Original Article

Changes in RNA, DNA and protein contents of laboratory-reared Japanese flounder *Paralichthys olivaceus* during metamorphosis and settlement

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**ABSTRACT:** By means of biochemical analyses on an individual basis, developmental changes in nutritional status during metamorphosis and settlement were examined in laboratory-reared Japanese flounder *Paralichthys olivaceus*. Although whole-body DNA content increased gradually throughout metamorphosis and settlement, the RNA content generally remained constant during the late metamorphosing stage and postmetamorphic phase. Subsequently, the RNA : DNA ratio decreased drastically from the late metamorphosing stage to the postmetamorphic phase following a continuous elevation in the ratio during early and mid-metamorphosing stages. The changes in protein content coincided well with RNA content. The protein : DNA ratio also peaked at the postmetamorphic phase and decreased for several days thereafter, suggesting the occurrence of hypertrophy until the postmetamorphic phase, followed by hyperplasia. It is speculated that the nutritional status of Japanese flounder becomes lower when associated with entry to the final phase of metamorphosis and/or settlement at which feeding habits drastically change. Poor nutritional status of the newly settled juveniles may increase their vulnerability to predators in the nursery ground.

**KEY WORDS:** Japanese flounder, metamorphosis, protein content, RNA : DNA ratios, settlement.

INTRODUCTION

Understanding the early life history of marine fish species is of primary importance because intense mortality is usually concentrated during the embryonic and larval periods.¹² Much research has been done to elucidate interannual recruitment variabilities by focusing on the early developmental stages, during which fish are extremely sensitive to environmental variables.⁴⁻⁵

Japanese flounder *Paralichthys olivaceus* is a commercially important species for mariculture and stock-enhancement as well as for coastal fisheries in Japan. It has also been scientifically interesting as a typical model for a marine fish that undergoes true metamorphosis which can be characterized by asymmetrical body transformation coupled with eye migration.⁵⁻⁸ During metamorphosis, habitat shift occurs from the water column to the bottom substrate, resulting in a food shift from plankton to benthos. From an ecological viewpoint, severe mortality associated with the completion of metamorphosis and/or settlement has been reported in plaice *Pleuronectes platessa*, as well as Japanese flounder and summer flounder *Paralichthys dentatus*.⁹⁻¹² Metamorphosis associated with drastic ecological shifts could be regarded as an another potential critical period controlling recruitment in flatfish. However, little is known about mortality associated with metamorphosis and settlement, notwithstanding the late-larval and early juvenile stages during which Japanese flounder settle, and which have apparently drawn much attention as an influential period in determining year-class strength.¹³⁻¹⁵

Tanangonan et al.¹⁶ and Gwak and Tanaka¹⁷ have done preliminary studies on the biochemical

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Changes occurring in laboratory-reared Japanese flounder. However, more detailed experiments are needed to assess and clarify the biochemical changes in Japanese flounder associated with development, especially during metamorphosis and settlement. The overall objective of the present study was to confirm and understand changes in the nutritional status of Japanese flounder during metamorphosis and settlement on a cellular level by determining RNA, DNA and protein contents.

**MATERIALS AND METHODS**

**Incubation and rearing**

To confirm the changes in RNA : DNA ratios during metamorphosis and settlement, a rearing experiment lasting 21 days from stages E to I8 (8 days after reaching stage I) was designed and carried out under rearing conditions that were similar to previous experiments.17,18 Japanese flounder larvae and juveniles were reared at the Fisheries Research Station of Kyoto University, located in Maizuru. Fertilized eggs, provided by the Japan Sea-Farming Association (Miyazu Station), were stocked in 500 L polycarbonate tanks, with running seawater and kept at 15°C until hatching. Water temperature was then raised to 18°C and kept constant throughout the rearing. Rearing was conducted under a natural photoperiod. From 3 days after hatching (DAH), the larvae were fed rotifers (Brachionus plicatilis) that had been cultured by Nannochloropsis oculata, and were fed brine shrimp (Artemia salina) nauplii enriched with squid liver oil from 15 DAH. Rotifers were supplied at a density of 5/mL until 25 DAH, and brine shrimp were provided at a density of 6/mL from 20 DAH.

To initially reduce individual variance, approximately 3000 larvae at stage E were carefully selected from the 500 L stock tanks and transferred to 100 L rearing tanks. The development of Japanese flounder larvae and juveniles was classified according to Minami,5 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.

**Sampling and dry weight measurements**

Sampling was performed daily prior to feeding. To determine the quantities of RNA, DNA, and protein contents in the whole body, 10 fish were individually sampled, rinsed, pipetted into Eppendorf microtubes, immediately frozen at –25°C, and stored at –87°C until required for later analysis.

For measurements of dry weight, individual fish were placed on preweighed pieces of foil and dried to a constant weight at 60°C. To obtain a precise value of dry weight, especially for the smallest larvae, freshly activated silica gel was placed on the balance in order to control for relative hygrometry. Time of removal from the desiccator to weighing was strictly controlled.

**Determination of RNA, DNA and protein quantity**

Measurements of RNA, DNA and protein contents were carried out for individual fish samples. The quantity of RNA and DNA in the whole body was determined by the fluorescence technique using ethidium bromide (Nacalai Tesque Co. Ltd, Kyoto, Japan), as described by Clemmesen,19 but slightly modified by Sato et al.20 Salmon sperm DNA (Wako Chemicals, Osaka, Japan) and yeast RNA (Kanto Chemicals, Tokyo, Japan) were used as standards. RNA and DNA contents are both expressed as mg/larva.

Total protein (dissolved in NaOH) was determined by a Bio-Rad protein kit (Bio-Rad, Tokyo, Japan) using bovine serum albumin as a standard. Results are expressed as g of protein per fish; the ratio of RNA to protein and those of protein to DNA content are cited as indices of protein synthesis capacity and cell size, respectively.21

**RESULTS**

Dry weight (DW) of larvae and early juveniles increased exponentially with developmental progress, and individual variance increased during stage I (Fig. 1).

Expressed as µg/fish (Fig. 2a), DNA content increased rapidly with aging from the onset of metamorphosis (stage E) until the mid-metamorphosing F stage, at which the rate of increase became gradual, and then again increased rapidly from the settlement I stage. RNA content (µg/fish) showed a consistent increase from the early to late metamorphosing H stage, followed by an apparent decrease for several days, and then a re-increase in content was confirmed until the end of the experiment (Fig. 2a).

Ontogenetic changes in DNA and RNA contents in terms of µg/mg DW (Fig. 2b) showed a quite different pattern from those on a fish basis. DNA content (µg/mg DW) at the early and mid-metamorphosing stage appeared rather stable.
compared with RNA content (µg/mg DW), with an evident peak. After that, both DNA and RNA contents showed a marked decrease until the postmetamorphic phase (stage I), and then remained at a stable level.

The protein content, expressed as µg/fish, showed an overall increase from the early to late metamorphosing stage, and then stayed at a stable level until reaching the I₂ stage (Fig. 3a). After the postmetamorphic phase, the protein content of newly settled juveniles showed a marked increase.

A significant increase in protein content, expressed as µg/mg DW, from the early metamorphosing to mid-metamorphosing stages was followed by a consistent decrease until the postmetamorphic phase (Fig. 3b). Thereafter, the protein content showed a gradual increase.

RNA:DNA ratios showed two peaks, which
flounder metamorphosis is its apparent asymmetrical body transformation from a compressed to a depressed type, accompanied by eye migration from the right to the left side of the head, and atrophy of the elongated anterior dorsal fin rays as well as completion of all fin rays. The metamorphosing larvae (stages F and G) migrate naturally from offshore into near-shore shallow nursery areas to settle on the sandy substrate and then complete metamorphosis a few days after settlement. As recent studies have addressed predation-induced mortality during metamorphosis and settlement to some extent, information on the biochemical changes occurring could contribute to understanding starvation-induced vulnerability to predation in the nursery grounds.

Changes in the RNA : DNA ratio

Late larval and early juvenile Japanese flounder show largely fluctuating RNA : DNA ratios during metamorphosis and post-settlement, mainly as a result of fluctuating RNA content values compared with a relatively gradual increase in DNA content. DNA content increased rapidly until 24 DAH, slowly increased during the mid- and late metamorphosing stages (G and H stages), followed by an accelerated rate of increase. This ontogenetic pattern in DNA content appears to be amplified by an ontogenetic pattern in RNA content, resulting in a more dynamic pattern in the RNA : DNA ratio, as shown in Fig. 4. This pattern indicates that the early ontogeny of Japanese flounder is composed occurred after the onset of metamorphosis and during the metamorphic climax of stage H, with a maximum value of 5.07±0.66 (Fig. 4). RNA : DNA ratios dropped markedly to 2.41±0.85 at the I₆ stage, and then tended to increase again. A gradual increase in DNA content compared with a constant level of RNA content was observed between the late metamorphosing and I₆ stages (Fig.2a), causing an overall significant decrease in the RNA : DNA ratio. However, the ratio increased again from the I₆ stage because of a relatively higher increase in RNA content compared with DNA content.

The protein : DNA ratio also peaked at stage I, resulting mainly from a higher increasing rate in protein content. The ratio then decreased markedly until completion of metamorphosis, probably due to a slow increase in protein content and a higher increase in DNA content (Fig.5). RNA : protein ratio showed a drastic decrease at the early metamorphosing stage and remained constant between the mid- and late metamorphosing stages, followed by a slight decrease at the post-metamorphic phase (Fig. 5).

DISCUSSION

Much attention has been given to understanding metamorphosis during the early life history of marine fish mainly because of its importance as an essential developmental strategy. One of the most conspicuous characteristics of Japanese flounder metamorphosis is its apparent asymmetrical body transformation from a compressed to a depressed type, accompanied by eye migration from the right to the left side of the head, and atrophy of the elongated anterior dorsal fin rays as well as completion of all fin rays. The metamorphosing larvae (stages F and G) migrate naturally from offshore into near-shore shallow nursery areas to settle on the sandy substrate and then complete metamorphosis a few days after settlement. As recent studies have addressed predation-induced mortality during metamorphosis and settlement to some extent, information on the biochemical changes occurring could contribute to understanding starvation-induced vulnerability to predation in the nursery grounds.
of cyclic phases of hyperplasia and hypertrophy. Fukuda et al. have reported that this kind of cyclic phase is related to the larval growth of cresthead flounder Limanda schrenki. Takii et al. have also described a similar result in striped jack Caranx delicatissimus. Based on these results, it could be speculated that both RNA and DNA contents are generally linked more closely to developmental stage in larval and juvenile Japanese flounder than to age. Richard et al. have made similar observations for Solea solea, and Ehrlich and Love support the theory that chemical changes are more closely dependent upon larval size than upon age.

A marked increase in RNA content from the early to late metamorphosing stages resulted in a marked increase in protein content and a peak in the RNA : DNA ratio. After peaking at the late metamorphosing stage (stage H), the ratio decreased abruptly until stage I₆, primarily as a result of the marked increase in DNA compared with the decrease in RNA content. A similar change has also been observed by the end of metamorphosis in plaice larvae. Increases in the RNA : DNA and protein : DNA ratios were observed repeatedly and reflected by the increased dry weight. Higher increasing rates in RNA content during days 19–28, and in DNA content during days 30–39 corresponded to hypertrophy and hyperplasia phases of growth in the Japanese flounder. During increases in the RNA : DNA ratio, it could be speculated that larval body growth chiefly occurs through cell enlargement (hypertrophy) resulting from active protein synthesis.

Similar changing patterns in both protein and RNA contents were also confirmed during metamorphosis. Protein content leveled off as RNA content decreased between the late metamorphosing stage and the postmetamorphic phase. The ontogenetic pattern in protein content during metamorphosis and post-settlement corresponds fundamentally with those of RNA content, indicating that RNA content reflects protein synthesis. A steep increase in both RNA and protein content after the postmetamorphic phase was also reflected well by the weight gain in dry weight. This concomitant fluctuation in the contents of protein and RNA has also led to a marked fluctuating pattern in the protein : DNA ratio around 30 DAH and in the RNA : DNA ratio. This decrease in the protein : DNA ratio during settlement coincides well with the result of cresthead flounder. Moreover, a marked decrease in the RNA : protein ratio, which occurred between the late metamorphosing stage and the postmetamorphic phase, suggests that the protein synthesis rate subsequently dropped during the non-feeding period that occurred with settlement. Thus, energy reserves would be required to complete metamorphosis. These findings indicate that active cell enlargement (hypertrophy) may occur between the early and late metamorphosing stages, and is followed by higher cell proliferation (hyperplasia) and reduced protein synthesis during the postmetamorphic phase. Evidently, these results give strong supportive evidence for a characteristic cyclic phase of hyperplasia and hypertrophy during metamorphosis, and are supportive of a previous study done by Gwak and Tanaka. In addition, high levels of accumulated glycogen in hepatic tissues were observed during the early metamorphosing stage, in contrast with the vacuolated hepatocytes found during the postmetamorphic phase. Consequently, it is possible that Japanese flounder larvae save energy by actively synthesizing protein until their metamorphic climax (stage H) so as to cope with a tentative non-trophic period for the few days following settlement—a time when additional energy will be needed to complete metamorphosis.

Conversely, decreases in the RNA : DNA ratios from stages H to I₆ could be explained by higher cell proliferation (hyperplasia) and/or a period of no change in RNA content resulting from the non-trophic phase. Metamorphic transformation mainly involves tissue degeneration caused by the eventual loss of larval structure, proliferation from larval tissues and organs into those of the adult, and the formation of new adult tissues and organs from primordium. RNA and DNA dynamics during metamorphosis would correspond well to these degeneration–proliferation developmental events.

**Relationship between ratios and ecophysiological findings**

The values in RNA : DNA ratios associated with metamorphosis and post-settlement confirm the results of Gwak and Tanaka, and are in agreement with other studies. The mean RNA : DNA ratios were 3.4 and 3.3 in previous experiments, 3.5 for the present study, and ranged between 3 and 4 in other studies. In addition, the lowest ratios of both previous experiments (2.49±0.14, stage I₅) and the present results (2.41±0.85, stage I₆) were similar, and demonstrate that juvenile Japanese flounder have a ‘minimum threshold ratio’ only during the post-settlement phase. Decreases in the RNA : DNA ratios from stages H to I₆ are also closely related to other morphological and histological findings. For example, an ‘arrested growth phase’ in body length is the result of reduced and non-feeding periods, as well as an abrupt decrement in
the epithelial heights in both the intestine and rectum immediately after settlement. In particular, the voluminal changes of the gallbladder, which is one of the most indicative signs among histological characteristics reflecting starvation status, is well correlated with a reduction in RNA:DNA ratios between stages H and I. These biochemical findings also correspond to physiological evidence of a drastic reduction in trypsin-like enzyme activity.\textsuperscript{8,35}

Additionally, the minimum threshold ratio at the postmetamorphic phase coincides well with ecological findings. The settlement of Japanese flounder larvae appears to occur mainly at stage H for both laboratory-reared\textsuperscript{36} and wild fish.\textsuperscript{5,37} Following metamorphosis, laboratory experiments indicate that the newly settled juveniles stop moving and decrease their feeding activities for approximately two days.\textsuperscript{11} Gwak and Tanaka have reported previously that more than 65% of starving wild flounder were classified into stages H and I.\textsuperscript{17}

Because changes in the RNA : DNA ratios during the H stage occur before the completion of eye migration and the resorption of elongated dorsal fin rays, one might suppose that such marked changes are related mainly to ecological changes and not to morphological changes. When rabbit fish \textit{Siganus lineatus} initiate metamorphosis, they spend less time swimming in search of live food, and instead stop at large to gaze.\textsuperscript{38} Similarly, less ability to search for food has been noted for plaice and sole during metamorphosis, which is related directly to feeding success.\textsuperscript{39} A high incidence of empty stomachs was observed among newly settled juveniles.\textsuperscript{5} Compared to other marine fish species that undergo metamorphosis, the non-trophic phase (due to temporal feeding cessation) of Japanese flounder is shorter and may have less severe effects on their survival. However, abnormal feeding activities induced by both a tentative non-trophic period\textsuperscript{8} and an undeveloped food-searching potential caused by drastic habitat change, would increase their vulnerability to predation.

Veer and Bergman have reported that flatfish may be more prone to predation during and shortly after metamorphosis\textsuperscript{15} due not only to new predators on the bottom, but also to reduced escaping ability.\textsuperscript{39} The finding that an individual-based biochemical change occurs may demonstrate that newly settled juveniles are likely to be exposed to severe predation-induced mortality as a result of poor nutritional status. Subsequently, it is evident that there is a second critical period occurring a few days after the settlement of Japanese flounder. Although mortality that occurs shortly after settlement does not seem to be related directly to starvation, both the non-feeding period caused by transitional events and their reduced adaptability in newly settled nursery grounds might deteriorate their nutritional status and increase the possibility of vulnerability to predation.

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**REFERENCES**

12. Witting DA, Able KW. Effects of body size on probability of predation for juvenile summer flounder based on


