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RNA content of copepods as a tool for determining adult growth rates in the field

Enric Saiz, Albert Calbet, Angela Fara, and Elisa Berdalet
Institut de Ciències del Mar, CSIC, P. Joan de Borbó s/n, E-08039 Barcelona, Catalonia, Spain

Abstract

We propose a method to determine growth rates of adult copepods based on their nucleic acid contents. We investigated the suitability of this method on the marine copepod *Acartia grani* in a range of food concentrations and temperatures. There was a good linear relationship between growth rate (estimated as egg production) and RNA contents of the adult copepods. The slope of the relationship was dependent on temperature. We also built a multipleregression model to predict egg production rates of *A. grani* as a function of RNA contents and temperature. This method allows very intensive field sampling and does not require incubations onboard. Samples are preserved frozen and analyzed in the laboratory using a very sensitive fluorometric technique that allows working with small numbers of individuals of the desired species.

The estimation of growth rates of copepods in the field is still not a completely solved problem. Very often zooplanktologists search for methods that allow reliable estimates of zooplankton growth rates at temporal and spatial scales short enough to cope with micro- and mesoscale hydrographic variability. For this purpose, approaches based on following development of well-defined cohorts, if ever possible in the field, are precluded. The method currently more accepted is the egg production method, which is species and site specific (Kiørboe and Johansen 1986; Berggreen et al. 1988). This method is based on the determination of egg production rates of adult female copepods and on the assumption (validated in the laboratory for several species) that juvenile growth rates of copepods are similar to the rates of egg production in adults (Berggreen et al. 1988; Fryd et al. 1991).

However, the egg production method has tradeoffs that suggest a search for alternative/complementary methods for the determination of in situ growth rates of copepods. The main drawbacks of the egg production method are that (1) egg production is usually estimated from onboard incubations, which can be flawed by changes in food availability during the incubation or by not reflecting properly the in situ food (patchiness) and temperature conditions (Saiz et al. 1997); (2) it is tedious and time consuming because adult females (in good condition) of dominant species must be sought, whereas natural populations are usually composed mainly of juvenile stages; (3) it has been shown that this assumed correspondence between growth rates of juveniles and adult copepods might not always be valid (Peterson et al. 1991); and (4) in practice, the method does not allow intensive sampling with short temporal and spatial scales or when working with more than one or two species. We herein propose and test an alternative to the egg production method that addresses some of these drawbacks. RNA is the molecule mainly responsible for the synthesis of both structural and catalytic proteins. Therefore, the amount of RNA per cell or individual must be directly related to the intensity of growth of the cell/individual. This feature has encouraged use of the RNA content (or RNA : DNA ratios) as a method for estimating growth rates of organisms in phyla as diverse as bacteria, microalgae, and fish. In general, a good correlation between RNA content and growth rate has been observed (*see* Sutcliffe 1970; Båmstedt and Skjøldal 1980; Clemmesen 1994 and references therein).

In the case of copepods (or other zooplankters), determination of growth rates based on nucleic acid content has been attempted, but with variable success. Båmstedt and Skjøldal (1976) reported dependency of nucleic acid contents of *Euchaeta norvegica* on season and body mass, and indirectly determined a good correlation between growth rates of late copepodites and RNA contents. Also, Båmstedt (1983) found a good agreement between the seasonal peaks of RNA content and the occurrence of the breeding season of high-latitude zooplankton.

However, data opposing the suitability of methods based on nucleic acid content have also appeared. Dagg and Littlepage (1972) found fairly good relationships between growth rate and RNA content for *Artemia salina* and *Euchaeta elongata*, but these relationships appeared to be species specific and the data showed high variability. They concluded that any general relationship between RNA content and growth rate would lack sufficient accuracy to be used as a predictive tool. Ota and Landry (1984) also tried to establish a correlation between growth rates and nucleic acid content of the copepod *Calanus pacificus*. They concluded that the dependencies of nucleic acid content on temperature and stage (size) would act as confounding effects and make methods based on nucleic acids difficult to use as a predictive tool for growth rates of zooplankton.

The scenario following attempts to establish a relationship between growth rates and nucleic acid contents of zooplankton showed very little promise. However, we think that some of the poor results obtained stem from the fact that research sought to obtain a general predictive relationship for the whole zooplankton community. Some of the poor correlations observed could have actually been a consequence of working with mixed species (each with specific nucleic acid contents) or of the heterogeneous demographic composition of samples (Dagg and Littlepage 1972; Baudouin and Scop-

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pa 1975; Ota and Landry 1984). An additional problem for the development of methods based on nucleic acid contents was that RNA and DNA were measured by low-sensitivity methods and required a considerable amount of biomass for analysis.

However, the perspectives for use of nucleic acid techniques to determine in situ growth rates of zooplankton seem to have changed substantially in recent years and warrant further work in this field. New spectrofluorometric methods have been developed in the last few years that permit much higher sensitivities (Clemmesen 1993; Fara et al. 1996). Furthermore, Nakata (1990), following a species- and age-specific approach, found a good association between the RNA : DNA ratio of *Paracalanus* sp. and phytoplankton abundance in the water column. More recently, Nakata et al. (1994) observed a good correlation between egg production rates and RNA contents of *Paracalanus* sp. and other species (Nakata pers. comm.). Stemming from this work, we herein present a laboratory study of the suitability of nucleic acid content as a tool to determine adult copepod growth rates. We have used a very sensitive spectrofluorometric method for the quantification of nucleic acids, recently developed for microplankton (Fara et al. 1996). Also, as Ota and Landry (1984) suggested, and we have tested the potential interaction of temperature with the correlation between nucleic acid contents and growth rates. Owing to the enhancement effect of temperature on chemical reactions, we predict that the amount of RNA per individual characteristic of a determined growth rate will be higher at lower temperatures. Our final aim is to develop a method for the estimation of copepod growth rate alternative to the egg production method, with the same reliability but lacking the inconveniences of sorting individuals onboard and of incubations. These features would allow higher time and space resolution in intensive sampling schedules.

Materials and methods

Description of the experiments—The experiments were designed to test the effects of temperature and food concentration on the nucleic acid content of the marine copepod *Acartia grani*. Three experiments were conducted. In Exp. 1, we tested the relationship between egg production rate and RNA content of adult copepods, as well as interaction with temperature. The experimental design consisted of two crossed factors, food concentration and temperature. Adult females from a culture of *A. grani* (cephalotorax length of 979 ± 6 (SE) μm, $n = 34$; this culture has been kept in our laboratory for >20 generations and is routinely fed the flagellate *Rhodomonas baltica*; *see* Calbet [1997] for further details) were picked out and preconditioned in groups of 21 females to the experimental conditions in 1.2-liter screw-cap bottles for ~48 h. A few males (4 per bottle) were also introduced to ensure fertilization of females. Six different nominal food concentrations were tested (0.2, 0.4, 0.7, 1.0, 2.0, and 3.0 ppm of the diatom *Thalassiosira weissflogii*; 1 ppm of *T. weissflogii* is ~1,131 cells ml^{-1}) at 17.8 and 23.3°C. The bottles were left standing during the incubation, being periodically turned upside down (twice a day) to prevent settling of the algae. We used a 12 : 12 L/D cycle. After the first day of preconditioning, the contents of the bottles were gently filtered onto submerged 180-μm sieves and the animals transferred to new suspensions of algae at the corresponding concentrations. At the end of the second day of preconditioning, copepods were checked and any dead animals or animals in bad shape were taken out. The remaining copepods (males included) were introduced into new suspensions of algae at the corresponding food concentrations and temperatures. The experimental bottles (two replicates for each food concentration) were incubated for 24 h, and their contents were then filtered through 180- and 20-μm sieves. The number of copepod eggs retained on the 20-μm sieve were counted under a stereomicroscope. The adult females were checked, and one group of 18 females for each bottle was transferred to precombusted GF/F filters and frozen in liquid nitrogen until analysis of their nucleic acid contents. This procedure allowed determination of the egg production and nucleic acid content of the same individuals.

We tested the linear relationship between RNA content and egg production rate and the effect of temperature on this linear relationship by covariance analysis. We applied a multiple-regression model to predict egg production rate as a function of RNA content and temperature.

In Exp. 2, we tested the coupling between the time response of changes in RNA content and of changes in egg production rate. The aim of the experiment was to expose the copepods to extreme changes in food availability and to determine to what extent the fast response of egg production to food availability (Calbet and Alcaraz 1996; Saiz et al. 1997) was matched by similar changes in the RNA content of the copepods.

The overall procedure was similar to that of Exp. 1. Groups of 20 adult females and 4 males of *A. grani* were introduced in 10 1.2-liter bottles filled with a suspension of 3.5 ppm of *T. weissflogii*. The bottles were left standing at 17.8°C in a 12 : 12 L/D cycle. After 1.5 d of acclimation, the animals were transferred to new suspensions and the time of the transfer was logged. After 24 h (day 1), two bottles were taken and the contents filtered through 180- and 20-μm sieves. The eggs laid were counted, and the females were processed for RNA analysis as explained above. For the remaining bottles, the animals were put in starving conditions by transferring to 0.2-μm filtered seawater, which was changed daily. During the next 2 d (days 2 and 3), two bottles were processed every day for the determination of egg production rates and nucleic acid contents of the female copepods. After day 3, the animals were transferred again to 3.5 ppm suspensions of *T. weissflogii* (renewed every day), and for the following 2 d (days 4 and 5) two bottles were taken daily for egg production and nucleic acid contents determinations. The daily change of water (either filtered or with algae) allowed us to obtain daily determinations of egg production.

In Exp. 3, we examined the effect of Formalin preservation on the determination of nucleic acid contents of copepods. Adult females of *A. grani* were picked out from the laboratory culture and six batches of 20 females prepared. Three batches were frozen in liquid nitrogen and the remaining three were preserved in 4% Formalin and kept for

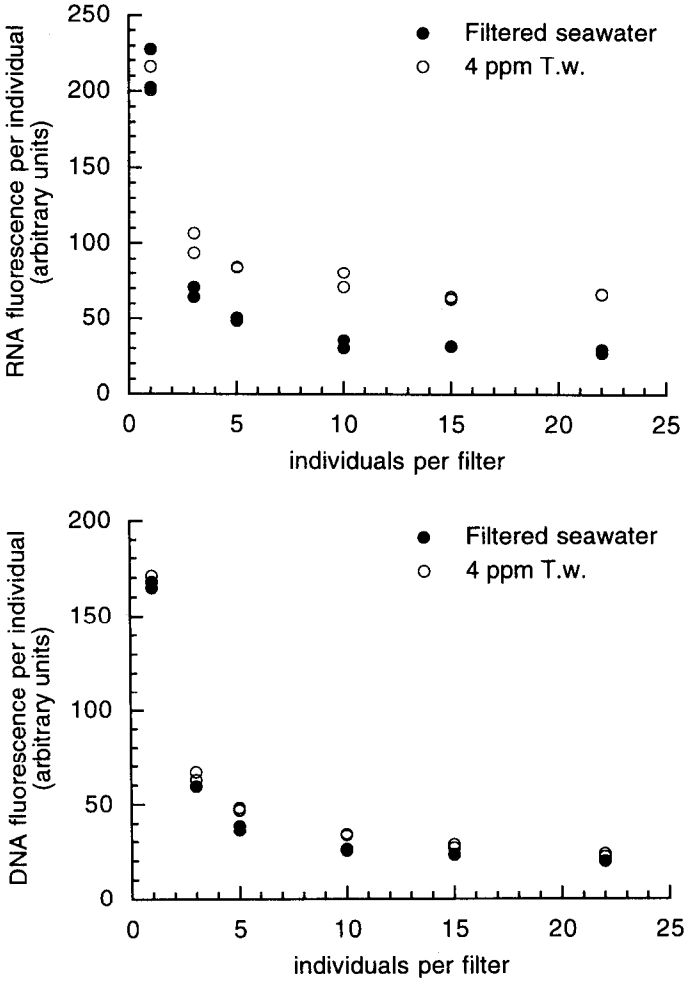

Fig. 1. Plot of RNA and DNA fluorescence per copepod as a function of the number of copepods on the analyzed filter. Data correspond to a preliminary experiment conducted to determine the minimum number of copepods required for the nucleic acid content analysis. Adult *Acartia grani* females were incubated either in filtered seawater (●) or in a 4 ppm suspension of *Thalassiosira weissflogii* (T.w.) (○); afterwards, groups of individuals were transferred to precombusted filters and frozen until analysis. The fluorescence values for both RNA and DNA become linear (i.e. independent of the number of copepods) after a minimum of 5–10 females per filter.

~3.5 d before nucleic acid content analysis. This experiment sought to determine whether nucleic acid contents could be conducted in Formalin-preserved samples.

RNA and DNA quantification—Analyses were conducted following the fluorometric method of Fara et al. (1996). Copepods were placed onto precombusted (450°C, 6 h) GF/F glass fiber filters and frozen in liquid nitrogen. At the moment of the analysis the filters were taken from the liquid nitrogen and immediately ground in 5 ml Tris buffer (0.9 mM $CaCl_2 \cdot 2H_20$, 0.9 mM $MgCl_2 \cdot 6H_20$, 100 mM NaCl, 100 mM *tris*(hydroxymethil)-aminomethane, pH 7.5) in a Potter-Elvehjem tissue-homogenizing tube for 2 min at 0°C. The homogenates were then centrifuged at 3,500 rpm for 10 min at 0°C, and the supernatant fluid was divided into three sub-

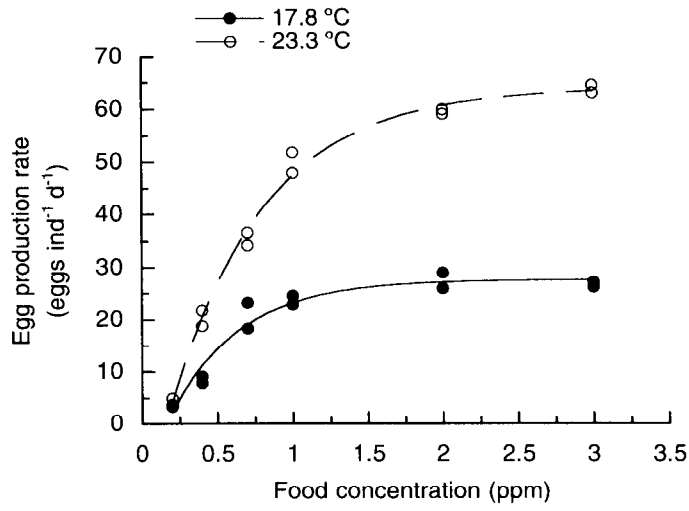

Fig. 2. Functional response of egg production rate (EPR) of *Acartia grani* to food concentration (in abcissae) and temperature. Ivlev's fits to the data were $EPR = 27.6(1 - e^{-2.18(food - 0.16)})$ at 17.8°C and $EPR = 64.1(1 - e^{-1.61(food - 0.16)})$ at 23.3°C.

samples. One aliquot was used to quantify blank fluorescence. For the nuclease digestion, one aliquot was incubated 20 min at 37°C with DNase-free RNase (0.5 μg ml^{-1}, final concn in the assay) and the other was incubated for 20 min at 25°C with RNase-free DNase (10 units ml^{-1}, final concn in the assay). After the enzymatic digestion, the subsamples were stained with Thiazole Orange. Fluorescence values of the aliquots treated with either RNase or DNase (previously corrected for the blank fluorescence) were used to determine DNA and RNA concentrations, respectively. For calculations, DNA and rRNA from *Escherichia coli* were used as standards. A sample-size test indicated that the optimum number of *A. grani* individuals required for analysis ranged from 15 to 20 (Fig. 1). Smaller sample sizes led to artificially high RNA content per copepod because the RNA content per filter was below detection and the response was not linear; when the fluorescence values per filter were divided by the number of copepods per filter, the high RNA per copepod values are obtained for samples below the detection limit. A preliminary test (data not shown) indicated that large sample sizes (>50 *A. grani* per filter) might induce a quenching effect and require dilution of the sample. We advise conducting this simple sample-size test for further use of this technique on species other than *A. grani*; the optimum sample size for the analysis might depend not only on biomass but also on the specific nucleic acid content of each species.

RNA and DNA contents were expressed as μg RNA or μg DNA ind.$^{-1}$. The DNA content is an estimator of cell number/biomass (analogous to dry weight); thus, the RNA : DNA ratio normalizes the RNA content to biomass.

Results

Figure 2 depicts the relationship between egg production rate and food concentration at the two experimental temperatures. An increase in temperature of ~5°C resulted in maximum egg production rates more than doubling (28 and 64 eggs

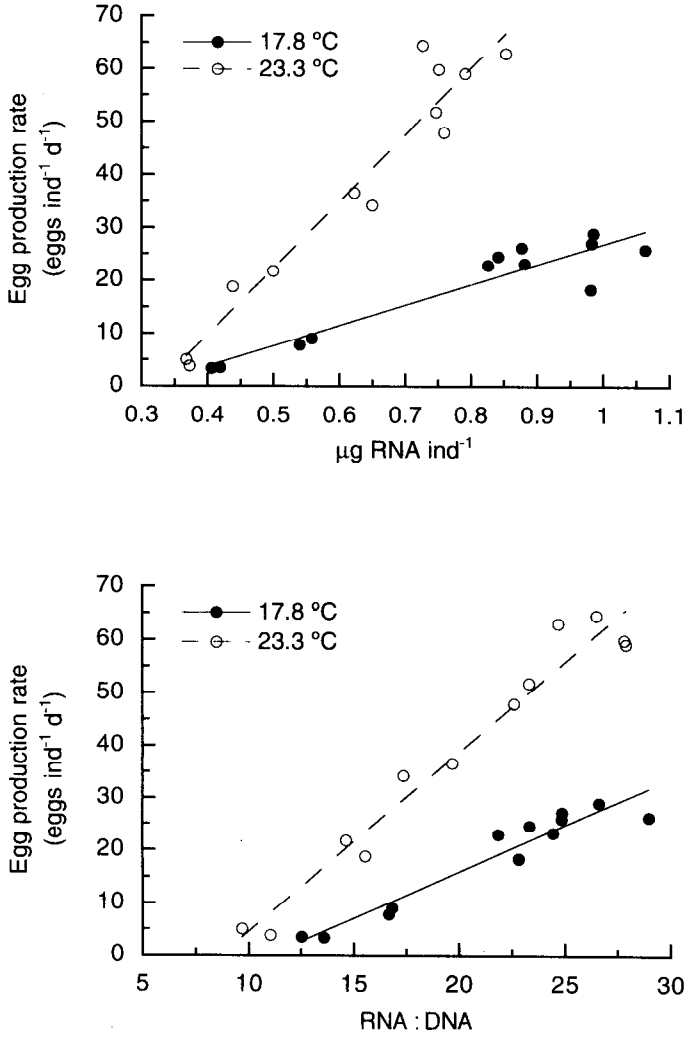

Fig. 3. Linear relationship between egg production rate and RNA content per copepod (top panel, µg RNA ind.$^{-1}$) and RNA: DNA ratio (bottom panel) at both experimental temperatures.

ind.$^{-1}$ d^{-1} at 17.8 and 23.3°C respectively). The Q_{10} value for the maximum egg production rate in that range of temperatures was 4.4.

Figure 3 shows the linear relationship between egg production rate and both RNA content per copepod and RNA: DNA ratio. The relationship was highly significant for the experiments at both temperatures using either RNA per copepod or RNA: DNA as regressors (see Table 1). A slightly better fit was obtained using the RNA: DNA ratio as a regressor (which takes into account the biomass of the individuals analyzed). In the following we use only the RNA: DNA ratio as regressor.

The effect of temperature on the linear relationship between egg production rate and the RNA: DNA ratio resulted in a significant change in the slope of the linear relationship (ANCOVA, $F_{1,20} = 32.6$, $P < 0.001$) but not for the intercept ($F_{1,20} = 2.66$, $P < 0.12$). For instance, a RNA: DNA ratio of 20 would result in rates of 16 eggs ind.$^{-1}$ d^{-1} at 17.8°C and 39 eggs ind.$^{-1}$ d^{-1} at 23.3°C.

We have also built a predictive model of egg production rate by multiple regression by using temperature, the RNA: DNA ratio, and their product as variables. The effect of temperature alone proved not significant ($P > 0.1$) and was eliminated from the model. Consequently, the best model (Table 2) included only two regressors: the RNA: DNA ratio and the product of temperature and RNA: DNA (interaction factor in the ANCOVA).

Table 1. Egg production rate of *Acartia grani* (EPR, eggs ind.$^{-1}$ d^{-1}) as a function of either RNA content (µg RNA ind.$^{-1}$) or RNA: DNA ratio at each experimental temperature. Values for the parameters (±SE) of simple linear regression fits (EPR = $a + bX$) are shown (*, $P < 0.001$).

	17.8°C	23.3°C
a. RNA content as regressor		
a	−11.8±3.66	−40.8±6.95
b	38.7±4.50	126.0±10.65
r^2	0.88*	0.93*
b. RNA: DNA as regressor		
a	−19.4±3.52	−29.6±4.69
b	1.76±0.159	3.41±0.223
r^2	0.92*	0.96*

Figure 4 shows the temporal evolution of egg production rates and RNA: DNA ratio in Exp. 2. It takes 2 d for well-fed *A. grani* females to reduce their egg production to almost zero values when transferred into filtered seawater. The recovery of egg production, once the copepods were put back in plenty of food, is slower, as already shown for the same species by Calbet and Alcaraz (1996). The overall trend followed by the RNA: DNA ratio is very similar to the one shown by egg production. However, although for egg production the recovery due to food presence (days 4 and 5) was 30% slower than its decline due to food privation (days 2 and 3), the RNA: DNA ratio responded 60% slower. The use of formaldehyde as a fixative resulted in loss of fluorescence of the samples, i.e. in negative or very low values of RNA and DNA per copepod. The RNA content (±SE) estimated in the Formalin-preserved samples was −0.02 ± 0.011 µg RNA ind.$^{-1}$, compared to the 0.49 ± 0.017 µg RNA ind.$^{-1}$ measured in the frozen samples. In the case of DNA, the two treatments gave 40 ± 0.8 and −4 ± 3 ng DNA ind.$^{-1}$, respectively, for liquid nitrogen and Formalin preservation. This result precludes the use of formaldehyde

Table 2. Multiple regression model to predict egg production rates (EPR, eggs ind.$^{-1}$ d^{-1}) of *Acartia grani* adult females as a function of the variables temperature and RNA: DNA ratio. Parameters (±SE) are shown.

Model

EPR = $a + b$ RNA: DNA + c (temp × RNA: DNA)
$r^2 = 0.96$, $n = 24$
$F_{2,21} = 264.6$, $P < 0.001$

Parameters

$a = -25.7 \pm 3.14$, $P < 0.001$
$b = -1.67 \pm 0.305$, $P < 0.001$
$c = 0.21 \pm 0.013$, $P < 0.001$

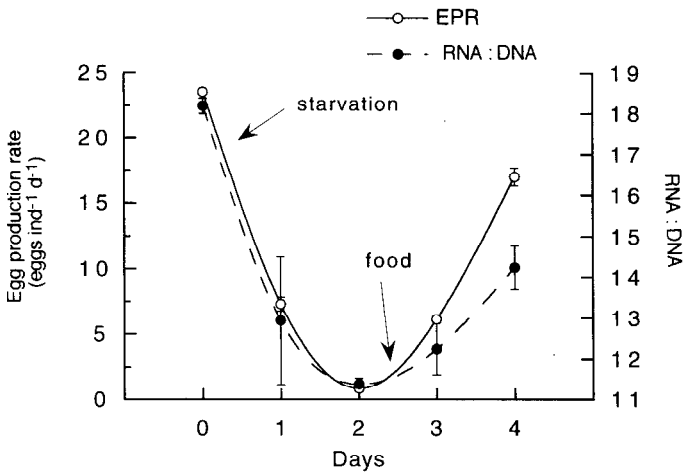

Fig. 4. Time response of egg production rate and RNA : DNA ratio of *Acartia grani* to changes in food availability (Exp. 2). The arrows indicate when the copepods were first transferred from saturating food conditions to filtered seawater (starvation), and were finally put back into plenty of food ("food").

as a fixative for samples to be procesed for nucleic acid contents. Formaldehyde probably does not allow Thiazole Orange to stain the nucleic acids.

Discussion

We have shown a significant relationship between the RNA content of adult copepods and their growth rate (estimated as egg production rate). This finding confirms the study by Nakata et al. (1994) for *Paracalanus* sp., and sets the base for further application of this method to determine growth rates of copepods in the field. Laboratory experiments would permit development of powerful regression models relating RNA content with egg production of targeted species, which could be further applied to field-preserved (frozen) samples.

There are two basic aspects of the method that make it useful. First, the relationship between RNA content and growth rate should be unique for a particular copepod species. Second, although copepod growth rates are affected by factors such as food quantity and quality (*see* Jónasdóttir [1994] and references therein), past food history, and lipid reserves (Attwood and Peterson 1989), the amount of RNA required to build proteins and enzymes at a particular rate should be the same independent of the nutritional conditions. That is, the slope of the relationship between growth rates and RNA content should be unique for one species independent of food quality and previous food history. For instance, if certain past and present nutritional conditions result in high growth rates for a particular zooplankter, the RNA content required for that growth should be accordingly high; these nutritional conditions would affect both the growth rate and the RNA content, but not their ratio (slope). Because in our experiments we only used one type of food item (*T. weissflogii*), further research with different food items is required to confirm this premise.

Our data also showed that the linear relationship between RNA content and egg production rate was highly dependent on temperature. Metabolic processes are dependent on temperature, and this holds also for the amount of RNA per copepod. This result emphasizes the fact that temperature must be taken into account when applying biochemical methods to determine in situ growth rates of all organisms. Our investigation showed that the changes induced by temperature on the linear relationship between nucleic acid content and growth can be quantified and are predictable. Although our data are restricted to two temperatures, we have been able to build a significant, predictive multiple regression model that explains 96% of the variability in our data. The consideration of three or four temperatures would improve the model. Note that the specific RNA content should be related to a biomass unit (e.g. dry weight or DNA content) rather than per individual.

Two main limitations were noted by Ota and Landry (1984) for using nucleic acids content as an estimator of growth rates. First, they argued that such a method might not be appropriate in the case of juvenile copepods. Developmental and physiological changes during the period to adulthood could induce variations in the RNA content not strictly related to growth (change in biomass). This fact has been proven for fish larvae during the first days after hatching (Clemmesen 1994), and one might expect that the process of molting (ecdysis) in crustaceans might result in peaks of high cell activity and high RNA content not associated with growth. Because our study was conducted on adult females, the question about the suitability of using the nucleic acid method for juvenile growth should be addressed in further studies.

The second objection pointed out by Ota and Landry (1984) was that differences in growth rates due to food limitation might be poorly represented by changes in the RNA content (*see* also Dagg and Littlepage 1972). In contrast, our experimental data showed that the RNA content of adult *A. grani* females reflected changes in egg production rates even under extreme food conditions (Fig. 3). This fact warrants the validity of the method.

Our experiments show that under steady food conditions (after 2 d of preconditioning) the RNA : DNA ratio was well correlated with growth rate (estimated as egg production). In addition, we investigated the time response of RNA content changes related to variations of growth induced by food availability (Fig. 4). Although RNA should respond quickly to changes in growth rates, some authors (Dagg and Littlepage 1972; Sapienza and Mague 1979) have argued that due to differences in stability of the different pools of RNA, total RNA content might not respond quickly to sudden reductions in growth rates. In contrast, our experiments showed that under slow-down conditions, the RNA contents paralleled the decline in egg production (growth) rates. Unexpectedly, when food was supplied, the egg production response was faster than the RNA building up. This result may have arisen from the experimental design. For this test, the animals were maintained in abundant food for a few days in order to achieve saturated and stable conditions. Then, the transfer to filtered seawater for 2 d induced the observed reduction in RNA content and egg production rate. After these 2 d of starvation, food was resupplied. We think that

the recovery of egg production was faster than the building up of RNA during this last period because the egg production very much reflected the maturation of oocytes produced during the previous feeding history. Had the starvation period been longer, a better coupling in the time responses of RNA and egg production would have very likely appeared.

Our study offers encouraging perspectives for an alternative method for the estimation of in situ growth rates of adult copepods. Most previous attempts to apply the nucleic acid method to estimate growth rates of zooplankton probably failed because of using poor (low sensitivity) biochemical methods and working with mixed populations. The use of new high-sensitivity spectrofluorometric techniques for nucleic acid determination and a species- and stage-specific approach have shown the method to be valid. For the application of this method, a calibration in the laboratory under temperature- and food concentration-controlled conditions is required. The quantification of RNA content or RNA : DNA ratios allows good time- and space-specific resolution. Although it can only be applied to selected targeted species, not to the whole community, this problem also pertains to other methods like the egg production one itself. Furthermore, it can alleviate some drawbacks of the egg production method in the field, e.g. food availability during the incubation (Saiz et al. 1997). It may also be used for copepods that carry their eggs in sacs (e.g. *Oithona*). For these organisms, the determination of their in situ growth rates is usually based on the quantification of the interval between clutches from temperature-dependent relationships (Uye and Sano 1995), although this interval can depend also on food availability during incubation (Saiz et al. 1997).

Our aim in the near future is to apply this methodology to field populations of selected species and to compare our predictions of in situ growth rates with the standard procedures. However, further studies should first extend the observed relationship in *A. grani* to other copepod genera. Also, the capacity of the method to detect fast increases in egg production rate due to sudden increases in food availability should be further studied.

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