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The Effects of Temperature, Starvation, and Kind of Foods on Growth, RNA/DNA Ratios and Protein Contents during the Early Life Stages of Olive Flounder (*Paralichthys olivaceus*)

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The effects of temperatures, starvation, and kind of foods on growth, RNA/DNA ratio and protein contents during metamorphosis and early juvenile stage of olive flounder (*Paralichthys olivaceus*) were examined. During metamorphosis, warm-acclimated fish showed higher RNA and DNA content than those of the cold-acclimated fish, excepting H stage (28 DAH) at which the ratio was higher at cold temperature. RNA/DNA ratio during metamorphosis showed similar values at two temperatures tested. However, after 42 DAH warm-acclimated juveniles had higher DNA content compared with cold-acclimated fish, resulted in marked decreases in RNA/DNA ratios. Higher RNA content at H stage of cold-acclimated fish was consistent with an increase in protein content. Growth of fish rearing at warm temperature was higher than those of fish at cold temperature during all experiments. In starvation experiment, contents of DNA, RNA and protein significantly decreased. Even though there were no significant differences in total length (TL) and body weight between the live mysid-fed and artificial pellet-fed fish at 35 mm TL, both RNA/DNA and protein/DNA ratios of the former group was significantly higher than those of the latter due primarily to lower DNA content of the live mysid-fed group. The results from this study suggest that temperature, starvation and kind of foods should be considered when RNA/DNA ratio applied to assessing the cultured larval and juvenile fish condition.

Key words: RNA/DNA, Protein, Temperature, Starvation, Mysid, *Paralichthys olivaceus*

Introduction

RNA/DNA ratio has proven to be a useful indicator of nutritional condition and growth for a variety of larval and juvenile fishes (Bulow, 1970; Wright and Martin, 1985; Clemmesen, 1989; Houlihan, 1991; Gwak and Tanaka, 2001). However, environmental factors which could affect those of indices have to be considered before applying them to the wild fish which live in different abiotic and biotic environmental conditions. Temperature is one of the most important abiotic factors because it is, together with starvation, a fundamental factor influencing the physiological and biochemical processes of fish (Elliot, 1982; Buckley, 1984; Hawkins et al., 1985). An acute change of environmental temperature reflected in the rates of oxygen consumption, the activity of RNA and protein synthesis in the cells of ectothermal animals (Pannevis and Houlihan, 1992). Buckley (1984) found that a positive relation between RNA/DNA ratios and protein growth rates, taking into account temperature as an additional independent variables. Gwak and Tanaka (2002) also showed that olive flounder has species-specific patterns in RNA/DNA ratio, and the RNA content was closely correlated with the protein content during metamorphosis. Since metamorphic size and period of olive flounder are mainly dependent upon temperature (Seikai et al., 1986), biochemical characteristics such as RNA, DNA and protein content might be highly variable under different temperatures especially during meta-

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morphosis coupled with settlement.

Many marine larval and juvenile fishes could encounter a critical period when food supply is inadequate for maintenance and growth. Therefore, fish have to store their residual energy in the tissues to prepare for the time of food scarcity (Elliot, 1982; Buckley, 1984; Hawkins et al., 1985; Seikai et al., 1986; Pannevis and Houlihan, 1992; Tanaka et al., 1996; Gwak and Tanaka, 2001, 2002). However, storing energy in the tissues and its utilization during critical period would be largely depended upon temperature, starvation and kind of foods (Ehrlich, 1975; Buckley, 1982). In addition, RNA/DNA ratio was influenced directly and/or indirectly by starvation as shown by close relationships between RNA and protein content (Buckley, 1980; Clemmesen, 1987, 1994; Gwak and Tanaka, 2001). The kind and quantity of the available food have also been often shown to affect the RNA activity levels and RNA/DNA ratio in teleosts (Buckley, 1984; McMillan and Houlihan, 1992). Furthermore, the changes in temperature and starvation are often concurrent in natural habitats, therefore it is needed to consider the connections between thermal acclimation and feeding. In fact, the spawning season of olive flounder ranges from January in the most southern part to July in the most northern part of Japan, that is, spawning continues for almost six months along the coastal waters of Japan and the temperature varies regionally during that period (Minami and Tanaka, 1992). Additionally, olive flounder undergoes metamorphosis associated with their approaching and settling down to nursery ground, this suggesting the larvae are brought to physically and biologically different environments. Subsequently, understanding the correlation of biochemical indices with temperature, starvation and kind foods would be crucially important when the indices were applied to wild fish.

The aim of the present study was to investigate the effects of sea water temperature, starvation and kind of foods on growth, RNA/DNA ratios and protein content of olive flounder at different stages and sizes, from the initiation of metamorphosis to early juvenile stage at 35 mm TL.

Materials and Methods

Incubation and rearing

Olive flounder larvae and juveniles were reared at the Japan Sea Farming Association (Miyazu Station, Kyoto). The eggs were incubated under natural photoperiod in large stock 100 t tanks at 15℃. Water temperature was kept at 18℃ in early days of yolk-sac larvae. The larvae were maintained with constant aeration using air stones. The larvae were fed rotifers (*Brachionus plicatilis*) cultured by the alga (*Nannochloropsis oculata*), from three days-after-hatching (DAH), brine shrimp (*Artemia salina*) nauplii from 15 DAH, and artificial feed (composed of 50.2% crude protein and 11.0% crude lipid, Otohime, Marubeni Nisshin Feed Co., Japan) from 20 DAH. During the experimental period, larval and juvenile olive flounder were fed to satiation three times per day (07:00, 12:00 and 17:00 hours). Development of olive flounder larvae and juveniles was classified according to Minami (1982), whereby stages F, G, and H correspond to early-, mid- and late-metamorphosing stages respectively. Metamorphosis was grouped into three phases: (i) premetamorphic (up to stage E); (ii) metamorphic (stages F-H); and (iii) postmetamorphic (stage I) phases.

Temperature experiment

Prior to the temperature experiments, two large 15 t water baths were prepared for the cold (14℃) and warm (22℃) acclimated experiments. Nine hundreds fish at each developmental stage of E to I and at each 20, 30, and 35 mm TL were carefully selected from the stock tanks and transferred evenly to 100 and 500 L polycarbonate aquaria in each water baths in which water temperature was kept at 18℃ and constant aeration was supplied. Each treatment was imposed on fish in three replicate aquaria. After transferring, the water temperature was lowered and raised to 14℃ and 22℃ at a rate of 1℃/3 hrs and the fish were fed a similar ration as the stock tank during 8 days of experiment.

Starvation and kind of foods experiments

For the starvation experiments, three hundreds fish at each 20, 30, and 35 mm TL juveniles were selected from the stock tanks and transferred to the 500 L polycarbonate aquarium with aeration. Each treatment was imposed on fish in three replicate aquaria. After transferring to the 500 L aquaria, the juveniles were completely deprived of food for the 8 days of experiment.

Nine hundreds of 35 mm TL juveniles were carefully selected from the stock tanks for the kind of foods experiment, then transferred evenly to three replicates of 500 L aquaria. Mysid, *Archaenomysis kokuboi* as a food for juvenile flounder was caught

with a net at night and kept alive in the 30 L tanks with aeration. Adequate amounts of the live mysids were added to the aquaria everyday and the juveniles always could prey on them ad libitum.

Sampling and preservations

Sampling was performed prior to feeding in the morning. For morphometric measurements, 10 fish were randomly sampled from each aquarium and anaesthetized in 200 ppm benzocaine (MS222), measured total length then preserved in 5% formalin. For biochemical analyses, 10 fish were randomly sampled from each aquarium, rinsed in distilled water, and frozen individually at -80℃ in 1.5 mL eppendorf tubes for both metamorphosing larvae and 20 mm TL juveniles and in vinyl bags for 30 and 35 mm TL juveniles.

Morphometric measurements and biochemical analyses

Morphometric measurements: Total length was measured to the nearest 0.1 mm under a profile projector for the metamorphosing fish and using vernier caliper for fish over 20 mm TL. For 30 and 35 mm TL juveniles, individual body weight was also recorded.

Biochemical analyses: Measurements for the RNA, DNA and protein content were performed on the same individual for all the metamorphosing stages from E to I and 20, 30, and 35 mm TL juveniles which were reared under two different temperatures, and for 20, 30, and 35 mm TL juveniles which were deprived of food. Whole body for the metamorphosing fish and 20 mm TL juveniles, and dorsal muscle in 30 and 35 mm TL juveniles were blotted, weighed (mg) and utilized to determine the RNA, DNA and protein content. All chemicals used in the RNA/DNA analyses were analytical grade: DNA from salmon sperm (Wako Chemical Co. Japan), RNA from yeast (Tokyo Chemical Co. Japan), RNase from bovine pancreas, and Ethidium bromide (Nacalai Tesque Co. Ltd, Kyoto, Japan). The methodology described in Clemmesen (1988) was used with slight modifications. Tris-EDTA buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, adjusted to pH 8.0 with HCl) was added to 10 mL glass homogenizer, and whole body and muscle sample were completely homogenized. After homogenization, one part of aliquot was used to determine RNA and DNA content, and the other part was used for protein content. The aliquot for RNA and DNA content was centrifuged at 6,000 rpm for 10 min at 4℃ after Phenol treatment.

Quantification of nucleic acid and protein content

Total nucleic acid contents (RNA+DNA) were determined fluorometrically with Hitachi 650-10LC (Japan) at excitation, 360 nm; emission, 590 nm by adding 50 μL EB-buffer solution. DNA content were determined in the same way after another 150 l aliquot was enzymatically digested with 20 μL RNase for 30 min at 37℃. Results were expressed as μg nucleic acids·fish^{-1} for the metamorphosing stages and 20 mm TL juveniles, and μg nucleic acids·mg body weight tissue^{-1} for 30 and 35 mm TL juveniles and as the ratio of RNA and DNA content. Total protein was dissolved in NaOH and determined by a Bio-Rad protein kit (Bio-Rad, Tokyo, Japan) using bovine serum albumin as a standard. Results were expressed as mg protein·fish^{-1} for the metamorphosing stages and 20 mm TL juveniles, and mg protein·mg body weight tissue^{-1} for 30 and 35 mm TL juveniles and as the ratios of protein to DNA content (an index of cell size).

Statistical analysis

Student's t-test was used to make comparisons of parameters for each experiment using STATISTIX 7.0 for Windows (Analytical Software, Tallahassee, FL, USA). Probabilities <0.001 were considered to be significant.

Results

Temperature effects on growth, RNA/DNA ratios and protein content

Significant differences ($P<0.001$) were found in total length and body weight between 14℃ and 22℃ (Fig. 1). The warm-acclimated fish showed higher growth rates with increases in total length and body weight than the cold-acclimated fish during metamorphosis, and at 30 and 35 mm TL juveniles. During metamorphosis DNA, RNA and protein content of the whole body were generally found to be significantly higher in the warm-acclimated fish than in the cold-acclimated one (Figs. 2, 3, 4). An exceptional case was found at H stage, where the biochemical parameters showed significantly higher values ($P< 0.001$) in the cold-acclimated fish. Significantly higher values in the protein content of warm-acclimated fish at early- and mid-metamorphosing stages and post-metamorphic phase were consistently ac-

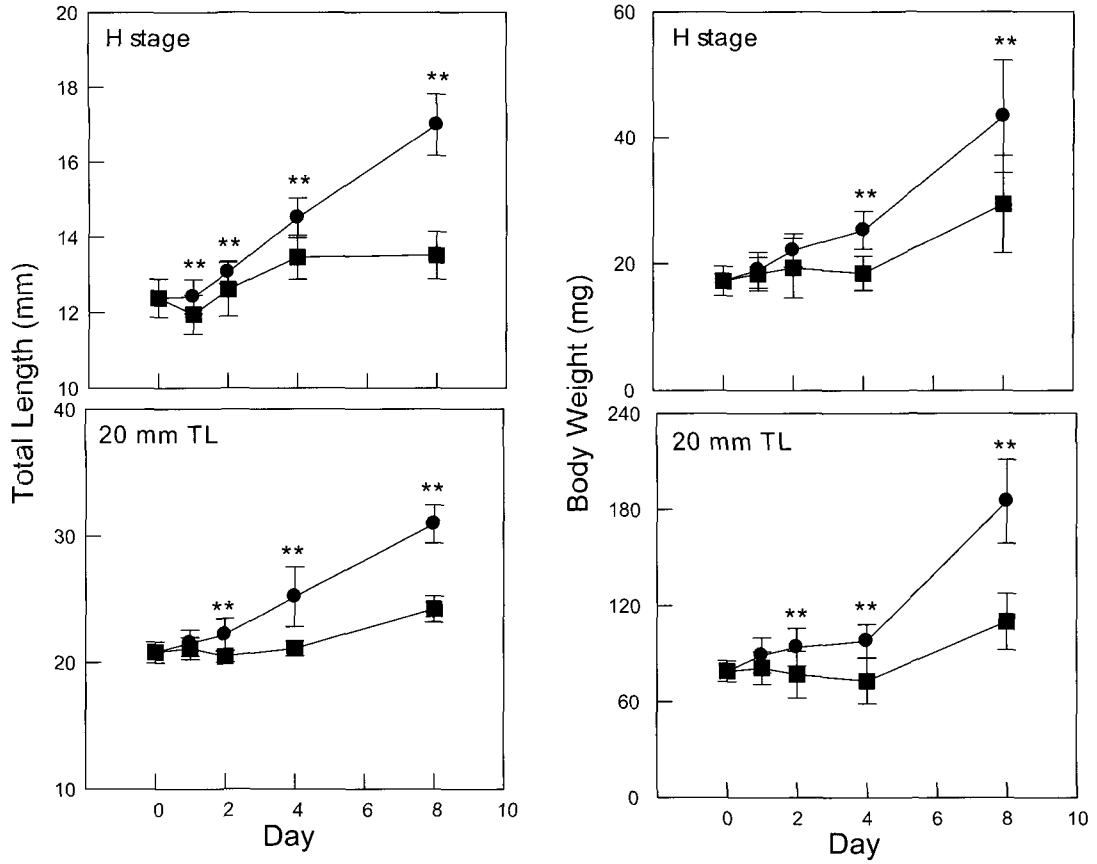

Fig. 1. Variation in total length and body weight for H stage larvae, and total length and body weight for 20 mm TL juveniles maintained at 14℃ and 22℃. Values for fish maintained at 14℃ (■) and 22℃ (●). Values are given as the mean±SD of thirty individuals.
**, significant difference ($P<0.001$) between 14℃ and 22℃.

companied with higher RNA content. Higher protein/DNA ratios during early- and mid-metamorphosing stages of the warm-acclimated fish indicated a relative increase in cell size (Fig. 5). However, there were no significant differences in RNA/DNA ratios of all the metamorphosing stages between the two temperatures tested (Fig. 6). RNA content is a better predictor in growth than RNA/DNA ratios during metamorphosis. Variations in RNA/DNA ratios were due to changes in RNA content related to developmental stage and also to changes in DNA content.

In contrast, the warm-acclimated fish at late-metamorphosing stage (H stage) showed significantly lower protein content ($P<0.001$) which was mainly due to the relatively lower RNA content (Figs. 2, 4). Decrease in contents of DNA, RNA and protein of the warm-acclimated fish resulted in significantly lower values in the ratios of protein/DNA at late-metamorphosing stage, indicating a relative decrease in cell size (Fig. 5). On the contrary, for 20, 30, and 35 mm TL juveniles, RNA, DNA and protein content were higher in the warm-acclimated fish. Subsequently, the protein/DNA ratios of the warm-acclimated fish were higher than those of the cold-acclimated fish (Fig. 5). However, RNA/DNA ratios of the warm-acclimated fish showed significantly lower values than those of the cold-acclimated fish, which was mainly due to more rapid increase in DNA content compared with RNA content (Figs. 2, 3, 6). A drastic increase of protein content in the warm-acclimated fish reflected an increase in RNA content (Figs. 2, 4).

Effects of starvation and kind of foods on growth, RNA/DNA ratios and protein content

Significant differences in RNA/DNA ratio, RNA and protein content between the starved and fed fish were found in 20 mm TL juveniles (Fig. 7). For

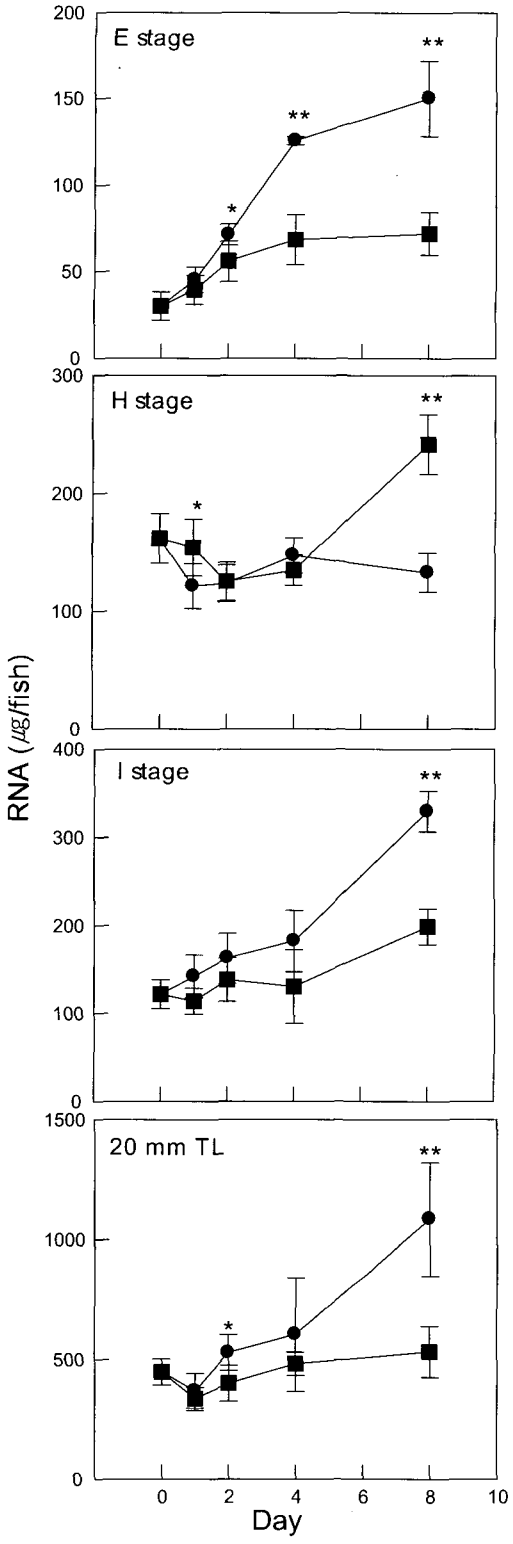

Fig. 2. Variation in RNA content for metamorphosing fish of E, H, I stages, and 20 mm TL juveniles maintained at 14℃ (■) and 22℃ (●). Values are given as the mean±SD of fifteen individuals. *, $P<0.05$; **, $P<0.001$.

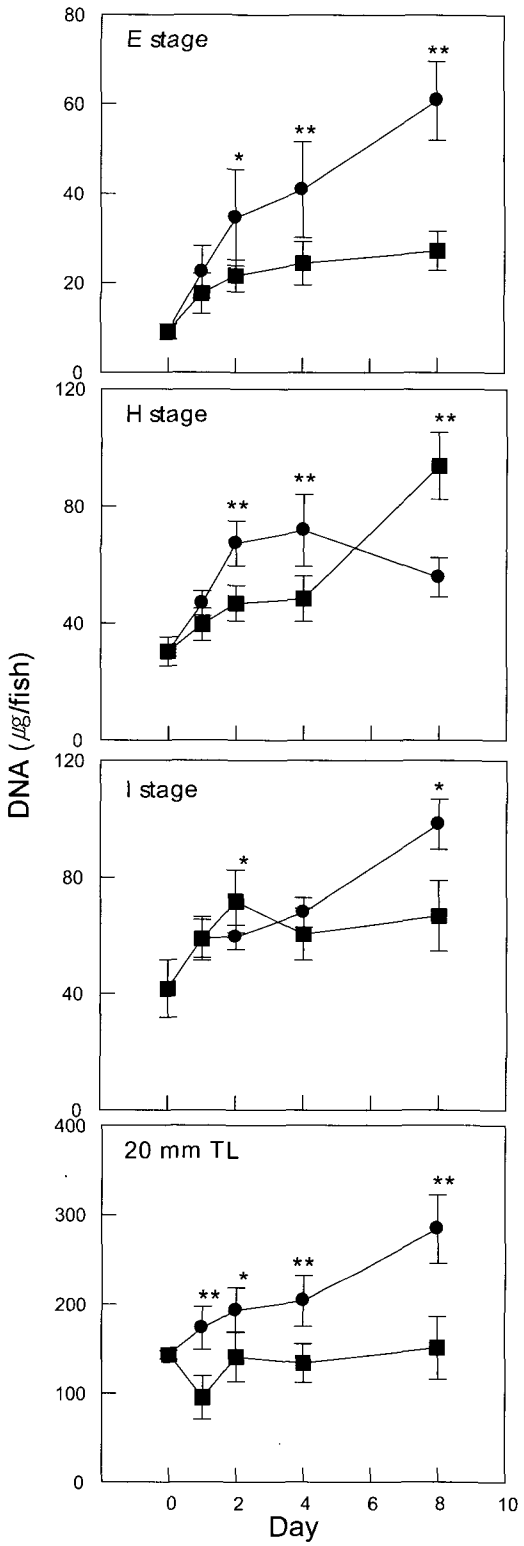

Fig. 3. Variation in DNA content for metamorphosing fish of E, H, I stages, and 20 mm TL juveniles maintained at 14℃ (■) and 22℃ (●). Values are given as the mean±SD of fifteen individuals. See Figure 1 legend for details.

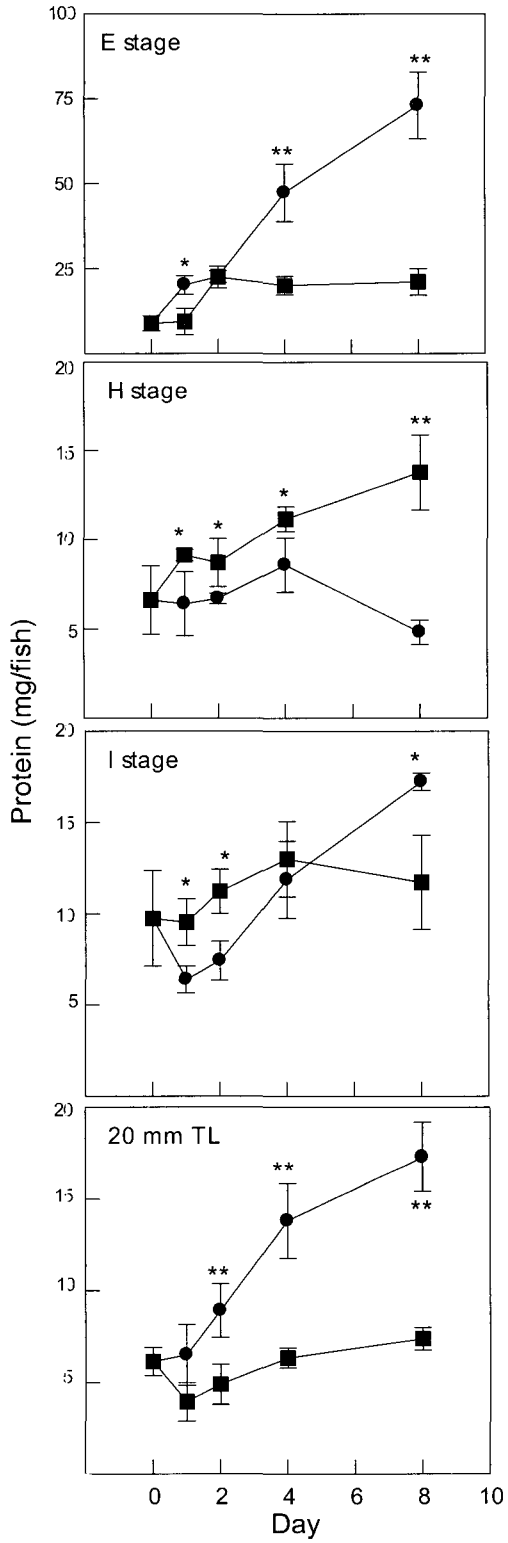

Fig. 4. Variation in protein content for metamorphosing fish of E, H, I stages, and 20 mm TL juveniles maintained at 14℃ (■) and 22℃ (•). Values are given as the mean±SD of fifteen individuals. See Figure 1 legend for details.

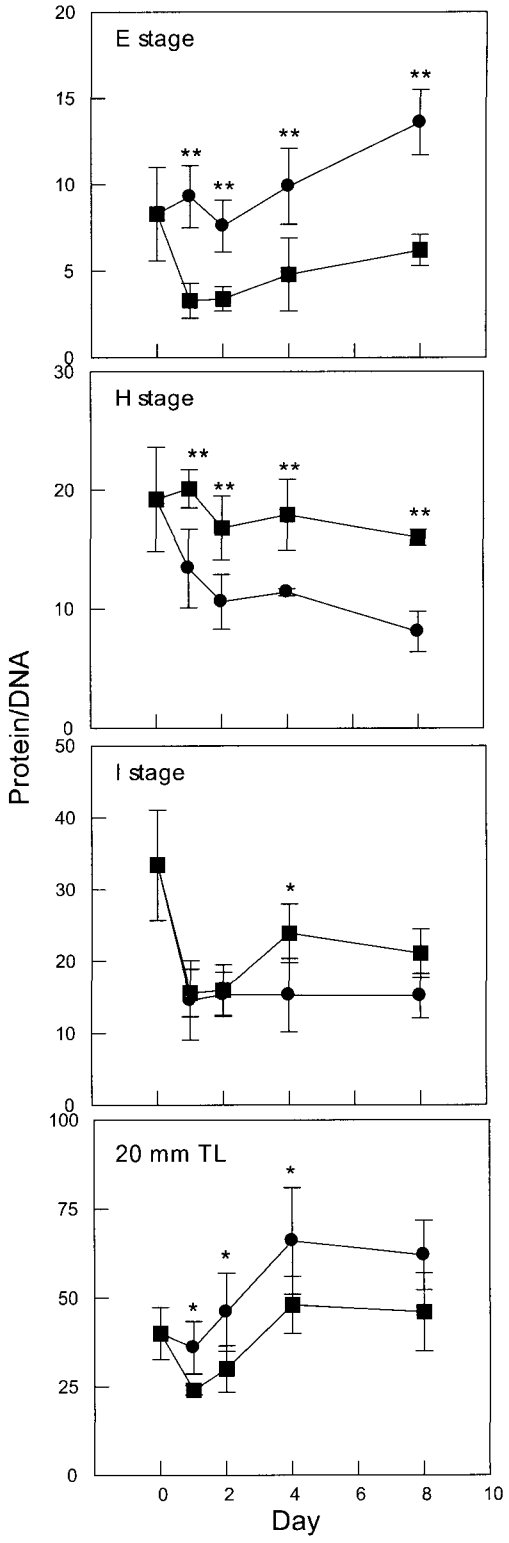

Fig. 5. Variation in Protein/DNA ratio for metamorphosing fish of E, H, I stages, and 20 mm TL juveniles maintained at 14℃ (■) and 22℃ (•). Values are given as the mean±SD of fifteen individuals. See Figure 1 legend for details.

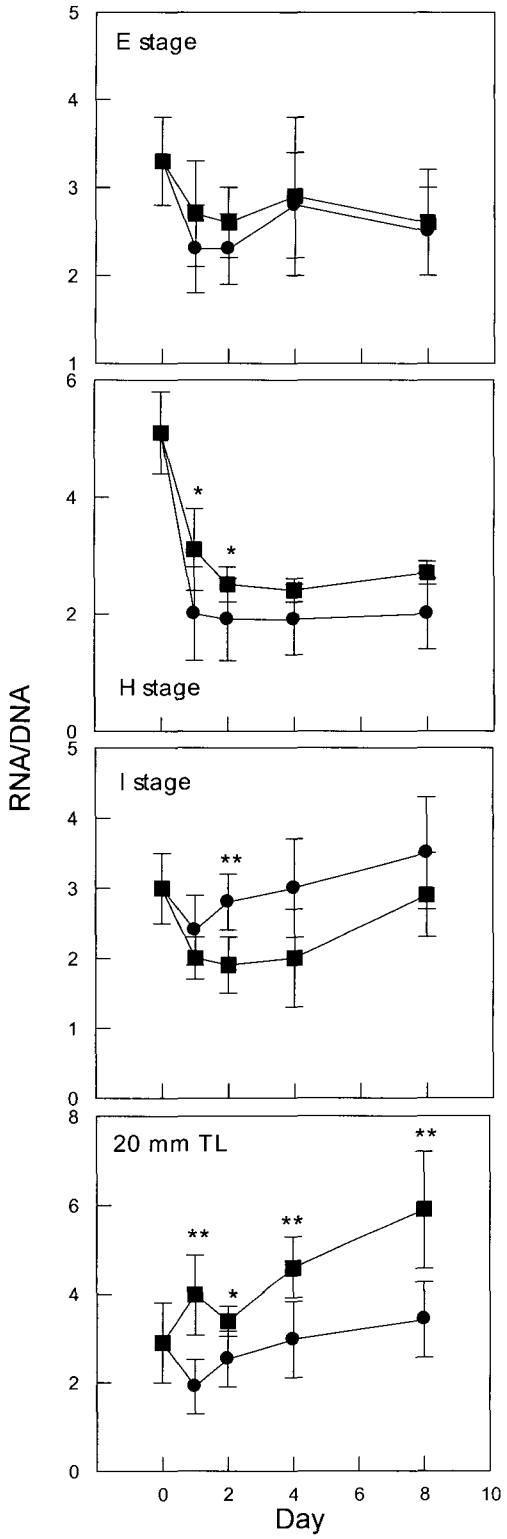

Fig. 6. Variation in RNA/DNA ratio for metamorphosing fish of E, H, I stages, and 20 mm TL juveniles maintained at 14℃ (■) and 22℃ (●). Values are given as the mean±SD of fifteen individuals. See Figure 3 legend for details.

measurement at 30 and 35 mm TL juveniles, there sults were similar to those for 20 mm TL juveniles. The starved fish had lower values of RNA, protein content and protein/DNA ratio, which were significant at the end of 8 days experiment ($P<0.001$). As starvation proceeded, the contents of DNA and RNA decreased, indicating a decrease in cell number and a parallel decrease in the total content of RNA available for protein synthesis. In addition, drastic decreases in both RNA and DNA content induced by 8 days of starvation resulted in the starved fish having significantly lower RNA/DNA ratios (Fig. 7). Furthermore, drastic decrease in RNA content of the starved fish was reflected in protein content, which was significantly lower than in the fed fish (Fig. 7). The relatively lower protein content of the starved fish led to significantly lower protein/DNA ratios (Fig. 7). The ratios of RNA/DNA and protein/DNA in the starved fish were significantly lower, and are reflected in somatic growth. The total length of fed fish was significantly longer than those of the starved fish ($P<0.001$, Fig. 7).

RNA/DNA ratios were considerably affected by kind of foods from 4 days after the start of experiment (Fig. 8). The ratios of RNA/DNA and protein/DNA in fish fed the live mysid were significantly higher than those of the fish fed the artificial pellet ($P<0.001$), which resulted from significantly lower DNA content of the fish fed live mysid (Fig. 8). However, no significant differences in total length, body weight and RNA content between the live mysid- and artificial pellet-fed fish were found (Fig. 8). Neither difference in the protein contents nor the ratios of RNA/DNA and protein/DNA of both the groups were significantly correlated with the whole body growth rate.

Discussion

Temperature effects on growth, RNA/DNA ratios, RNA and protein content

At every metamorphosing stage, excepting H stage, the cold-acclimated larval and juvenile flounder had lower content of RNA and DNA, which contrasted with the findings of previous studies in which cold-acclimated fish had higher content of RNA (Goolish et al., 1984; Ferguson and Danzmann, 1990; Foster et al., 1992). No significant difference in RNA/DNA ratios during all metamorphosing stages between the two experimental temperatures was found, being comparable with the results of other studies that showed

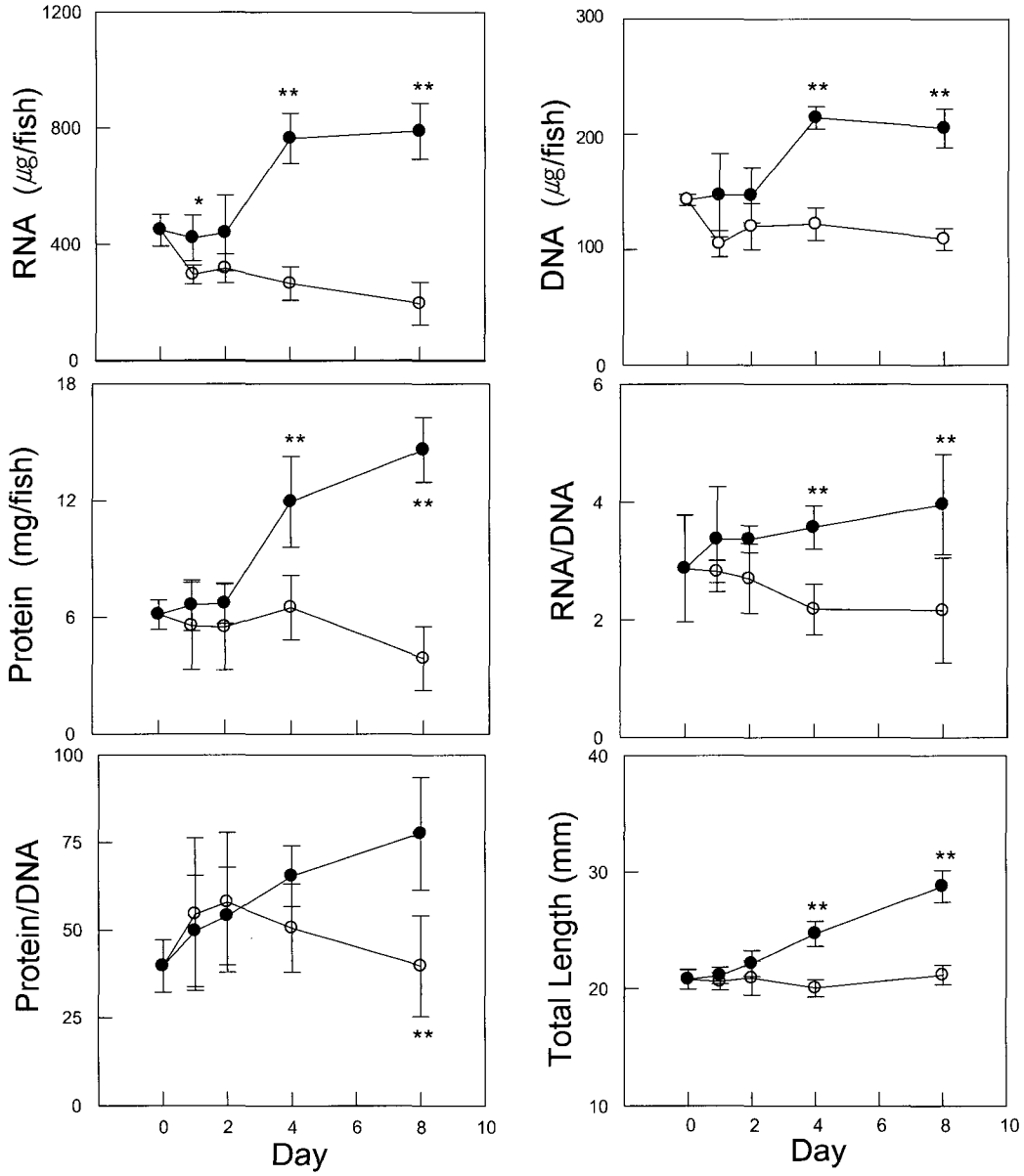

Fig. 7. Variation in RNA content, DNA content, protein content, RNA/DNA ratio, protein/DNA, and total length for fed and starved 20 mm TL juveniles. Values for fed fish (○) and starved fish (•). Values are given as the mean±SD of fifteen individuals. *, $P<0.05$; **, $P<0.001$.

RNA/DNA ratios decreased significantly with increasing temperature (Mathers et al., 1992). However, lower RNA/DNA ratios in the warm-acclimated fish over 20 mm TL were quite consistent with the results of other studies (Foster, 1992; Mathers et al., 1992). The comparable results of RNA, DNA and protein content, and RNA/DNA ratios between the two temperatures were observed during metamorphosis. It might be mainly induced by an increase in cell size or hypertrophy related to an increase in protein content combined with cell division from early- to late-metamorphosing stages and continued active cell division, or hyperplasia, related to an increase in DNA content from late- to post-metamorphic phase (Gwak and Tanaka, 2002).

As Seikai et al. (1986) reported, the early-metamorphosing fish reared at a warmer temperature reached to the post-metamorphic phase within 8 days, whereas the cold-acclimated fish remained in the early-metamorphosing stages during the same days.

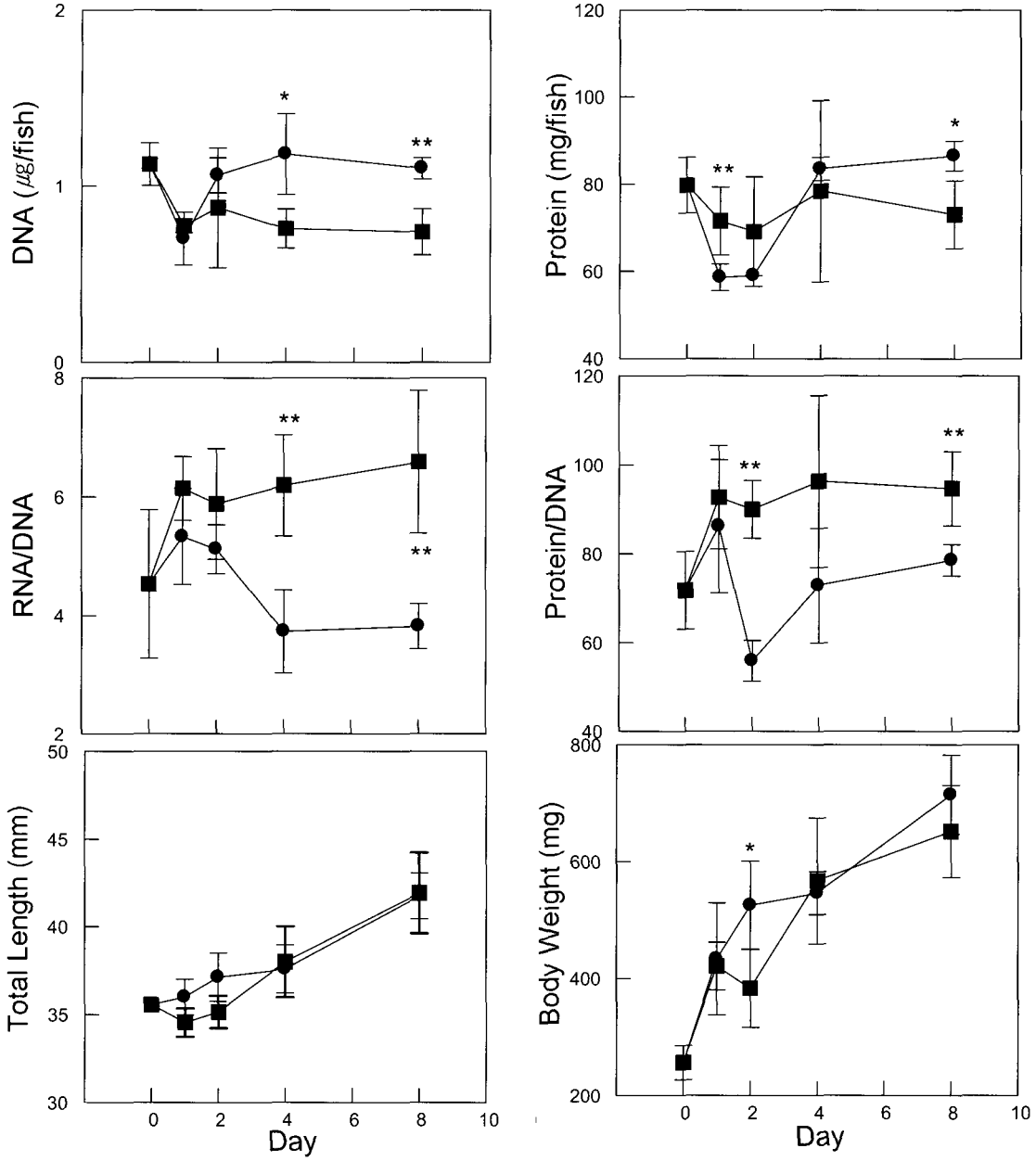

Fig. 8. Variation in DNA content, protein content, RNA/DNA, protein/DNA, total length, and body weight for 35 mm TL juveniles which fed live mysid and artificial food. Values for fish fed live mysid (■) and artificial food (•). Values are given as the mean±SD of fifteen individuals. *, $P<0.05$; **, $P<0.001$.

The present results of relatively higher increasing rate in DNA content of metamorphosing larval and juvenile flounder at the warm temperature also indicated that higher cell proliferation (hyperplasia) and faster completion of metamorphosis could occur compared with those of the cold-acclimated fish. It seems likely that part of this apparent variation in biochemical parameters is due to interspecific differences. Foster et al. (1992) observed that 4℃ decrease in water temperature for 4 weeks resulted in cod having higher white muscle RNA content than fish held at a constant 10℃, even though the fish at each temperature had similar growth rates, and the tissue DNA content was unaltered by temperature acclimation. However, RNA/DNA ratios of olive flounder during metamorphosis seemed to be influenced mainly by substantial increases in DNA content instead of RNA content with increasing temperature.

Higher protein content and protein/DNA ratios during early- and mid-metamorphosing stages in the warm-acclimated fish suggest that they have larger cells than the cold-acclimated fish. Higher protein content in the warm-acclimated fish was caused by an increase in the rate of protein synthesis and also by increased retention of synthesized protein (reduced protein turnover). Gwak and Tanaka (2002) showed that olive flounder larvae actively accumulate energy until the late-metamorphosing stage (H st.) to prepare for a tentative non-feeding period associated with settlement and to finish metamorphosis. Positive linear relationships have previously been observed between tissue protein synthesis rates and RNA content, or RNA/protein ratio (Garlick et al., 1976; McMillan and Houlihan, 1988; Houlihan et al., 1989). Furthermore, the changes in protein content of flounder larvae and juveniles during metamorphosis were closely related to those of RNA content related to the results that the protein/DNA ratios reached to the peak at the post-metamorphic phase (Gwak and Tanaka, 2002). Higher RNA and protein content of the warm-acclimated fish during early- and mid-metamorphosing stages (E-G stages) could reflect a higher rate of protein synthesis stimulated by the need for energy accumulation. Higher growth rate in the warm-acclimated fish was closely associated with marked increase in RNA and protein content, and the difference in growth rate between experimental temperatures become larger as fish grew.

No significant difference in RNA/DNA ratios was found between the two experimental temperatures at the late-metamorphosing stage (H stage). The contents of RNA, DNA and protein in the warm-acclimated fish were exceptionally lower than those in the cold-acclimated fish, being in contrast to the other metamorphosing stages. Westerman and Holt (1988) suggested that highly variable RNA/DNA ratios may be due to the periods of cell proliferation accompanied by high rates of protein synthesis during development of organs, alternating with periods of hyperplasia associated with significantly lower rates of protein synthesis. The late-metamorphosing stage for olive flounder is a substantial transition of metamorphosis at which morphometric, histological and biochemical changes of body reached a peak (Minami, 1982; Fukuhara, 1986; Gwak et al., 1999). Gwak and Tanaka (2002) showed that no increase in protein content corresponding to a decrease in RNA content right after the late-metamorhpic stage when the fish temporarily stop feeding. More energy is needed to finish metamorphosis and keep metabolism constant at higher temperature, and the non-feeding period may concurrently accelerate a degradation of protein.

The results presented here indicate that the effects of water temperature on RNA/DNA ratio have to be ascertained prior to application to wild fish growing at different temperatures and the fish undergoing metamorphosis.

Effects of starvation and kind of foods on growth, RNA/DNA ratios, RNA, and protein content

Somatic growth results mainly from the balance between protein synthesis and degradation (Houlihan, 1988). The relationship between RNA content in the tissue and protein synthesis rates may be explained by stimulation of food uptake on RNA content and is affected by both feeding and the degree to which the fish is fasted (Smith, 1986; McMillan and Houlihan, 1992). Different daily growth rates of wild juvenile flounder, which are mainly caused by the amount of available food organisms, from 3 different nursery areas in Japan were reported (Tanaka et al., 1989). There is evidence that food reduction could reduce the content of RNA in fish tissues much as food reduction determine protein synthesis rates, with white muscle being the tissue most sensitive to fasting (Smith, 1981; Loughna and Goldspink, 1984; Fauconneau et al., 1989 Houlihan, 1991). In addition, a number of studies have revealed that RNA content in the tissues are directly related to the protein synthesis rates (Houlihan, 1991; Mathers et al., 1993). In terms of RNA/DNA ratios, RNA and protein content, the results of starved olive flounder juveniles agree fundamentally with those of the previous studies (Mathers et al., 1994; Gwak and Tanaka, 2001). It is clear from the present study that significantly lower protein and DNA content of the starved fish resulted in smaller cell size (protein/DNA).

Examination of the effects of different food kinds on RNA/DNA ratios and DNA content using live mysid-fed fish is of particular interest. Blaxter (1976) and Theilacker (1986) showed that the morphological, behavioral and histological differences between laboratory-reared and wild fish. Several recent studies suggest that the biochemical difference between the laboratory-reared and wild fish (Balbontin et al., 1973; Buckley, 1981). Significantly higher DNA content of the artificial pellet-fed fish and no difference in RNA content between the live mysid-fed and artificial food- fed fish result in lower RNA/DNA and protein/

DNA ratios of the artificial pellet-fed fish. Mathers et al. (1994) showed similar results indicating no difference in RNA content between laboratory-reared and wild fish, with as contrast to the present results that indicated higher DNA content of wild herring larvae comparing with the laboratory-reared fish.

In the present study, with particular reference to the nucleic acid and protein content, we compared juveniles of olive flounder fed live mysid and artificial food. The results of higher RNA/DNA ratios of live mysid-fed juveniles were similar to the results of Buckley et al. (1984), who observed relatively higher RNA/DNA ratios from sand lance (*Ammodytes* spp.) caught at sea, and Raae et al. (1988) with cod (*Gadus morhua*) larvae. Clemmesen (1989) also showed that there is a great variability in RNA/DNA ratios of the wild larvae as compared to the laboratory-reared one. Similarly, olive flounder larvae and juveniles caught at sea showed significantly higher RNA/DNA ratios compared to laboratory-reared fish at a same stage (Gwak and Tanaka, 2001). Regardless of higher RNA/DNA and protein/DNA ratios of the live mysid-fed fish, no difference in both total length and body weight was found between the two groups. These results are comparable with the results that the wild larvae gain weight more quickly(Buckley, 1981; Raae et al., 1988). It is likely that the nutritional difference in diet component between the live mysid food and artificial food may influence more on the RNA/DNA and protein/DNAratios.

The results presented here have demonstrated the potential usefulness of RNA/DNA ratios for assessing larval and juvenile olive flounder and higher sensitivity of RNA/DNA ratios to both starvation and kind of foods. Further experiments with different ration size are needed to evaluate the growth and nutritional conditions of wild fish.

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