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CHANGES IN RIBONUCLEIC ACID, DEOXYRIBONUCLEIC ACID, AND PROTEIN CONTENT DURING ONTOGENESIS IN WINTER FLOUNDER, *PSEUDOPLEURONECTES AMERICANUS*, AND EFFECT OF STARVATION

Normal development of most embryonic and prolarval (yolk-sac) teleosts depends on material stored in the yolk for a source of both energy and biosynthetic precursers. After hatching there is a transition period when larvae shift from dependence on yolk to an exogenous food supply. The availability of sufficient prey of the proper quality and the ability of larvae to capture and assimilate it are critical to survival during the larval stage. Since differential mortality during the larval stage could be important in determining the year-class size of marine fish, a method for determining the nutritional condition of fish larvae in plankton samples could aid in determining larval survival and prediction of subsequent year-class size. In the past, weight-length relationships (Blaxter 1971), morphometric (Ehrlich et al. 1976), chemical (Ehrlich 1974a, b), and histological (O'Connell 1976; Theilacker 1978) methods have been used with varying degrees of success. All four approaches have limitations and diagnosis of the starving condition in sea-caught larvae is difficult.

Bulow (1970) used RNA-DNA (ribonucleic acid-deoxyribonucleic acid) ratios as indicators

of recent growth rates in golden shiner, *Notemigonus crysoleucas*, adults. He reported that RNA-DNA ratios were very sensitive to changes in feeding levels. The RNA content of a wide variety of organisms have been related to growth rate (Sutcliffe 1970). Sutcliffe (1965) was able to predict quite accurately the growth rates of laboratory populations of brine shrimp, *Artemia salina*, and mud snail, *Nassarius obsoletus*, larvae using a growth-RNA relation determined on whole amphipod *Orchestia platesis*. Dagg and Littlepage (1972), however, concluded that the general positive relationship between growth rate and RNA content lacked sufficient specificity for determination of growth rate. A positive relationship is expected since growth in marine teleosts is accompanied by and is partially a function of protein synthesis, and certain types of RNA are directly involved in protein synthesis serving as both a template and organizer. The DNA content of an organism has been used as an indication of cell number since the DNA content of somatic cells is generally constant for a given species. Some eggs and early larvae, however, have been shown to contain large amounts of cytoplasmic DNA, greatly exceeding the amount of nuclear DNA (Neyfakh and Abramova 1974). This study was undertaken to determine the relationships between changes in DNA, RNA, and protein content and events in the development of winter flounder, *Pseudopleuronectes americanus*, and to determine if measurements of these classes of biochemicals could be used to determine the nutritional condition of winter flounder larvae.

Methods

Pseudopleuronectes americanus adults were caught by trawl net off Rhode Island and kept in a 1,900 l aquarium. Eggs were obtained, fertilized, and incubated according to methods previously described (Smigielski 1975). Larvae were maintained at 8° C in 38 l all black glass aquaria. Commencing 4 days after hatching the larvae were fed zooplankton collected in the Narragansett Bay area in excess of 2 organisms/ml according to the methods of Laurence (1975). On days 3 and 28 a portion of the larvae were transferred to an identical aquarium containing seawater filtered through a 0.45 μm Millipore[1] filter. Wild winter flounder larvae were collected in Narrow River, R. I., with a 505 μm 0.5 m plankton net.

About 40 eggs or larvae were pooled per sample through day 11 after hatching; thereafter 10 larvae were pooled per sample except on days 43, 50, and 58 when only 5 larvae were used for the largest size group. All samples through day 36 were run in triplicate; thereafter samples were run in duplicate. Starting on day 28 the standard length of each larva sampled was determined using an ocular micrometer and 10 larvae were taken on each sampling day for dry weight determinations.

Eggs and larvae were homogenized in 2 ml of ice-cold distilled water immediately after sampling. Protein was determined on duplicate 0.1 or 0.05 ml samples of homogenate using a modification of the Lowry method (Hartree 1972). RNA and DNA were extracted and partially purified from 1.7 ml of homogenate using a modification of the Schmidt-Thannhauser method (Munro and Fleck 1966) adapted for the micro quantities present in larval fish and eggs. RNA concentration was estimated from the absorbancy at 260 nm of the acid-soluble, alkali-hydrolyzed fraction. The DNA content of larvae was determined from the absorbency at 260 nm of the alkali-stable, acid-hydrolyzed fraction. Because of the very small quantities of DNA in winter flounder eggs their DNA content was determined on the alkali-stable fraction using a modification of the 3,5-diaminobenzoic acid dihydrochloride florometric assay described by Holm-Hansen et al. (1968) and Hinegardner (1971). At the beginning of this study RNA was also determined using the orcinol method (Sutcliffe 1965) and DNA was determined using the indole method (Ceriotti 1952). These values were in good agreement with the values reported.

Results

DNA content per egg increased rapidly between fertilization and hatching (Figure 1). Upon hatching 10 days after fertilization, larvae contained slightly more RNA and DNA than unhatched eggs and retained 78% of the protein. During the period from hatching to the end of the yolk-sac stage (day 5), DNA and RNA content remained essentially constant while a decrease in protein was observed. Although plankton was added on day 4 to aquaria containing fed larvae, visual observation of the gut indicated that the majority of the larvae had

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

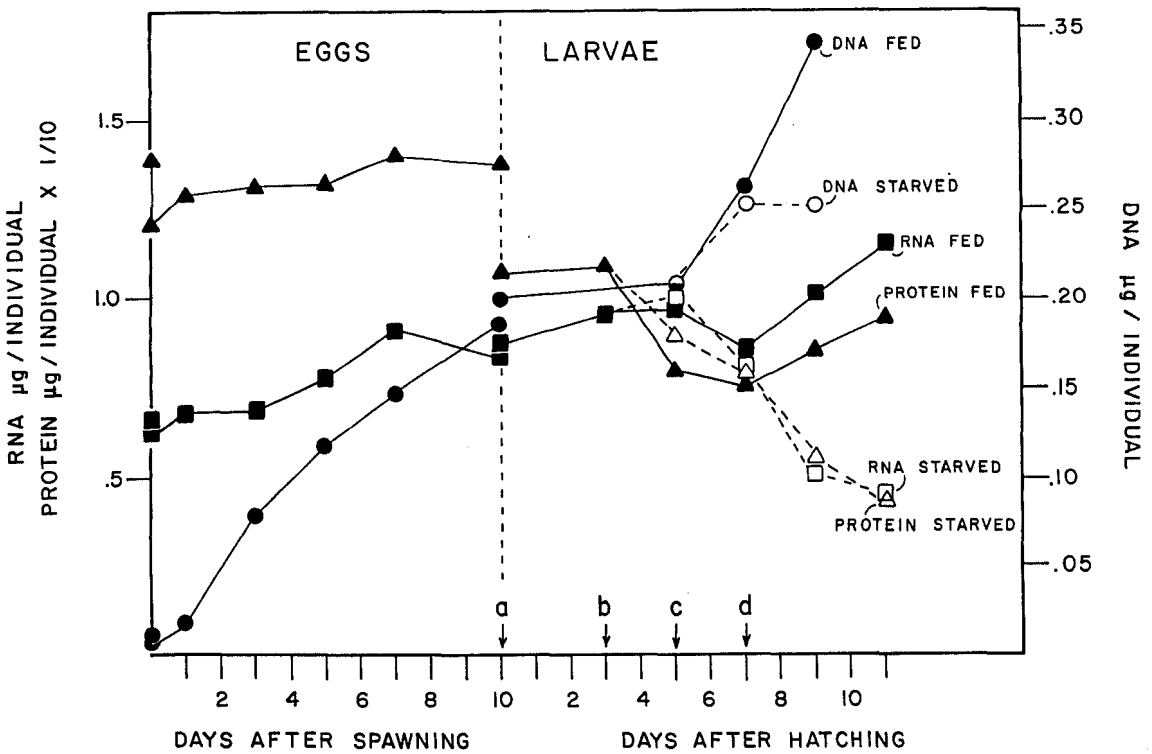

FIGURE 1.—Time course of development of DNA, RNA, and protein content per individual winter flounder egg or larva. a) 50% hatch; b) starved larvae transferred to filtered seawater; c) most larvae showed no visible yolk; d) food visible in gut of fed larvae.

not begun feeding until day 7. Between the end of the yolk-sac stage and initiation of feeding (day 7) DNA content increased sharply while RNA and protein decreased. Of the protein present in the egg just prior to hatching, 45% was lost by the time of feeding initiation. After feeding initiation DNA, RNA, and protein content increased steadily (Figure 1; Tables 1, 2).

The RNA and protein content of winter flounder larvae transferred to filtered seawater prior to feeding initiation continued to decrease until a 100% mortality was observed between day 11 and 14 (Figure 1). Starved larvae did show an increase in DNA content on day 7 similar to that observed in fed larvae. The RNA-DNA ratio of both starved and fed larvae decreased from the end of the yolk-sac stage through day 9. However, the RNA-DNA ratio was significantly higher in fed larvae than starved larvae on day 9.

The RNA content of a second group of larvae transferred to filtered seawater 28 days after hatching decreased within 2 days, while both DNA and protein content appeared to increase (Table 1). After 4 days a decrease in all three components was observed. A 50% mortality, consisting almost entirely of the smaller individuals

TABLE 1.—RNA, DNA, and protein content of starved and fed winter flounder larvae 28 to 36 days after hatching.

Age (days)	Starvation time[1] (days)	Standard length[2] (mm)		RNA[3] (µg/larva)		DNA[3] (µg/larva)		Protein[3] (µg/larva)		RNA/DNA[3]	
		Fed	Starved	Fed	Starved	Fed	Starved	Fed	Starved	Fed	Starved
28	0	5.61±0.60		3.85±0.67		0.91±0.21		36±7		4.27±0.42	
30	2	5.83±0.72	5.64±0.56	4.74±0.73	3.32±0.51	0.99±0.13	1.04±0.11	37± 3	44±5	4.81±0.56	3.16±0.26
32	4	5.84±0.66	5.68±0.54	4.75±0.14	2.59±0.61	1.21±0.16	0.93±0.13	79± 4	36±7	3.99±0.50	2.78±0.29
	C[4]	5.44±0.80		3.95±0.22		0.99±0.05		43± 4		3.98±0.06	
36	8	6.53±0.92	6.41±0.45	8.91±0.66	3.70±0.50	2.17±0.41	1.73±0.18	94± 4	60±8	4.19±0.43	2.14±0.16
	C[4]	6.23±0.94		8.15±2.67		1.92±0.46		86±24		4.20±0.34	

[1]Number of days starved fish were in filtered seawater.
[2]Data are means ±1 SD for 40 to 50 larvae.
[3]Data are means ±1 SD of three replicates containing 10 larvae each.
[4]Fish removed from fed population and transferred to filtered seawater 18 h prior to sampling to clear stomach contents.

TABLE 2.—RNA, DNA, and protein content of wild and cultured (fed) winter flounder.

Age (days after hatching)	Standard length (mm) Range	Mean	SD	RNA (μg/larva)	DNA (μg/larva)	Protein (μg/larva)	RNA/DNA
Cultured larvae[1]:							
42	4.98–6.07	5.60	0.39	3.55±0.23	—	44± 1	—
	6.27–6.89	6.50	.20	9.19±0.75	1.99±0.06	99±10	4.61±0.09
	7.00–8.77	7.77	.57	21.83±1.98	3.91±0.70	200±15	5.62±0.52
43	5.26–6.35	5.76	.30	3.35±0.61	0.82±0.13	44± 5	4.10±0.07
	6.36–7.26	6.82	.26	7.75±0.98	1.85±0.25	91± 5	4.18±0.03
	7.29–8.54	8.19	.33	27.42±0.28	4.84±0.00	274± 5	5.67±0.06
50	4.10–6.54	6.01	.55	5.32±0.40	1.27±0.08	49± 4	4.19±0.03
	6.31–7.69	7.00	.34	11.08±0.29	2.52±0.02	115± 1	4.40±0.08
	[2]7.50–8.67	8.83	.56	32.82±2.83	6.19±0.69	370±37	5.31±0.14
58	5.79–6.91	6.31	.27	5.70±0.03	1.35±0.07	64± 1	4.23±0.20
	6.67–8.35	7.27	.47	14.33±1.03	3.18±0.08	153± 1	4.50±0.21
	[3]7.40–8.49	7.33	.31	37.08±1.02	7.05±0.10	434± 3	5.26±0.07
Wild larvae[4]:							
Group I	7.29–7.45	7.34	.09	19.05	4.19	212	4.55
II	5.80–6.71	6.41	.53	—	2.48	131	—
III	6.54–7.54	6.95	.41	25.36±3.20	5.45±0.34	376±43	4.65±0.41

[1]Standard length data for cultured larvae are means ±1 SD for 50 to 150 larvae. Chemical data are for two replicates consisting of 10 larvae each except for the largest size group on days 43, 50, and 58 when only 5 larvae were used per replicate.
[2]In this sample, 6 of the 10 fish had metamorphosed.
[3]In this sample, all fish had metamorphosed.
[4]Data for Groups I and II represent values for pools of three larvae each. Data for Group III are means ±1 SD for five larvae analyzed individually.

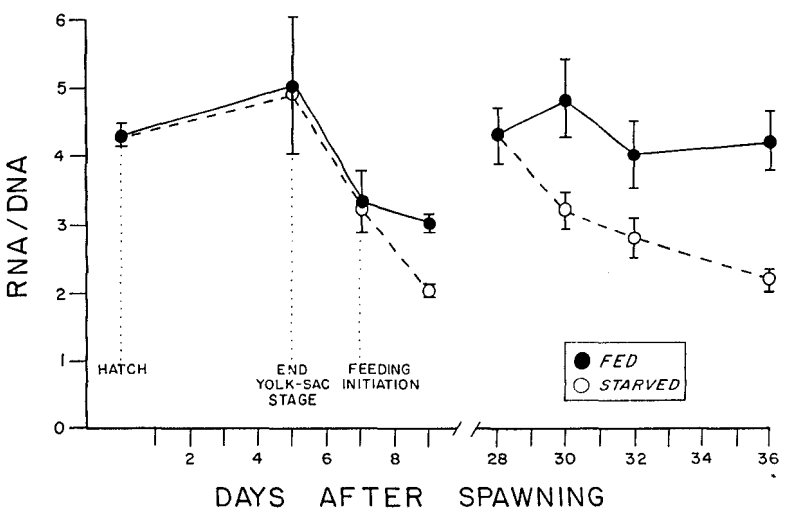

FIGURE 2.—RNA-DNA ratios of starved and fed winter flounder larvae. Open circles indicate values for larvae transferred to filtered seawater on day 3 and day 28. Brackets indicate ±1 SD.

in the group, occurred 7 days after transfer to filtered seawater, accounting for the high DNA, RNA, and protein values observed on the final day of sampling (day 36). The RNA-DNA ratio of larvae transferred to filtered seawater decreased continually until a 100% mortality was observed. No significant change in the RNA-DNA ratio of fed larvae was observed during the same period (Figure 2). The DNA, RNA, and protein content of different size groups of wild and cultured larvae through metamorphosis is shown in Table 2.

Discussion

The DNA, RNA, and protein content of winter flounder eggs reported in this study are total values for the yolk plus the embryo. Any increase in the amount of a particular component must therefore result from synthesis rather than transfer from the yolk to the embryo. The continual net accumulation of DNA from fertilization to hatching is probably correlated with an increase in cell number (Regnault and Luquet 1974) although the content of DNA per cell may decrease (Neyfakh and Abramova 1974). The small increase in protein content during the same period is evidence that protein is not an important energy source during early development in winter flounder. The 46% decrease in protein content between the maximum 3 days prior to hatching and the

minimum at initiation of feeding on day 7 indicates that protein is probably an important energy source during this period although this includes a 22% decrease in protein upon hatching, the majority of which may be lost with the chorion. Two periods of decrease in RNA content were observed. One occurred just prior to hatching; the other just prior to feeding initiation. No significant net decrease in the DNA content of eggs or fed larvae was observed between any sampling periods.

The decrease in protein and RNA content (Figure 1) as well as the decrease in the RNA-DNA ratio (Figure 2) prior to feeding initiation resembles the pattern observed for starved larvae. Even in the presence of excess food the RNA-DNA ratio fell from 4.9 at the end of the yolk-sac stage to 3.0 at initiation of feeding on day 7. The critical importance of food availability at the initiation of feeding capability was demonstrated 2 days later when fed larvae contain almost 100% more RNA and 55% more protein than larvae held in filtered seawater.

The RNA-DNA ratio was the most reliable and sensitive index of nutritional state evaluated in this study which included relationships between RNA, DNA, protein, standard length, and dry weight. RNA content was the most labile, decreasing within 2 days after removal of food. DNA content was generally conserved except in the final stages of starvation prior to death. The protein-DNA ratio, which is an index of the amount of protein per cell, generally decreased as starvation progressed and the protein-RNA ratio generally increased. The RNA-DNA ratio was particularly useful as an indicator of condition since unlike other indices it fell within well-defined limits throughout most of the period studied. Winter flounder larvae established a mean RNA-DNA ratio of between 4.0 and 4.8 3 wk after initiation of feeding (Figure 2). This range is similar to the RNA-DNA ratio values reported by Bulow (1970) for golden shiners. The RNA-DNA ratio was not greatly affected by either the age or size of the larvae until metamorphosis when the RNA-DNA ratio increased to between 5.3 and 5.7 and remained at this level until the experiment was terminated on day 58 (Table 2). This is particularly important since the age of sea-caught larvae is difficult, if not impossible, to establish and a large size range is observed in larvae of the same age. This point was demonstrated on day 36 when a large mortality of smaller larvae resulted in an increase in the mean DNA, RNA, and protein content of starved larvae. The RNA-DNA ratio, however, was unaffected by the change in size distribution and continued to decrease. Results from larvae transferred to filtered seawater 18 h prior to sampling and allowed to empty their stomachs (Table 1) indicate that the RNA-DNA ratio is not significantly affected by stomach contents at the time of sampling.

The RNA-DNA ratios of winter flounder larvae captured in Narrow River fell within the range of values observed for fed winter flounder in the laboratory. The high RNA-DNA ratios indicated that the larvae were in good nutritional condition. This observation is supported by visual examination of the larvae and the high growth and survival rates of laboratory-reared winter flounder held in situ in Narrow River with a semiopen environmental chamber (Laurence et al. 1979).

Before measurements of RNA-DNA ratios are useful in the field, the effect of changing environmental conditions such as temperature, salinity, and possibly various pollutants as well as low prey concentrations and intermittent feeding should be evaluated. Although adult golden shiners, larval winter flounder, and larval cod, *Gadus morhua*, (Buckley unpubl. data) showed a similar decline in RNA-DNA ratio when food is withheld, the response of other species should be determined.

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EGGS AND EARLY LARVAE OF SMALLMOUTH FLOUNDER, *ETROPUS MICROSTOMUS*

Smallmouth flounder, *Etropus microstomus* (Gill), ranging from early postlarvae to adult were described and illustrated in detail by Richardson and Joseph (1973). Eggs and larvae through yolk-sac absorption had yet to be identified.

During a 1975-76 ichthyoplankton survey of Block Island Sound conducted by Marine Research, Inc. small unidentified planktonic fish eggs were taken. Through subsequent rearing of a number of these eggs and completion of a length series with larger, known larvae, we identified the specimens as *E. microstomus* eggs. Our descriptions of eggs and yolk-sac larvae together with the work of Richardson and Joseph (1973) provide a complete developmental series for identification of this species.

Methods

Sampling was conducted in Block Island Sound at five stations along each of three transects running from Charlestown and East Beach, R.I., to Block Island, a distance of approximately 14.8 km. Collections were made with 60 cm, 0.505 mm mesh, bongo nets. All tows were made obliquely, bottom to surface at approximately 2.5 kn for about 5 min. Digital flowmeters provided volume estimates and quantitative density estimates. Periodically, a 30 cm, 0.505 mm mesh, plankton net was fixed above the bongo net to collect samples of live eggs. These were returned to the laboratory in aerated 4 l thermos jugs and incubated at 20°-21° C. *Etropus microstomus* eggs and larvae were stored in 3-5% buffered Formalin[1] solutions before examination.

Descriptions of the Egg

Etropus microstomus eggs (Figure 1, Table 1) are small, 0.561-0.740 mm in diameter ($\bar{x}$ = 0.64) with a single small oil globule, 0.051-0.165 mm ($\bar{x}$ = 0.12). The egg is spherical with a transparent, unsculptured chorion. The oil globule is also spherical. Occasonally two oil globules were noted or a single one with several surrounding oil particles were found. This condition has commonly been noted for other species (Ahlstrom and Ball

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.