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Biochemical proxies for growth and metabolism in *Acartia bifilosa* (Copepoda, Calanoida)

Towe Holmborn[1], Kristin Dahlgren[2], Claire Holeton[3], Hedvig Hogfors[1], and Elena Gorokhova[1]*
[1]Department of Systems Ecology, Stockholm University, SE-106 91 Stockholm, Sweden
[2]Department of Ecology and Environmental Science, Umeå University, SE-901 87 Umeå, Sweden
[3]Department of Plant Ecology, Evolutionary Biology Centre, Uppsala University, SE-752 36 Uppsala, Sweden

Abstract

Biochemical proxies are becoming increasingly common for growth assessment in zooplankton. Their suitability is often unknown, however, and proper calibration is lacking. We investigated correlations between physiological variables (ingestion, egg production, and respiration rates) and biochemical indices related to protein synthesis (RNA content, RNA:DNA ratio, RNA:protein ratio, and protein specific aminoacyl-tRNA synthetases [spAARS] activity) in copepods *Acartia bifilosa* exposed to different algal concentrations (0–1200 µg C L^{-1}). All variables assayed increased with increasing food concentration either linearly (spAARS) or nonlinearly (all other variables). Egg production and ingestion rates were significantly and positively correlated with RNA content and RNA:protein ratio, whereas correlations with spAARS and RNA:DNA ratio were weaker or nonsignificant. However, when RNA:DNA ratio and spAARS activity were used as predictors of ingestion, together they had higher explanatory value than did either variable separately. As there were substantial differences in saturating food concentrations among the assayed variables, applicability of biomarkers as proxies of physiological rates will be more reliable if restricted to the nonsaturated phase of the functional response of either variable, unless both variables saturate simultaneously. These findings contribute to methodology of zooplankton growth assessment and to our understanding of biochemical processes underlying growth and metabolism in copepods.

Introduction

Zooplankton, particularly copepods, are an important link between phytoplankton and higher trophic levels, such as zooplanktivorous fish. Because of this key position, zooplankton productivity assessment is important for understanding energy flows in pelagic food webs and ecosystem effects of both natural and human-induced perturbations. Therefore, methodological issues in this field have been addressed in many theoretical and empirical studies (Runge and Roff 2000 and references therein).

Currently, methods commonly used for assessing copepod production include the physiological method, the temperature-dependent models, and the egg production method (all reviewed by Runge and Roff 2000). Direct measurement of the egg production rate is the most common way to access in situ growth of copepods, whereas respiration and ingestion rates are also commonly used as metabolic activity indicators (Runge and Roff 2000). All these variables (i.e., egg production, ingestion, and respiration rates) respond positively to changes in feeding conditions, reaching a plateau at saturating food concentrations (Gaudy 1974; Kiørboe et al. 1985; Støttrup and Jensen 1990). Although these traditional methods are of great importance for understanding key mechanisms of physiological responses, the methods are by nature labor intensive and often difficult to use in field studies.

There is an increasing recognition that biochemical indices can provide a suitable alternative and/or complement existing methods, thus facilitating assessment of in situ growth and physiological condition in zooplankton (Runge and Roff 2000). It is also recognized that biochemical indices can provide reliable endpoints in toxicological assays (Dahl et al. 2006; Gardeström et al. 2006). Therefore, the rules that could be used to assess physiological variables from biochemical measurements need to be defined. Biochemical indices of

*Corresponding author: E-mail: towe@ecology.su.se

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physiological variables are based on key components of synthetic or metabolic pathways directly or indirectly linked to cell proliferation and synthesis of important biological macromolecules. In particular, concentration of ribonucleic acid (RNA) has been widely used as a sensitive indicator of the rate of copepod growth, both reproductive (Nakata et al. 1994; Saiz et al. 1998; Holmborn and Gorokhova 2008a, 2008b) and somatic (Wagner et al. 2001), and as a measure of metabolic activity in response to nutritional conditions (Wagner et al. 1998; Gorokhova et al. 2007) and environmental stress (Calliari et al. 2006), with the RNA concentration generally being expressed as the ratio of RNA to DNA or as the ratio of RNA to protein. The rationale is that total RNA content in a somatic cell is primarily a function of ribosome number correlating with protein synthesis, whereas the amount of DNA is quasi-constant and, therefore, an indicator of the cell number; the RNA:DNA ratio is thus an indicator of the amount of RNA per cell. The RNA:protein ratio is proportional to the number of ribosomes per amount of protein and, therefore, is a measure for the cytoplasmic ribosome concentration (Bremer and Dennis 1996). Metabolic enzymes, such as trypsin (Lemos et al. 2002), citrate synthase (Cullen et al. 2003), lactate dehydrogenase (Cullen et al. 2003), phosphofructokinase (Mayrand et al. 2000), and moulting enzyme chitobiase (Sastri and Roff 2000; Richards et al. 2008), have also been suggested as relevant proxies for somatic growth, physiological stress (chitobiase and phosphofructokinase), and metabolic activity (trypsin, citrate synthase, and lactate dehydrogenase) in crustaceans. The rationale of testing these enzymatic indicators is that elevated growth requires high ATP production, which in turn depends on the activity of glycolytic and mitochondrial enzymes.

Recently, another indicator of zooplankton growth based on the activity of the aminoacyl-tRNA synthetases (AARS) has been introduced and applied for fish (Vinayak and Sarkar 1999), cladocerans (Yebra and Hernández-León 2004), and copepods (Yebra et al. 2005). The AARS are a family of heterogeneous enzymes whose primary function is to catalyze an important step of protein synthesis: the aminoacylation of the tRNA, i.e., the activation and union of the amino acid to the tRNA (Ibba and Söll 2000). Thus, both AARS activity and RNA-based indices are biochemically related to protein synthesis.

Defining biochemical correlates of growth-related variables will facilitate appropriate application and interpretation of observed growth responses to environmental factors. Studies on this subject and cross-evaluations of different biochemical indices and physiological variables would also improve our understanding of cellular mechanisms underlying growth performance as well as linkages between different biochemical indices (Couture et al. 1998; Mayrand et al. 2000). Furthermore, to the best of our knowledge, no study has so far explored the possibility of using multiple biochemical indices to describe growth and metabolic activity in zooplankton. This approach, however, has recently been introduced in ecotoxicology for physiological stress evaluation (Guerlet et al. 2007). Also, in fish, multiple regressions significantly explained (*a*) variance in egg viability from ovarian fluid pH, protein levels, and activities of aspartate aminotransferase (Lahnsteiner et al. 1999) and (*b*) somatic growth from hepatosomic index, citrate synthase activity and water content in brain, liver, and muscle (Couture et al. 1998). In line with this, Weber et al. (2003) suggested that use of multiple indices (i.e., muscle RNA:DNA ratio, individual lipid and triglyceride contents, and muscle protein concentration) would provide a more complete assessment of the short-, medium-, and long-term growth potential in individual fish. In this study, we attempted to test if this approach, i.e., use of multiple biochemical indices, may improve assessment of growth and physiological condition in zooplankton.

The broadcast spawning calanoid copepod *Acartia bifilosa* (Giesbrecht) is one of a few dominant mesozooplankton species in the northern Baltic proper and a preferred prey for zooplanktivorous fish (Arrhenius 1996). Previously, application of RNA-based indices for egg production assessment in *A. bifilosa* has been attempted and found to be useful (Gorokhova 2003; Holmborn and Gorokhova 2008a, 2008b). Here, using *A. bifilosa* as a model species, we aimed to further investigate usefulness of some commonly used biochemical indices for growth and metabolic activity and provide evidence for their differential ability to respond to changes in copepod feeding and growth. Using adult females incubated at different food concentrations, we established correlations between physiological variables (ingestion, egg production, and respiration rates) and biochemical indices related to protein synthesis (RNA content, RNA:DNA ratio, RNA:protein ratio, and protein specific AARS activity [spAARS]) to evaluate usefulness of each proxy for predicting physiological variables. Further, we identified noncorrelating indices and used them jointly in multiple regression analysis to explore the possibilities of this approach.

Materials and procedures

Sampling and sorting of copepods—Sampling was carried out in the Himmerfjärden Bay, in the northwestern Baltic proper (58°59′N 17°44′E), in June 2007. Zooplankton were collected from the upper 10 m by vertical hauls using a WP-2 net (mesh size 200 µm, ∅ 57 cm) equipped with a cod-end. Surface water temperature during sampling was 16°C, and salinity was 6.3‰. The contents of the tows were placed in large (~20 L) insulated containers, diluted with surface water, and brought to the lab within a few hours. Containers were gently aerated until sorting commenced. The zooplankton were sieved, washed into a Petri dish, and sorted under a dissecting microscope (×40) using a wide-mouth pipette.

Algal cultures—The alga used in the experiment was *Tetraselmis suecica* (Prasinophyceae; CCMP908; National Center for Culture of Marine Phytoplankton) grown on f/2 medium with constant illumination (90 µmol photon PAR m^{-2} s^{-1}) at 16°C and 7‰ salinity in artificial seawater (Instant Ocean™,

Aquarium Systems). Cultures were maintained in extended exponential growth through a semicontinuous harvesting regimen (~30% exchange every other day). The algal concentrations (cells mL^{-1}) were determined using a laser particle counter, Spectrex PC-2000 (Spectrex).

Experimental setup—A timeline showing all steps of the experimental design is shown in Fig. 1. In the feeding experiment, ~30 adult females of similar size (prosome length 660 ± 4 µm [mean ± SD]; $n = 15$) and one to five males (to ensure fertilization) were placed in 1.2-L bottles, prepared in triplicate with dilutions of algal culture to nominal concentrations of 0, 30, 150, 600, and 1200 µg C L^{-1}. Concentrated algal growth medium was added to each bottle, sufficient to prevent nutrient limitation of algal growth during incubations. The copepods were acclimated to the respective food concentrations for 22–24 h. All incubations were carried out on a rotating plankton wheel (0.5 rpm) in an environmental chamber at the ambient temperature and salinity. After the acclimation period, the contents of the bottles were gently poured onto a submerged 200-µm sieve and rinsed with GF/F-filtered seawater into Petri dishes, where they were inspected using a dissecting microscope (×40). A few dead copepods were removed, and three females from each replicate were sampled for biochemical analyses. The remaining copepods were transferred to bottles with fresh algal suspensions at the respective concentrations. Two bottles of each food concentration without copepods served as controls. After another 24 h of incubation, the content of the bottles were sequentially filtered through submerged 200-µm (to collect copepods) and 35-µm (to collect eggs) sieves; the filtrate was collected, and algal concentration was measured by the particle counter. During the feeding experiment, the algal concentrations decreased by no more than 20%. The copepods were again inspected and mortality was recorded; survivorship was on average 96%, with no apparent trend among the food concentrations. From each replicate, three females were taken for biochemical analyses, while for respiration measurements females from two replicates per food concentration (10 individuals per replicate) were used.

Ingestion rate—The ingestion rate (I, µg C ind^{-1} d^{-1}) was calculated according to Frost (1972) using the algal concentrations (µg C L^{-1}) determined before and after the feeding experiment. The cell concentrations (cell mL^{-1}) measured by the particle counter were converted to carbon equivalents using the regression $\log_{10}C = 0.76 \times \log_{10}V - 0.29$ (Mullin et al. 1966), where C is carbon content in pg $cell^{-1}$ and V is cell volume in $µm^3$, obtained from the particle counter.

Egg production rate and viability—All eggs produced during the experiment were counted using a microscope (Leica DM IRB, ×100, or Wild M8 Heerbrugg, ×50), and for each replicate egg production rate (EPR, eggs ind^{-1} d^{-1}) was calculated as number of eggs produced per female over 24 h (Runge and Roff 2000). No signs of egg cannibalism were observed during the experiment.

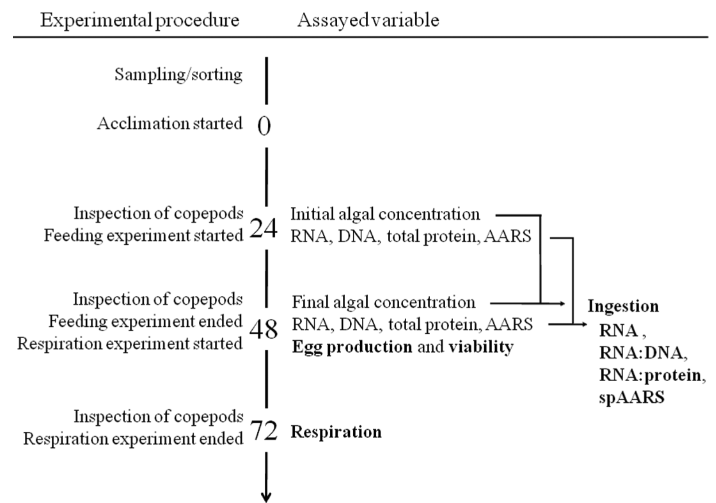

Fig. 1. Timeline showing different steps of the experimental setup and assayed variables. Numbers denote hours elapsed from the start of the experiment.

To assay egg viability, eggs were stained using fluorescence probes according to the method of Buttino et al. (2004). In replicates with <40 eggs, all eggs were used for staining, whereas for replicates with >40 eggs a random batch of 40 ± 5.5 (mean ± SE) eggs was used. The stained eggs were counted using an epifluorescence microscope with a blue filter (Leica DM IRB, ×100). The viability was calculated as percentage of viable eggs (% viable eggs).

Respiration rate—Measurement of respiration was carried out immediately after termination of the feeding experiment in vials filled with filtered seawater, as algae-free media was required to maintain stable conditions in the closed vials. Copepods (10 females from each of two replicates per food concentration) were incubated in 5-mL enclosed vials immersed in a water bath (15°C). Oxygen concentration in the vials was measured continuously over a 24-h period with point measurements every 5 min from each of 10 channels using a Fibox 3 fiberoptic oxygen meter (PreSens). Controls (five replicates) contained only filtered seawater and were monitored for 24 h to record the baseline. Respiration rates (R, nL O_2 ind^{-1} min^{-1}) were determined by regression analyses as the rate of change in oxygen concentration over 1 h, starting 1 h after the transfer to the respiration vials. Values from the first hour were disregarded, as they were likely to have been affected by handling stress and temperature artifacts, whereas measurements from the second hour were assumed to give the best representation of the respiration rates of copepods incubated at the respective food concentrations.

Biochemical assays (RNA, RNA:DNA, RNA:protein, and spAARS)—Females sampled at the end of the acclimation period and at the termination of the feeding experiment (three individuals per replicate on each sampling occasion), were snap-frozen and kept at –80°C until analyses. For extraction, the animals were placed in Eppendorf tubes containing

300 µL extraction buffer (1% *N*-laurylsarcosine in TE buffer) and subjected to a repeated (×3) sequence of ultrasound (30 s) and ice bath (1 min). Samples containing all chemicals without copepods served as negative controls. The samples were vortexed and centrifuged at 6000*g* for 10 min (0°C), and a 50-µL portion of the extract was subsampled and used for assessment of AARS activity. Subsequently, the remaining part of each sample was allowed to shake for 2 h at room temperature for nucleic acid and protein extraction.

Copepod RNA and DNA contents (µg ind^{-1}) were quantified using a microplate fluorometric high-range RiboGreen (Molecular Probes) assay according to Gorokhova and Kyle (2002). In brief, 10 µL of the extract was diluted with 60 µL TE buffer, and 70 µL RiboGreen reagent was added, followed by fluorescence measurement, incubation with 5 µL Rnase, and another fluorescence reading. Measurements were made in triplicate for each sample using FLUOstar Optima microplate reader (BMG Labtechnologies; filters: 485 nm for excitation and 520 nm for emission) and black solid flat-bottom microplates (Greiner Bio-One). Plates were scanned with 0.2-s well measurement time and 10 measurements per well. On each analytical occasion, wells containing samples, nucleic acid standards, and the negative controls were measured in parallel. The mean standard curve slope ratio (m_{DNA}/m_{RNA}), determined according to Caldarone et al. (2006), was 1.85.

Copepod protein content (µg ind^{-1}) was determined by a microplate fluorometric assay using the NanoOrange Protein Quantification kit (Molecular Probes) with bovine serum albumin standards (Jones et al. 2003) as directed by the manufacturer for microtiter assays. In brief, 10 µL extract was diluted in NanoOrange working solution to achieve a final volume of 130 µL. Samples were incubated at 95°C for 10 min and cooled to room temperature for 25 min (light protected). Then 120 µL sample was used for fluorescence measurements using FLUOstar Optima microplate reader (filters: 485 nm for excitation and 590 nm for emission) and black solid flat-bottom microplates (Greiner Bio-One) with an integration time of 1 s.

Protein specific AARS activity (spAARS, nmol PPi mg $protein^{-1}$ h^{-1}) was assayed following the method of Yebra and Hernández-León (2004). The assay measures the activity of different AARS based on the release of pyrophosphate (PPi) during aminoacylation of tRNA, assessed as the oxidation of NADH by PPi. The reaction was adapted to a microplate format by scaling all volumes back to a total reaction mixture of 150 µL $well^{-1}$. The 50-µL portion of the extract designated for AARS analysis was added to 60 µL Milli-Q water and 40 µL PPi reagent (Sigma) in UV-transparent flat-bottom microplates (Greiner Bio-One). The plates were incubated at room temperature for 1 min, and the extinction of the reaction mixture manifesting oxidation of NADH was measured using the microplate reader FLUOstar Optima at 340 nm (10 min, 37°C). The AARS activity in solution (nmol PPi mL^{-1} h^{-1}) was calculated using the equation given by Yebra and Hernández-León (2004) and normalized to protein concentrations, measured as described above, to arrive to spAARS activity.

Statistics—In all statistical analyses, the basic data unit for physiological variables was the postincubation point measurement, whereas for biochemical variables the basic data unit was the mean value of the bottle mean for pre- and postincubation. Our assumption was that the biochemical variables averaged over the incubation period would best represent the observed physiological rates. All significance levels were set at $P < 0.05$.

To evaluate responses in measured variables to increasing food concentration, functional response analyses, using Statistica version 8.0 (StatSoft, 2007), was applied. As there was high between-replicate variability, it was impossible to identify the exact type of functional response; therefore, after visual inspection of the data, curvilinear responses were assumed to follow a hyperbolic model: $y = y_{max} (1 - e^{-b [f + c]})$, where y is the response variable, e is the natural logarithm, y_{max} is the theoretical saturation value of the response variable, b and c are shaping constants, and f is food concentration. For I and EPR, the c constant was assumed to be 0, which implies $y = 0$, when $f = 0$. In this study, food concentrations corresponding to 95% or less of the y_{max} were considered as nonsaturating for a given response variable. The functional response for spAARS was not significantly different from linear, therefore, it was described using model $y = a \times f + b$, where a and b are constants and f is food concentration. As all variables, except for spAARS, showed a slight decline at the highest food concentration, the significance of this decline was tested. Because sample sizes were inadequate to determine adherence of the data to normality, Mann-Whitney *U*-tests with adjustments for small sample sizes (Statistica) were used. Differences in egg viability among food concentrations were evaluated using Fisher exact test in Stata Statistical Software, version 8 (StataCorp, 2003).

To investigate the relationships between biochemical indices and physiological variables, Pearson product moment correlation analyses (Statistica) was used. To improve normality and homogeneity, percentage of viable eggs were arcsine square root transformed, whereas all other data were $\log_{10}(x + 1)$ transformed. Pearson product moment correlations were also used to identify significant associations between RNA-based indices and spAARS. Consecutively, to explore the possibility of using multiple biochemical indices to describe a physiological variable, noncorrelating biochemical indices were used as independent variables and physiological variables as dependent variables in stepwise linear regression analysis (forward model, entry rule $F = 1.00$) on the $\log_{10}(x + 1)$–transformed data.

Assessment

Functional responses—All variables, except egg viability, responded positively to increasing food concentration, with linear (spAARS) or hyperbolic (all other variables) functional responses ($P < 0.05$; Fig. 2A–G, Table 1). The r values of the response curves ranged from 0.6 to 0.9, being highest for I, fol-

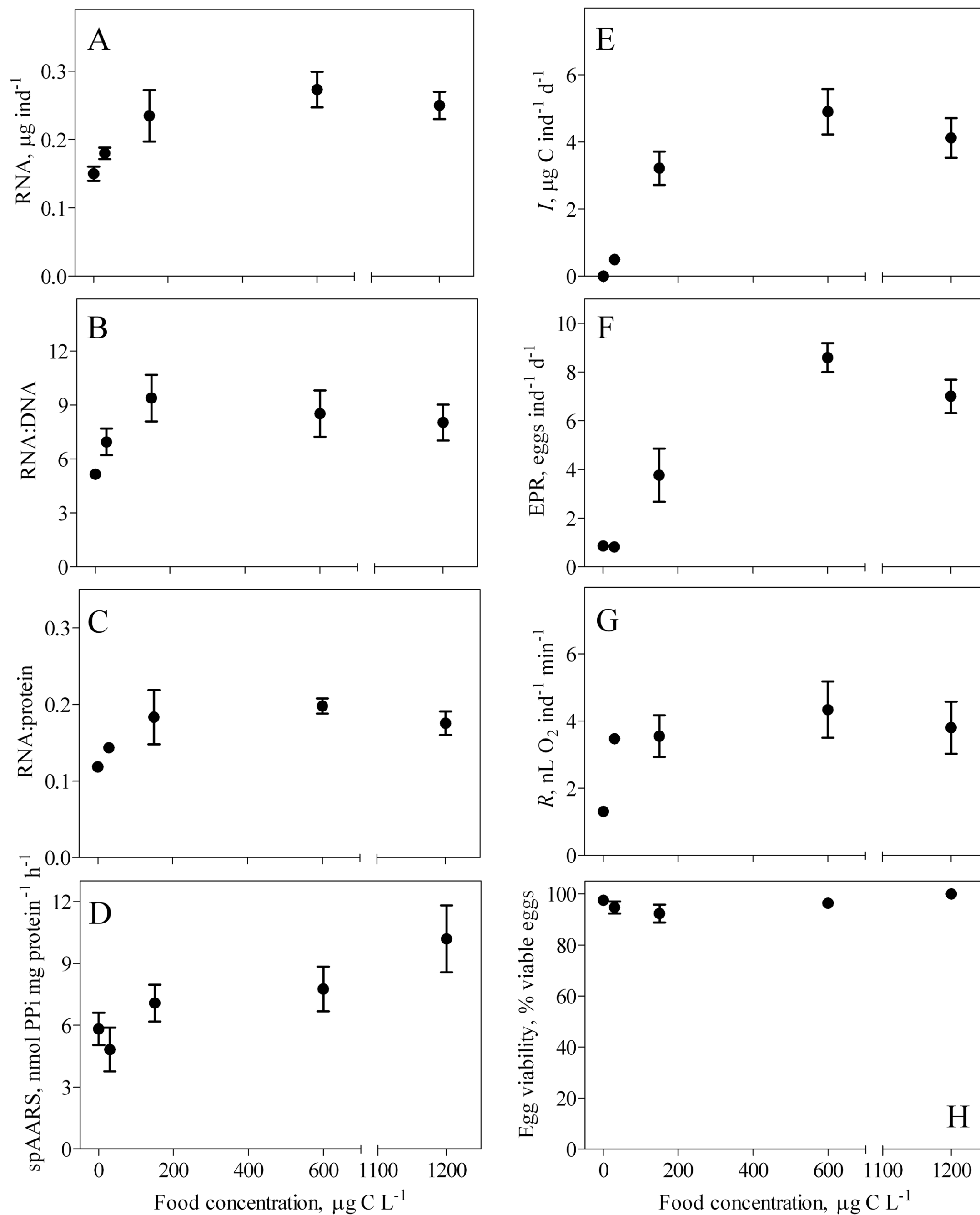

Fig. 2. *Acartia bifilosa*. Changes in biochemical (A–D) and physiological (E–H) variables in response to food concentration (µg C L^{-1}): RNA content, µg ind^{-1} (A), RNA:DNA ratio (B), RNA:protein ratio (C), spAARS activity, nmol PPi mg protein^{-1} h^{-1} (D), ingestion rate, *I*, µg C ind^{-1} d^{-1} (E), egg production rate, EPR, eggs ind^{-1} d^{-1} (F), respiration rate, *R*, nL O_2 ind^{-1} min^{-1} (G), and egg viability, % viable eggs (H). All data are presented as mean ± SE for each food concentration based on three replicates for all variables except *R* (two replicates).

lowed by EPR, *R*, RNA, RNA:protein, spAARS, and RNA:DNA. Although a slight decrease in all response variables, except for spAARS, was recorded at the highest food concentration compared to the next highest one, it was not statistically significant for any of the assayed variables ($P > 0.05$, Mann-Whitney *U*-test). Physiological variables reached 95% saturation at different food concentrations: $I_{95\%}$ (4.3 µg C ind^{-1} d^{-1}), $EPR_{95\%}$ (7.5 eggs ind^{-1} d^{-1}), and $R_{95\%}$ (3.7 nL O_2 ind^{-1} min^{-1}) were predicted to occur at algal concentrations of approximately 400, 600, and 45 µg C L^{-1}, respectively. RNA-based indices reached 95% saturation earlier than did *I* and EPR: $RNA_{95\%}$ (0.25 µg ind^{-1}), $RNA{:}DNA_{95\%}$ (8.2), and $RNA{:}protein_{95\%}$ (0.18) were predicted to

Table 1. *Acartia bifilosa*: functional response parameters.

	y_{max}/a	b	c	r
I	4.5 ± 0.40	0.0075 ± 0.0025	0	0.91
EPR	7.9 ± 0.72	0.0049 ± 0.0016	0	0.91
R	3.9 ± 0.42	0.060 ± 0.064	6.8 ± 8.9	0.76
RNA	0.26 ± 0.019	0.0099 ± 0.0076	87 ± 75	0.74
RNA:DNA	8.6 ± 0.69	0.028 ± 0.029	32 ± 39	0.61
RNA:protein	0.19 ± 0.014	0.016 ± 0.017	60 ± 69	0.66
spAARS	0.0038 ± 0.0012	5.6 ± 0.75	—	0.65

Ingestion rate (I, μg C ind^{-1} d^{-1}), egg production rate (EPR, eggs ind^{-1} d^{-1}), respiration rate (R, nL O_2 ind^{-1} min^{-1}), and biochemical indices (RNA content [μg ind^{-1}], RNA:DNA ratio, RNA:protein ratio, and spAARS activity [nmol PPi mg protein^{-1} h^{-1}]) as a function of food concentration (μg C L^{-1}). A hyperbolic functional response; $y = y_{max}(1 - e^{-b[f+c]})$ where y is the response variable, e is the natural logarithm, y_{max} is the theoretical saturation value of the response variable, b, and c are shaping constants (mean ± SE), and f is food concentration, was applied for all variables except for spAARS. The functional response of spAARS was described using a linear response ($y = a \times f + b$) where a and b are constants and f is food concentration. All analyses were significant ($P < 0.05$); n = 15 in all cases except R, where n = 10.

occur at algal concentrations of approximately 220, 80, and 120 μg C L^{-1}, respectively. The egg viability at all food concentrations was high, ranging from 85% to 100%, with an average of 96% (Fig. 2H), and no significant response to the increased food concentrations ($P > 0.05$, Fisher exact test) was shown. Therefore, no correction for egg mortality was applied to the egg production rates.

Correlations and regressions—Pearson product moment correlation analyses revealed that I was significantly and positively related to all measured biochemical indices ($P < 0.05$ in all cases), with r values being highest for RNA and RNA:protein (0.74 and 0.70, respectively; Table 2). EPR was significantly related to RNA, RNA:protein, and spAARS, with r values of 0.78, 0.68, and 0.59, respectively ($P < 0.05$; Table 2), whereas R and egg viability were not significantly related ($P >$ 0.05 in all cases) to any of the biochemical indices explored in this study (Table 2).

To achieve a better prediction of a physiological variable, a multiple linear regression model could be applied, provided that independent variables are not intercorrelated. The correlation analyses for biochemical indices assayed independently (i.e., RNA-based indices versus spAARS) indicated that RNA and RNA:protein significantly correlated with spAARS (r = 0.53 and 0.54 respectively; $P < 0.05$ in both cases, Table 2). This precludes, due to the multicolinearity, joint use of RNA and spAARS or RNA:protein and spAARS in a multiple regression model. By contrast, RNA:DNA was not correlated to spAARS ($P > 0.05$, Table 2), and therefore these two indices could be used in concert. Consequently, to explore the possibility of such combined models in describing ingestion from RNA:DNA and spAARS, stepwise linear regression analysis was performed. Together, these two independent variables explained 43% (adjusted r^2), of the variation in I ($P < 0.013$; $\beta_{RNA:DNA}$ 0.43 ± 0.21, β_{spAARS} 0.45 ± 0.21). Multiple regression analyses was not applied to EPR and R because there were no significant correlations between either RNA:DNA or spAARS and these physiological variables (Table 2).

Discussion

In *Acartia bifilosa,* RNA content and RNA:protein ratio correlated most strongly with ingestion and egg production rates, whereas no significant correlations were obtained for respiration and egg viability. Although with a much lower explanatory value, ingestion rate was also significantly and positively related to RNA:DNA ratio and spAARS activity; the latter also had a significant positive correlation with egg production rate. These results agree with previous studies on copepods showing that RNA content explains higher percentages of variation in egg production rate than does RNA:DNA ratio (*A. bifilosa,* 92% and 75% for RNA and RNA:DNA, respectively; Gorokhova 2003) and spAARS activity (*Calanus helgolandicus*, 40%; Yebra et al. 2005). Interestingly, RNA:DNA ratio, which was the least accurate indicator of ingestion and egg production rates, had by far the highest correlation to respiration rate, although only marginally significant ($P = 0.06$, $r = 0.61$). The lack of significance in all correlations for respiration rate does not imply that biochemical indices cannot be used for assessing respiration. For example, Donnelly et al. (2004) investigated several biochemical indices in a variety of crustaceans and found that protein, RNA and DNA contents, and their ratio were significantly related to respiration. In their study, however, a large variability in the predictive capacity of these indices was observed among different species, ranging from nonsignificant to highly significant. These results indicate complex and species-specific relationships between respiration and indices related to protein synthesis. It is also possible that other biochemical proxies are more suitable for assessment of respiration in copepods than the biochemical indices tested in present study.

As shown by the multiple linear regression analysis, it is possible to use several biochemical indices in concert to better describe a physiological variable. Indeed, together spAARS and RNA:DNA ratio significantly explained 43% of the variation in ingestion rate, which is more than did either RNA:DNA ratio or spAARS in univariate correlations with ingestion rate (32%

Table 2. *Acartia bifilosa*: Pearson product moment correlation coefficients (*r*) between different variables.

	RNA		RNA:DNA		RNA:protein		spAARS	
	P	*r*	*P*	*r*	*P*	*r*	*P*	*r*
I	0.0018[a]	0.74	0.026[a]	0.57	0.0037[a]	0.70	0.021[a]	0.59
EPR	0.0006[a]	0.78	0.070	0.48	0.0053[a]	0.68	0.020[a]	0.59
R	0.25	0.40	0.064	0.61	0.30	0.36	0.48	0.25
% viable eggs	0.69	0.11	0.60	–0.15	0.87	–0.045	0.21	0.35
spAARS	0.044[a]	0.53	0.26	0.31	0.039[a]	0.54	—	—

Variables used in the analyses: ingestion rate (*I*, µg C ind^{-1} d^{-1}), egg production rate (EPR, eggs ind^{-1} d^{-1}), respiration rate (*R*, nL O_2 ind^{-1} min^{-1}), RNA content (µg ind^{-1}), RNA:DNA ratio, RNA:protein ratio, and spAARS activity (nmol PPi mg protein^{-1} h^{-1}). n = 15 in all cases except correlations with *R*, where n = 10. [a]Significant correlations.

and 35% [r^2], respectively; Table 2). Although the explanatory value obtained in the multiple linear regression analysis is still lower than the squared correlation coefficients between ingestion and either RNA content or RNA:protein ratio (55% and 49%, respectively), these results support the use of multiple biochemical indices for growth and metabolic activity assessment in copepods—an approach previously suggested for assessment of egg viability (Lahnsteiner et al. 1999), physiological condition, and growth (Weber et al. 2003; Couture et al. 1998) in fish.

Studies on functional responses in *Acartia bifilosa* are scarce and provide little data for direct comparisons. Nevertheless, the ingestion rates observed in our study are similar to those reported for this species at similar food concentrations (Uriarte et al. 1998). Moreover, the ingestion rates as well as the food concentration at 95% of the theoretical maximal ingestion rate are within the range reported for this genus, albeit in the lower part of the range (*A. tonsa*, Thor et al. 2002 and Calliari et al. 2006; *A. clausi*, Calliari et al. 2006); the latter is most probably related to the generally smaller body size in *A. bifilosa*. The observed egg production rates correspond well to those described previously for this species (Koski and Kuosa 1999; Koski et al. 2002; Gorokhova 2003; Holmborn and Gorokhova 2008a, 2008b). Although in most of these studies the food media was natural plankton assemblage, whereas algal monoculture was used in our study, these similarities indicate relevance of our findings to in situ situations. For respiration rate, there are no comparable datasets for *A. bifilosa*, but a few exist on congeners *A. tonsa* (Thor et al. 2002; Kiørboe et al. 1985) and *A. clausi* (Calliari et al. 2006). The respiration rate measured in our study was in the same range, albeit in the higher part of the range, or slightly above the range reported previously for other *Acartia* species (*A. tonsa*, Kiørboe et al. 1985, Thor et al. 2002, and Calliari et al. 2006; *A. clausi*, Calliari et al. 2006). The resolution of this response to food availability was poorer, however, with the only apparent difference observed between the starved and fed animals (Fig. 2G). Moreover, an estimated 95% of the theoretical maximal respiration observed by Thor and co-workers (2002, *A. tonsa*) would occur at a food concentration of 295 µg C L^{-1}, whereas our estimate of this value was much lower, 45 µg C L^{-1}. It is possible that the generally high respiration and weak coupling to food availability in our study is related to the low number of treatments, particularly in the lower range of the food concentrations, and to the low number of replicates, which were constrained by limitations of the equipment.

The differences in functional responses to food availability between the variables (Fig. 2; Table 1) help explain the observed correlation patterns and the differential ability of biochemical indices to predict changes in specific physiological variables (Table 2). In particular, RNA-based indices were predicted to reach their 95% saturation at lower food concentrations (80–220 µg C L^{-1}) than did the egg production and ingestion rates (600 and 400 µg C L^{-1}, respectively). This implies that the best correlations should occur between those variables that saturate at most similar algal concentrations, which is indeed the case here. As saturating concentration of RNA (220 µg C L^{-1}) was the closest to that of ingestion (400 µg C L^{-1}) and egg production (600 µg C L^{-1}), their correlations with RNA were greater than with any other biochemical index (Table 2). Similarly, RNA:DNA ratio was the best correlate of respiration rate, as both variables saturated at low algal concentrations (80 and 45 µg C L^{-1}, respectively). Furthermore, there was a discrepancy between the saturating functional responses of the physiological variables and that of spAARS activity, with a positive slope throughout the entire range of the food concentrations. Consequently, the correlations and hence predictive capacity of the biochemical variables would be stronger if only the increasing parts of the functional response curves, representing nonsaturating food concentrations, were considered. Unfortunately, our experimental design had too few treatments in the nonsaturating food concentrations to make meaningful correlations based on data from only these parts of the functional responses.

To the best of our knowledge, our study is one of very few to compare the usefulness of different biochemical indices to assess feeding, metabolism, and egg production in copepods (see also Biegala et al. 1999) and to evaluate the use of multiple biochemical indices for this purpose. The results suggest that among the indices tested, individual RNA content and

RNA:protein ratio are the most useful indicators of ingestion and egg production rates in copepods, whereas spAARS activity and RNA:DNA ratio are less reliable. None of the tested indices, however, were efficient for identifying changes in respiration and egg viability under the experimental conditions used in our study. Moreover, joint use of biochemical indices could be beneficial to improve the predictive ability of the biochemical assays. This prompts the need for investigating other biochemical indices of growth, e.g., those related to ATP or DNA synthesis, as these will be less likely to intercorrelate with indices related to protein synthesis and, therefore, might be particularly useful as covariables in multiple linear regressions. However, an important consideration in application of these (and likely other) biochemical indices for feeding and growth studies is differences between predictor and outcome variables in their functional responses to food availability. In coastal areas of the northern Baltic proper, phytoplankton (>2 µm) biomass is typically in the range of 10–300 µg C L^{-1} (monthly means, 1977–1989, Hajdu et al. 1997), with the highest values occurring during diatom spring blooms (up to 800 µg C L^{-1}, Larsson et al. 2006 and Hajdu et al. 1997), whereas open Baltic areas generally have lower algal concentrations. Therefore, prior knowledge about the saturating conditions for both a predictor and an outcome variable is needed when interpreting biochemical indices obtained in situ, especially during spring and summer blooms, as well as in laboratory experiments simulating algal blooms. For example, the saturating food concentration for RNA (220 µg C L^{-1}) would set the upper limit for the applicability of this index to infer ingestion and egg production in *A. bifilosa*. Moreover, it is possible that natural food assemblages would provide a higher quality food, resulting in lower saturating food concentrations. Field observations support this notion: during a natural spring phytoplankton bloom in the northern Baltic proper, RNA levels of *A. bifilosa* reached their 95% saturation at an algal concentration of approximately 4.4 µg chlorophyll *a* (Chl *a*) L^{-1} (Gorokhova et al. 2007), i.e., about 130 µg C L^{-1}, assuming an average carbon:Chl *a* ratio of 30 as suggested by data from the same study (see their Figs. 2 and 3); this value is nearly twice as low as what was observed in the present study, where algal monoculture was used as food.

These findings contribute to the development of methods for in situ assessment of copepod physiological status in field and laboratory studies. Other potential applications of these findings include ecotoxicological studies and aquaculture, where correctly selected biochemical proxies could provide useful endpoints of a toxicological assay (Dahl et al. 2006; Gardeström et al. 2006) and give insight into mechanisms behind changes in population stocks and effects of different factors on production of a cultured species (Lemos et al. 2002). Several other factors, however, such as developmental stage, body size, food quality, temperature, and salinity, may affect the relationships between physiological and biochemical variables (Holmborn and Gorokhova 2008a, 2008b), and the effects are likely to be species-specific. The development of robust predictive models based on biochemical proxies requires a thorough validation and understanding of the mechanisms behind the prognostic values.

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