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Use of RNA:DNA ratios as an indicator of nutritional stress in the American oyster *Crassostrea virginica**

D. A. Wright & E. W. Hetzel

University of Maryland Chesapeake Biological Laboratory, Center for Environmental and Estuarine Studies, Solomons, Maryland 20688–0038, USA

ABSTRACT: Hatchery spawned oysters approximately 1½ yr old were subjected to starvation stress by exposing them to 1 µm filtered seawater over an 8 wk period. Control (fed) oysters were exposed to unfiltered flowing seawater. RNA:DNA ratios in mantle tissue from fed and starved oysters were monitored throughout, together with shell length, width, height, whole specimen displacement and shell displacement. Wet weight was estimated from whole weight determinations made before and at the end of the experiment. From Day −4 (before the start of the experiment) to Day 52, there was a 32 % increase in mean weight of fed animals and a 12.6 % loss in wet weight of starved animals. Rate of wet weight growth was significantly less in starved oysters than in fed oysters after Day 3. Shell height increased in fed oysters, but remained more constant in starved specimens. Shell lengths were significantly different from Day 42. Condition index, defined as ratio of wet tissue weight to cavity volume, decreased rapidly in starved oysters and became significantly less than in fed oysters. Two sample t-tests between treatments showed that there was a significant difference in RNA:DNA ratio between treatments on all sampling days except Day 0. By Day 55, starved oysters had 36.5 % lower ratios than fed oysters. Over the 55 day experimental period the RNA:DNA ratio correlated highly with condition index, but was lower in response to starvation than in reported studies on vertebrates.

INTRODUCTION

Several investigations have shown that a sub-lethal stress can reduce the amount of energy surplus to the animal's maintenance requirements at the expense of fecundity and larval viability (Bayne 1972, 1975, Helm et al. 1973, Bayne et al. 1975, 1978). Bayne and co-workers (Bayne & Widdows 1978, Bayne et al. 1979) have quantified this energy surplus as a 'scope for growth' i.e. the amount of energy available for somatic and germinal growth. 'Scope for growth' measurements have proved to be extremely accurate in assessing physiological condition, although they are relatively time consuming and there is an attraction towards comparatively quick cytological and biochemical observations such as lysosomal enzyme latency (Moore et al. 1978, 1982). Such measures of condition have proven effective under conditions of both starvation and pollution stress (Bayne et al. 1979).

The ratio of RNA to DNA present in tissues has been used as an index of growth rate in fish, crabs, and zooplankton (Sutcliffe 1965, Leick 1968, Regnault & Luguet 1974, Sulkin et al. 1975, Buckley 1979, 1980, 1982, 1984) but the application of this technique to bivalve molluscs has been limited. RNA serves as the machinery required for protein synthesis and the amounts of RNA present have been shown to be directly related to the rate of protein synthesis (Munro & Fleck 1966, Kayes 1979). Work on larval fish by Buckley (1979, 1980, 1982, 1984), Buckley et al. (1984) and Wright & Martin (1985) have shown the technique to be rapidly responsive to changes in growth rate, and capable of providing immediate data on growth rate. These properties render the technique particularly suitable for assessing stress in field populations although prior laboratory calibrating is necessary. The current study was designed to test the validity of the technique by comparing RNA:DNA ratios with mor-

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phometric growth parameters in 2 groups of oysters under well-fed and starvation-stressed conditions.

METHODS

Experimental. Oysters were obtained from Wildi Oysters Inc. of Shady Side, Maryland. These were cultchless hatchery-spawned, single-parent oysters, set in the summer of 1980. These oysters were selected due to their known genetic history and the extraordinary similarity of their physical characteristics. Oysters were kept in ambient flow-through seawater tables at ambient temperature and salinity prior to acclimation.

In order to acclimate the oysters to higher experimental temperatures, on January 12, 1982 they were placed into flowing heated seawater and the temperature gradually raised from 1 °C over 18 d to 14.5 °C. At the beginning and end of this acclimation period, 28 oysters were randomly selected and their shell length, width, weight, whole weight, tissue wet and dry weight, whole specimen displacement, and shell displacement measured. Displacement was measured after the method of Galtsoff (1964) and Roosenburg (1969). Cavity volume was calculated as the difference between the whole specimen and shell displacement. Four d prior to the start of the experiment (Day −4) the shell characteristics of all oysters were recorded, as before, with the exception that no tissue weights or displacements were measured. At this time the oysters' shells were cleaned and numbered. On Day −1, 25 oysters were selected and their dimensions measured as at the start of acclimation. A regression analysis equation of whole weight *versus* wet weight, from the post-acclimation oysters, was used to estimate the initial wet weights of the oysters.

Half of the oysters were randomly selected on Day 0, and separated into heated, filtered flowing seawater. Seawater was filtered through a 5 μm filter system followed by 2 AMF-Cuno Microwynd II, 1 μm filters. Flow rate through the filters was maintained at approximately 12 l h^{-1}, while the ambient tank received 360 l h^{-1}. Temperature in both seawater tables was maintained at 14.5 °C.

Particulate carbon and nitrogen levels were periodically measured in both the filtered and ambient tanks. This was performed by filtering the water through a pre-combusted Whatman GFC filter (<1.2 μm) to remove particulates. The filter was then analysed for carbon and nitrogen content by high temperature combustion, using a Perkin-Elmer elemental analyser (Model 240B).

Throughout this paper, oysters maintained in the heated filtered seawater tables will be referred to as 'starved oysters', while oysters from the heated ambient seawater tables will be referred to as 'fed oysters'. On Days 0, 3, 6, 9, 13, 17, 21, 28, 35, 42 and 55 (number of days from the start of the experiment), 4 starved and 4 fed oysters were randomly selected from the seawater tables. These had their morphometric dimensions measured as for acclimation measurements, except that no dry weights were determined. A condition index was calculated as ratio of wet tissue weight to cavity volume of each oyster. Oysters were cleaned, shucked and their mantle tissue excised and weighed. If there was gonadal intrusion into the mantle, this part of the mantle was not removed. Excised mantle tissue was immediately frozen in liquid nitrogen, then kept at <−20 °C. Mantle tissue was homogenized within 1 h of excision. Frozen mantle tissue was homogenized in enough <4 °C distilled water to yield an approximate tissue concentration of 10 mg wet tissue weight (WTW) ml^{-1}. The homogenate was placed immediately into plastic, screw-top scintillation vials, frozen in liquid nitrogen and stored at <−20 °C until analysis. RNA and DNA analyses were performed the following day on three 5 ml aliquots of homogenate from each oyster sampled. Other aliquots of homogenate were dried at 50 °C for 72 h to determine the dry weight of the homogenate. Protein assays were conducted 2 d following sampling using a 0.5 ml aliquot of homogenate.

On Day 52, 25 oysters from each treatment were randomly selected, and their morphometric dimensions measured as for acclimation analysis. On Day 55, 12 starved oysters were randomly selected and placed into the ambient seawater table to be re-fed. Re-fed oysters were sampled 3 and 7 d later as before (Days 58 and 62).

Assays. Analysis of RNA and DNA was performed using the Schmitt-Thanhauser procedure of Munro & Fleck (1966), as modified by Buckley (1979). The procedure is illustrated in Table 1. Protein was measured using the Lowry et al. (1951) procedure as modified by Hartree (1972). Absorbancies in both procedures were read with a Gilford Model 240 UV-Vis. spectrophotometer with 1 cm pathlength cuvets.

Mantle tissue was selected for analysis due to (1) its accessibility, and (2) the likelihood of it reflecting growth through its rapid production of shell in growing oysters. Mantle tissue samples were approximately 50 and 5 mg wet tissue weight (WTW) for all RNA:DNA and protein assays, respectively. The amount of tissue was kept constant to avoid possible changes in recovery using differing amounts of tissue. Triplicate analyses were performed on each of the 4 specimens randomly selected from each treatment.

Data analysis. The statistical analyses employed were as suggested in Sokal & Rohlf (1973) and Atchley et al. (1976).

Table 1. Analytical procedure for RNA : DNA determinations

Homogenize approximately 0.20 g mantle tissue in 20 ml cold (< 4 °C) distilled water

↓

Place homogenate in scintillation vial and freeze immediately in liquid nitrogen

↓

Store homogenate at < − 20 °C. Rapidly thaw homogenate and pipette 5 ml aliquots into centrifuge tubes. Cool tubes to < 4 °C. Refreeze remaining homogenate for later protein and dry weight determination

↓

Add 2.5 ml cold (< 4 °C) 0.6N perchloric acid (PCA) to each tube and let stand 10 min

↓

Centrifuge tubes at 6000 g for 10 min. Discard supernatant (contains acid-soluble free nucleotides and sugars)

↓

Wash (vortex) twice with 2 ml cold 0.2N PCA and centrifuge both times as before. Discard supernatants

↓

Resuspend pellet in 2 ml 0.3N KOH and heat at 37 °C for 1 h (hydrolyzes RNA)

↓

Cool to < 4 °C. Add 1.25 ml cold PCA. Stand 10 min at < 4 °C

↓

Centrifuge as before

↓

Collect supernatant in separate test tube (will now contain acid-soluble RNA)

↓

Wash pellet twice with 2 ml 0.2N PCA. Centrifuge after each washing and combine all 3 supernatants

↓

PELLET	SUPERNATANT
↓	↓
Mix pellet with 3 ml 0.6N PCA	Add 2.75 ml 0.25N KOH to combined supernatants
↓	↓
Heat at 85 °C for 15 min	Measure absorbance at 260 nm against suitable blank for RNA
↓	↓
Cool in ice water for 15 min	Remove 0.818 ml sample from cuvet and pipette into clean test tube
↓	↓
Centifuge at 27000 g for 5 min	Add 0.183 ml 1N NaOH to test tube (final volume 1 ml, normality 0.1 N base)
↓	↓
Read at 260 nm against 0.6N PCA blank for DNA	Analyze for protein to correct its absorbance at 260 nm
↓	
Remove 0.562 ml sample from cuvet and pipette into clean test tubes	
↓	
Add 0.474 ml 1N NaOH (final volume 1 ml, normality 0.1 N base)	
↓	
Analyze for protein to correct for its absorbance at 260 nm	

A significance level of 0.05 was used for all statistical analyses. Paired student's 2-tailed t-tests were performed between treatments of each day's results. Where student's t-tests 1- or 2-way ANOVAs were performed, the normality of the data was first determined using an F_{max} Test. Where data sets failed the F_{max} Test, a Wilson's non parametric ANOVA was performed.

Table 2. *Crassostrea virginica*. Morphometric measurements of acclimation and post-acclimation oysters

	Whole weight (g)	Wet weight (g)	Dry weight (g)	Height (mm)	Length (mm)	Width (mm)	Cavity volume (ml)
Pre-acclimation	21.70±1.66	3.96±0.30	0.83±0.062	65.49±1.93	41.13±0.74	16.87±0.66	9.36±0.81
Post-acclimation	22.06±1.05	4.27±0.24	0.93±0.056	65.74±1.43	42.82±0.96	16.61±0.53	9.41±0.65
Post-experimental (fed)	27.00±1.23	5.65±0.32	1.35±0.081	69.38±3.03	51.45±1.13	17.97±0.53	9.56±0.65
Post-experimental (starved)	22.29±1.04	3.73±0.21	0.65±0.041	64.12±1.23	42.62±0.97	17.29±0.52	8.70±0.63

No significant differences between any pre- and post-acclimation measurements. All fed post-experimental measurements significantly greater than starved measurements, except for shell width and cavity volume

RESULTS

Table 2 describes morphometric data for specimens before and after the acclimation and experimental periods. The regression equation used to estimate initial wet weights from whole weight measures (see 'Methods') was:

$$y = 0.197\ x - 0.834\ (r^2 = 0.776) \quad (1)$$

where y = wet weight (g); x = whole weight (g). A comparison of 6 different regression models indicated this to be the best fit to the data. With the exception of shell width and cavity volume, all morphometric measurements in fed animals were significantly greater than in starved animals. Little change was observed in starved oysters except for a decline in wet tissue weight during the experiment. Over the period Day −4 to Day 52 there was a 32.3 % increase in mean wet weight in fed animals and 12.6 % decrease in wet tissue weight in starved animals. In the regular sampling day data there was considerable variation in the wet tissue weight data (Fig. 1 A). Starved oyster wet tissue weight decreased significantly relative to fed oysters. Mean losses in wet tissue weight of starved oysters ranged from 1.0 to 35.4 %. Fed oysters gained wet weight during the experimental period, with increases ranging from 3.4 to 51.3 %. Shell height increased in fed oysters while remaining more constant in starved animals (Fig. 1 B). By Day 55, shell height had increased by 12.63 ± 3.00 and 4.73 ± 4.34 % in fed and starved oysters respectively, although differences in shell height were only significant on Days 23 and 58 as determined by paired t-tests. Shell lengths were significantly different after, and including, Day 42 (Fig. 1 C). By this day shell lengths had increased by 27.4 ± 8.3 and 4.9 ± 3.2 % in fed and starved groups respectively.

Regression equations expressing growth days *versus* associated mean percentage change in wet weight for fed and starved oysters were:

$$y = 0.546\ x + 4.106\ (r^2 = 0.62,\ n = 11) \quad (2)$$

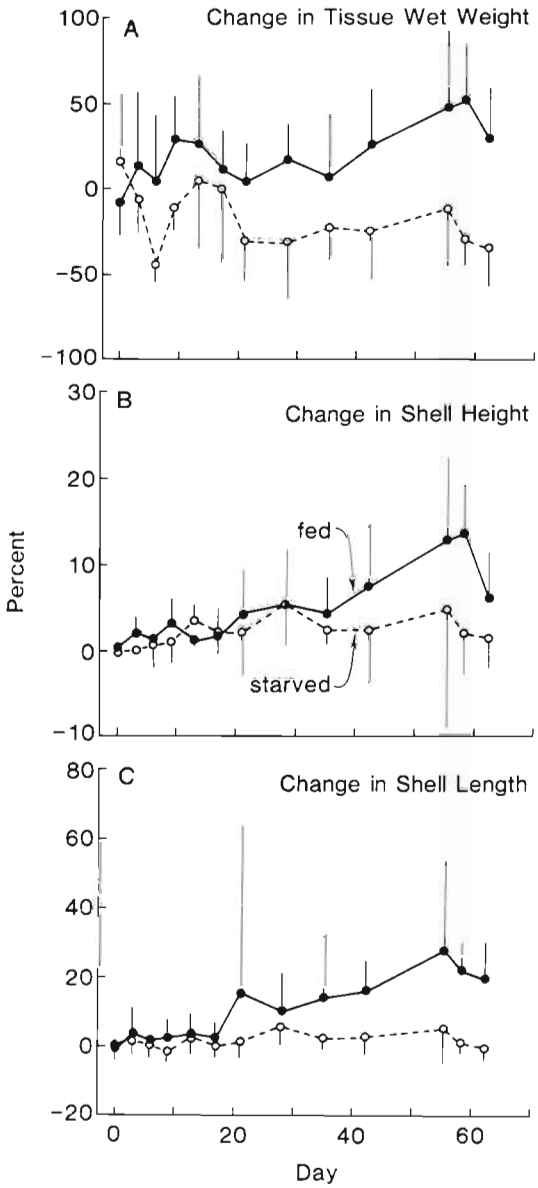

Fig. 1. *Crassostrea virginica*. Percent changes in wet weight and shell dimensions of oysters over experimental period. Vertical lines: ± 1 SD (n = 4)

and

$$y = 0.275\ x - 10.32\ (r^2 = 0.28,\ n = 11) \quad (3)$$

respectively; the slopes represented an average 'percent growth rate' of wet tissue from Day 0 to 55. Rate of wet weight growth was significantly less in starved oysters relative to fed oysters after Day 3 (Fig. 2 A). Rate of growth in the fed oysters initially fluctuated then leveled off at approximately 0.5 % d^{-1} after Day 17. Growth rates of starved oysters initially decreased then increased slightly, but also became fairly constant after Day 17, at approximately −0.5 % d^{-1}. Rates of percent increase in both shell height and length (Fig. 2 B, C) were significantly different between treatments.

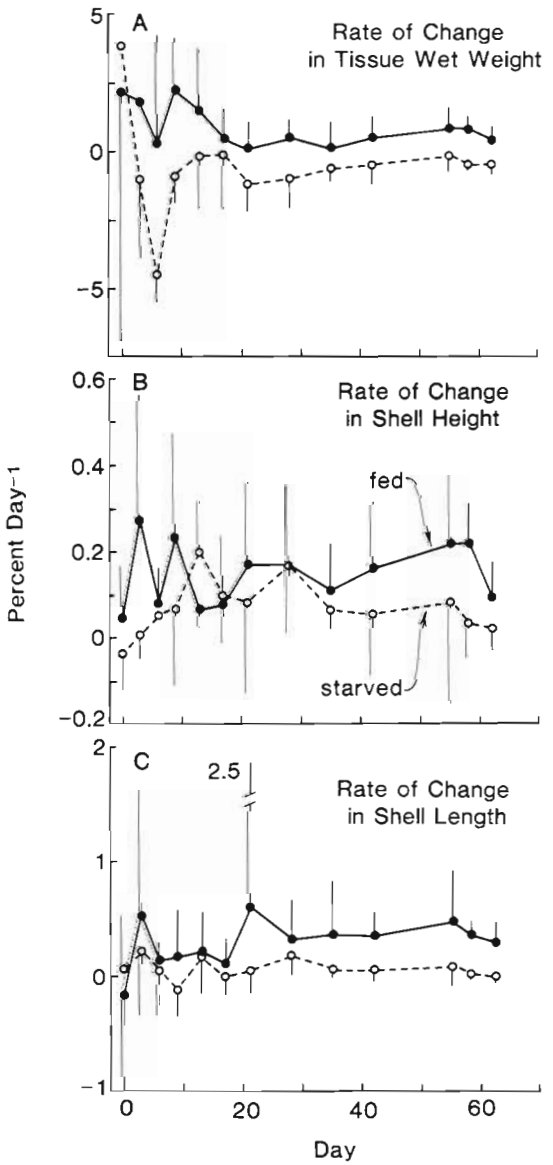

Fig. 2. *Crassostrea virginica*. Rate of growth of various morphometic parameters over experimental period. Vertical lines: ± 1 SD (n = 4)

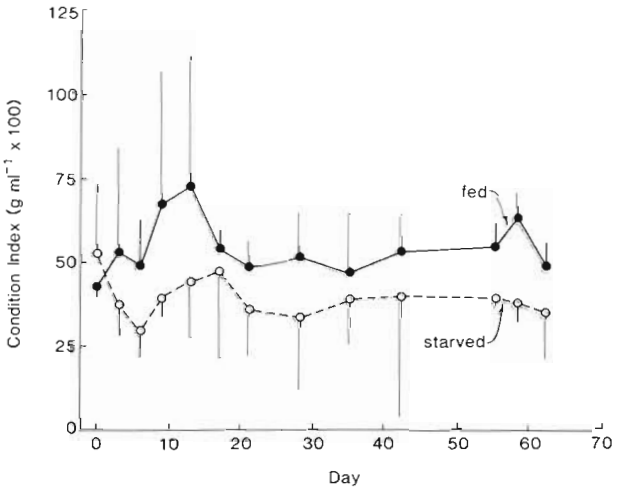

Fig. 3. *Crassostrea virginica*. Condition indices (ratio of wet tissue weight: cavity volume) in oysters over experimental period. Vertical lines: ± 1 SD (n = 4)

Condition index

Condition decreased rapidly in starved oysters, then rose slightly, followed by relative constancy after Day 17 (Fig. 3). Individual paired t-tests indicated that starved oysters had a significantly lower condition on Days 6, 9, 13, 21, 28, 55, 58 and 62. Two-way ANOVA indicated a significant difference between fed and starved animals. After Day 17, condition indices fluctuated at approximately 50 in fed oysters and between 35 and 40 in starved oysters. There was a 0.64 correlation between condition in all samples to Day 55 and rate of increase (%) of wet tissue.

Nucleic acids

Fig. 4 traces RNA and DNA levels and RNA:DNA ratios in oysters throughout the 55 d experimental period. Two sample t-tests between treatments showed that there was a significant difference in RNA:DNA ratio between treatments on all sampling days except for Day 0. Both starved and fed oysters showed a decline from initial ratios of 3.59 ± 0.04 and 3.37 ± 0.24 respectively, but starved oysters declined to a much greater extent. By Day 55, starved oysters had 36.5 % lower ratios than fed oysters. Refeeding starved oysters on Day 55 did not result in a noticeable change in ratios.

Protein accounted for approximately 7.08 % of the tissue wet weight. Levels of protein remained fairly constant throughout the experimental period. No significant differences between treatments were observed.

In Table 3 product-moment correlation coefficients between RNA:DNA ratios, DNA and various mor-

Table 3. *Crassostrea virginica.* Correlations between morphometric growth rates, condition index, and nucleic acid ratio. All points from Day 0 to 55: correlations significant above 0.217 (n = 86)

	RNA : DNA ratio	Net change shell height (% d^{-1})	Net change shell length (% d^{-1})	Net change shell width (% d^{-1})	Net change shell weight (% d^{-1})	Condition index (g ml^{-1})	DNA (µg mg^{-1})
RNA : DNA ratio	1.000	0.111	− 0.152	− 0.099	0.300	0.396	− 0.462
Net change shell height (% d^{-1})	0.111	1.000	0.486	0.091	0.174	0.182	− 0.029
Net change shell length (% d^{-1})	0.152	0.486	1.000	0.120	0.063	0.087	− 0.124
Net change shell width (% d^{-1})	− 0.099	0.091	0.120	1.000	− 0.168	− 0.021	0.000
Net change shell weight (% d^{-1})	0.300	0.174	0.063	− 0.168	1.000	0.643	0.195
Condition index (g ml^{-1})	0.396	0.182	0.087	− 0.021	0.643	1.000	− 0.289
DNA (µg mg^{-1})	− 0.452	− 0.029	− 0.124	0.000	− 0.201	− 0.289	1.000

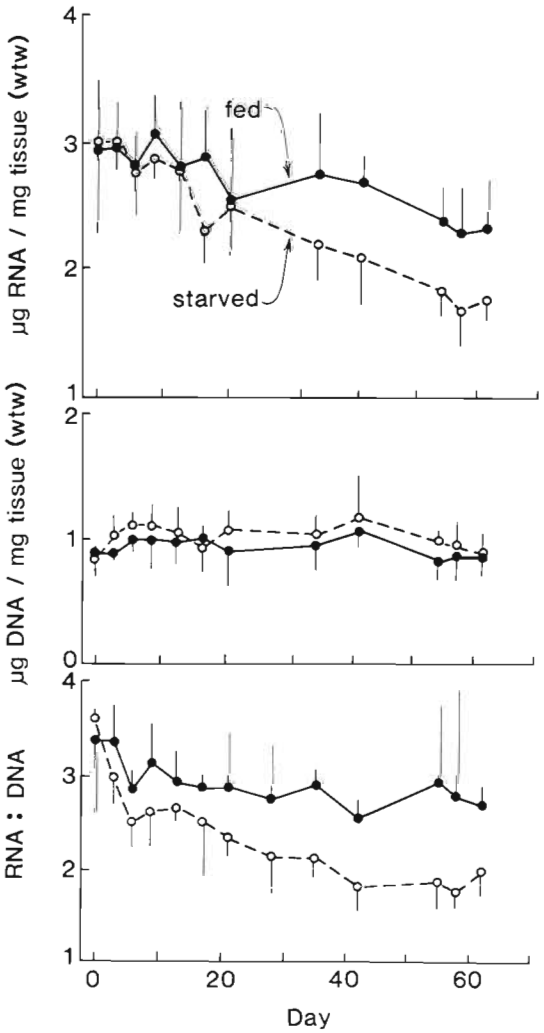

Fig. 4. *Crassostrea virginica.* Nucleic acid levels and RNA:DNA ratio mantle tissue of oysters throughout experimental period. Vertical lines: ± 1 SD (n = 4)

phometric growth parameters are presented in matrix form. All correlations above 0.217 are significant at the 5 % level (n = 86).

DISCUSSION

Results indicate that when oysters were placed in 1µm filtered, flowing seawater there was a significant decrease in the ratio of RNA to DNA in their mantle tissue, compared with oysters maintained in seawater containing ambient food levels. Concurrent with the decrease in RNA:DNA ratios there was a decreased rate of growth in starved oysters relative to fed oysters.

The decrease in RNA:DNA ratio recorded here was lower than that seen in similar experiments on fish. In Bullow's (1970) study of golden shiners there was a 33 to 35 % decrease in RNA:DNA ratio after 14 d of starvation, and 56 % after 45 d of starvation. Starvation of black bullheads for 26 d (Kayes 1979) resulted in a 46 % decrease in RNA:DNA ratios. By contrast, in our experiments the magnitude of decrease resulting from placing oysters in 1µm filtered seawater was 36.5 % after 55 d of starvation.

There are probably several explanations for this. Although the 1µm filtration was rigorously monitored, it is likely that oysters in this medium derived nutrients through absorption of dissolved organics and filtration of particulates less than 1µm in size. Probably a more important factor in determining the difference between fed and starved oysters was that even in fed oysters there was a significant decline in RNA:DNA ratio throughout the experimental period. This decline may indicate a stressed condition even in 'fed' oysters. This is possible since water temperatures were elevated to 14.5 °C, well above ambient temperatures. Particulate carbon levels in the ambient water were approximately 0.8 mg carbon l^{-1}, and particulate nitrogen, 0.1 to 0.2 mg l^{-1}; both normal values for January and February. However, at a water temperature of 14.5 °C, the experimental temperature, normal particulate carbon levels in the environment are approximately 1 to 2 mg carbon l^{-1}. It is possible, therefore, that there was insufficient energy contained in the seston in the experimental ambient water to meet

energy demands of the oysters. In addition to the above, a lower metabolic rate in oysters may also result in a slower response with respect to RNA:DNA ratios than would be observed in vertebrates.

After 55 d, starved oysters were re-fed, however RNA:DNA ratios in these re-fed animals only increased by 0.108 after 1 wk of feeding and failed to reach the value achieved by the fed animals.

These observations suggest that the RNA:DNA ratio in oysters responds more sluggishly to starvation than in vertebrate systems where significant response times may be as low as 12 h in the case of rat livers (Stocco 1976) or 1 d in fish (Bulow 1970, Wright & Martin 1985).

There is a significant but moderate correlation between RNA:DNA ratios for individual oysters and such morphological parameters as condition index and rate of wet tissue growth. This low correlation may be due to starved oysters actually displaying a weight loss, indicated by a negative growth rate, which does not correlate well with RNA:DNA ratios. In response to a stress, bivalves will initially utilize energy reserves to adjust to the stressor. After a period of time, the animal may acclimate itself to the stress, resulting in no further use of energy reserves, and no further energy loss. This probably explains the initial decline in tissue weight seen here, followed by relative constancy later. Shell growth did not appear to be highly correlated with either tissue growth rate or RNA:DNA ratios.

Given the above limitations, there is good reason to believe that RNA:DNA ratios may be used as an indication of nutritional stress in oysters. The comparatively slow response to a change in feeding status may in fact be an asset in this regard given the wide fluctuations of available food in the environment.

Current results contradict those of Pease (1976) who found little correlation between growth rate of oysters and the RNA:DNA ratios of whole animal or adductor muscle. There are, however, several differences between the methods of Pease (1976) and this study. In the current work, oysters of known initial weight were used so that an accurate measure of growth could be obtained. In Pease's study, samples were taken directly from the field and the growth rate obtained was an average over, in some cases, several years. In addition, the analytical methods employed by Pease were different from those of this study. In Pease's work, an ethidium bromide technique was used to measure RNA, and DNA was measured using the Ceriotti method. Although the ethidium bromide technique was not tested here, the Ceriotti procedure produced variable results even using standards. In this study, the more commonly used Munro & Fleck, Schmitt-Thanhauser procedure was employed, as modified by Buckley (1979).

Kayes (1979) suggested that skeletal-muscle RNA alone might provide a better index of growth than RNA:DNA ratios in black bullheads. In this study, however, the number of significant differences between paired treatments (fed and starved) was higher for ratios than for RNA alone. In starved oysters there was a 39.7 % decrease in RNA over 55 d, while there was a 48 % decrease in the RNA:DNA ratio. In this case the RNA may have had an apparently higher concentration due to cell atrophy, which was also reflected in the small but significant drop in DNA concentration in starving animals. High DNA concentrations in starved animals have also been reported by other investigators (Bulow 1970, Dagg & Littlepage 1972, Buckley 1979, Kayes 1979).

In view of the general plasticity of this species with respect to morphological characteristics, and lengthy data accumulation from growth studies, this study gives encouragement to the use of RNA:DNA ratios as a growth index for oysters in the environment. The difficulties involved in obtaining accurate morphometric growth data for oysters suggest that a comparison between RNA:DNA ratio and 'scope for growth' measurements (Bayne et al. 1979) may in future provide better information as to whether or not RNA:DNA ratios reflect the ecological fitness of the organism.

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