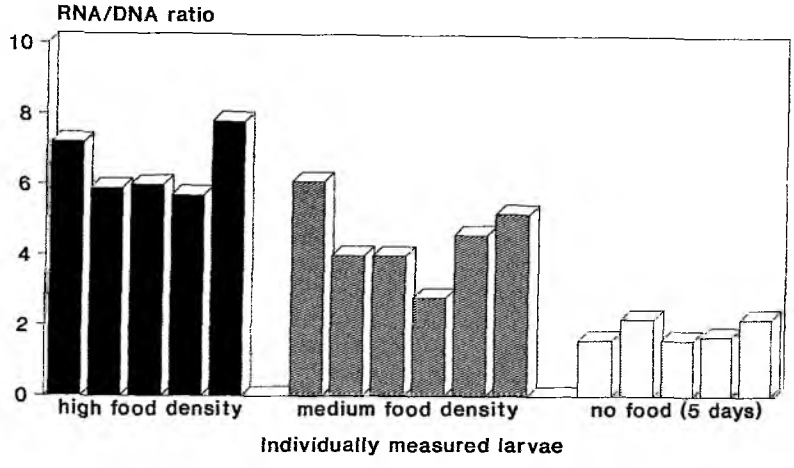

Fig. 4. RNA/DNA ratios of 18 days old turbot larvae reared in the laboratory at two different feeding levels and starving for 5 days

(1975), BOER (1975), KARSTEN and WOLLENBERGER (1972, 1977); LEPECQ and PAOLETTI (1966), and PRASAD et al. (1972) using ethidium bromide to determine DNA and RNA contents in cell and tissue homogenates. (Details of these trials are not given in the paper). The use of this very simple analytical procedure using whole-tissue homogenate resulted in unsatisfactory reproducability. Further the comparison of the DNA-bisbenzimidazole and ribonuclease treatment did not give compatible results. Strong fluorescence "quenching" effects due to contamination with cell substances other than nucleic acids caused problems in determining DNA and RNA contents accurately. Therefore purification steps had to be included to eliminate the substances causing "quenching". Similar variations between fluorescence readings on whole-tissue homogenate from tube worms which may have arisen from blood components or autofluorescence of tissue protein were found by DEBEVOISE and TAGHON (1988). They also had to include purification steps using chromatography on hydroxy-apatite columns. But this method is too time consuming and therefore not applicable for testing of a large sample number. With the use of phenol-chloroform/isoamylalcohol washes, SDS and proteinase K as described above a highly sensitive and reproducible method has been developed.

The comparison of the use of a DNA specific dye bisbenzimidazole and the ribonuclease treatment showed that the DNA dye bisbenzimidazole resulted in better reproducability. It seems that the degradation of RNA with ribonuclease is less reliable. It was decided to give the DNA dye bisbenzimidazole preference, since the procedure showed good reproducability and the variability between replicate aliquots was only about 2 %. It was demonstrated that the presence of RNA in the samples does not interfere in the determination of DNA and that the enhancement of fluorescence is linear with increasing amounts of DNA in purified homogenates (CESARONE et al. 1979; LABARCA and PAIGEN 1980; CLEMMESEN unpubl.). Preliminary results indicate that freeze-drying has no significant effect on the nucleic acid concentration in fish larvae.

The special advantage of the fluorescence method is its higher sensitivity compared to the conventional UV-absorbance test (BUCKLEY 1979). The UV-absorbance has a lower limit of 1–2 µg/ml DNA or RNA, whereas EB and bisbenzimidazole allow nucleic acid determinations down to 0.1 µg/ml. It was shown by CESARONE et al. (1979) that the use of bisbenzimidazole to determine chromatin samples in mouse liver cells was in perfect agreement with the UV-absorbance measurement (MUNRO and FLECK 1966). Results using

another fluorescence-method to evaluate the nutritional status of individual fish larvae using digestive enzymes, especially trypsin, (UEBERSCHÄR, this volume) will be compared with this RNA/DNA technique. It is assumed that the digestive enzyme content changes rapidly with the uptake of food organisms. Differences in the nutritional situation in the range of hours to a few days are detectable. The RNA/DNA ratio is more reliable for measuring effects caused by longer starvation intervals (BUCKLEY 1984; CLEMMESEN 1987; WANG and STICKLE 1986). Since fish larvae show a starvation potential in the range of several days before they reach "the point of no return", (BLAXTER 1981) it seems adequate to measure influences caused by a few days of food-deprivation.

It can be concluded that the presented analytical procedure is successful in determining nucleic acid contents of whole-body homogenates of individual marine fish larvae. It is proposed that the use of the RNA/DNA ratio determined from individual fish larvae might help in identifying starving larvae in the field, which should help to test the "starvation hypothesis" of larvae caught in ecologically different marine environments. Since the RNA/DNA ratio was proven valid for determining the nutritional status of a wide range of marine organisms (DEBOISE and TAGHON 1988; DORTCH et al. 1983; WANG and STICKLE 1986; WRIGHT and HETZEL 1985), the presented method should be applicable to species other than fish larvae, when only small sample quantities are available.

Acknowledgement

Part of this study was supported by a grant from the "Deutsche Forschungsgemeinschaft" Ne 99/19.

References

BEERS, P. C.; WITTLIFF, J. L., 1975: Measurements of DNA and RNA in mammary gland homogenates by the Ethidium bromid technique. Anal. Biochem. **63**, 433–441.

BLAXTER, J. H. S., 1981: The rearing of larval fish. In: HAWKINS, A. D. (Ed.): Aquarium systems. London: Academic Press, 303–323.

BOER, G. E., 1975: A simplified microassay of DNA and RNA using ethidium bromid. Anal. Biochem. **65**, 225–231.

BRUNK, C. F.; JONES, K. C.; JAMES, T. W., 1979: Assay of nanogram quantities of DNA in cellular homogenates. Anal. Biochem. **92**, 497–500.

BUCKLEY, L. J., 1979: Relationship between RNA/DNA ratio, prey density and growth rate in Atlantic Cod *(Gadus morhua)* larvae. J. Fish. Res. Bd. Can. **36**, 1497–1502.

BUCKLEY, L. J., 1980: Changes in the ribonucleic acid, deoxyribonucleic acid, and protein content during ontogenesis in winter flounder, *Pseudopleuronectes americanus*, and the effect of starvation. Fish. Bull. **77**, 703–708.

BUCKLEY, L. J., 1981: Biochemical changes during ontogenesis of cod *(Gadus morhua* L.) and winter flounder *(Pseudopleuronectes americanus)* larvae. Rapp. Proc.-v. Réun. Couns. Int. Explor. Mer **178**, 547–552.

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

BUCKLEY, L. J.; LOUGH, R. G., 1987: Recent growth, chemical composition and prey field of haddock *(Melanogrammus aeglefinus)* and cod *(Gadus morhua)* larvae and post larvae on Georges Bank, May 1983. Can. J. Fish. Aquat. Sci. **44**, 14–25.

CESARONE, C. F.; BOLOGNESI, C.; SANTI, L., 1979: Improved microfluorometric DNA determination in biological material using 33258 Hoechst. Anal. Biochem. **100**, 188–197.

CLEMMESEN, C., 1987: Laboratory studies on RNA/DNA ratios of starved and fed herring *(Clupea harengus)* and turbot *(Scophthalmus maximus)* larvae. J. Cons. int. Explor. Mer **43**, 122–128.

CUSHING, D. H., 1975: Marine ecology and fisheries. Cambridge: Cambridge University Press, 278 pp.

DEBOISE, A. E.; TAGHON, G. L., 1988: RNA:DNA ratios of the hydrothermal-vent vestimentiferans *Ridgeia piscesae* and *R. phaophiale* indicate variations in growth rates over small spatial scales. Mar. Biol. **97**, 421–426.

DEFLAUN, M. F.; PAUL, J. H.; DAVIS, D., 1986: Simplified method for dissolved DNA determinations in aquatic environments. Appl. Environ. Microbiol. **52**, 654–659.

DORTCH, Q.; ROBERTS, T. L.; CLAYTON, J. R. JR.; AHMED, S. I., 1983: RNA/DNA ratios and DNA concentrations as indicators of growth rate in planktonic marine organisms. Mar. Ecol. Prog. Ser. **13**, 61–71.

HUNTER, J. R., 1976: Report of a colloquium on larval fish mortality studies and their relation to fisheries research, Jan. 1975. NOAA Tech. Rep. NMFS Circ. **395**, 5 pp.

KARSTEN, U.; WOLLENBERGER, A., 1972: Determination of DNA and RNA in homogenized cells and tissues by surface fluorometry. Anal. Biochem. **46**, 135–148.

KARSTEN, U.; WOLLENBERGER, A., 1977: Improvements in the ethidium bromid method for direct fluorometric estimation of DNA and RNA in cell tissue homogenates. Anal. Biochem. **77**, 464–470.

LABARCA, C.; PAIGEN, K., 1980: A simple, rapid, and sensitive DNA assay procedure. Anal. Biochem. **102**, 344–352.

LASKER, R., 1975: Field criteria for the survival of anchovy larvae: The relation between inshore chlorophyll maximum layers and successful first feeding. Fishery Bull. Fish. Wildl. Serv. U.S. **73**, 455–462.

LE PECQ, J.-B.; PAOLETTI, C., 1966: A new fluorometric method for RNA and DNA determination. Anal. Biochem. **17**, 100–107.

MANIATIS, T.; FRITSCH, E. F.; SAMBROOK, J., 1982: Molecular cloning. A laboratory manual. New York: Cold Spring Harbor Laboratory Publications, 521 pp.

MARTIN, F. D.; WRIGHT, D. A.; MEANS, J. C.; SETZLER-HAMILTON, E. F., 1985: Importance of food supply to nutritional state of larval striped bass in the Potomac river estuary. Trans. Am. Fish. Soc. **114**, 137–145.

MAY, R. C., 1974: Larval mortality in marine fishes and the critical period concept. In: BLAXTER, J. H. S. (Ed.): The early life history of fish. New York: Springer Verlag, 1–19.

MUNRO, H. N.; FLECK, A., 1966: Recent developments in the measurement of nucleic acids in biological materials. A supplementary review. Analyst **91**, 78–88.

NELLEN, W., 1986: A hypothesis on the fecundity of bony fish. Meeresforsch. **31**, 75–89.

PRASAD, A. S.; DUMOUCHELLE, E.; KONIUCH, D.; OBERLEAS, D., 1972: A simple fluorometric method for the determination of RNA and DNA in tissues. J. Lab. Clin. Med. **80**, 598–602.

REGNAULT, M.; LUQUET, P., 1974: Study by evolution of nucleic acid content of prepuberal growth in the shrimp, *Crangon vulgaris*. Mar. Biol. **25**, 291–298.

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

UEBERSCHÄR, B., 1988: Determination of the nutritional condition of individual marine fish larvae by analyzing their proteolytic enzyme activities with a highly sensitive fluorescence technique. Meeresforsch. **32**, 144–155.

WANG, S. Y.; STICKLE, W. B., 1986: Changes in nucleic acid concentration with starvation in the blue crab *Callinectes sapidus* Rathbun. J. Crust. Biol. **6**, 49–56.

WRIGHT, D. A.; HETZEL, E. W., 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.

Author's address: Dipl. Biol. CATRIONA M. CLEMMESEN, Institut für Meereskunde an der Universität Kiel, Düsternbrooker Weg 20, D-2300 Kiel, F. R. Germany