Ontogenetic changes in nucleic acid, protein contents, and growth of larval and juvenile Japanese flounder

Xue Hong TONG1,2, *, Cheng Man BAO2, Xin Hui TANG2, Xiao Lan YANG2, Huan Li WANG2
1Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, Yancheng Teachers University, Yancheng, P.R. China
2College of Life Science and Technology, Yancheng Teachers University, Yancheng, P.R. China

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Abstract: In order to estimate relationships among growth potential and biochemical indicators of Japanese flounder, RNA and DNA as well as protein indices were measured from 5 days after hatching (DAH) to 45 DAH. Results showed that the RNA/DNA ratio and protein/DNA ratio had obvious relations with instantaneous growth rate ($G_M$) and length-based instantaneous growth rate ($G_L$) during the premetamorphic period. Significant negative correlations among protein/DNA ratio and $G_M$ and $G_L$ were then observed during metamorphosis. During the postmetamorphic phase, the RNA/DNA ratio was positively correlated with $G_M$ and $G_L$. Data indicated that body growth of Japanese flounder is mostly hyperplastic before 20 DAH, hypertrophic until 27 DAH, and hyperplastic until the end. In order to investigate the effect of diel periodicity on RNA/DNA ratio in larvae of the fed and starved groups, an experiment was conducted for 2 days. Results showed that the average RNA/DNA ratio in the daytime was higher than that in the dark and the ratio in fed larvae was higher compared with that in starved ones. In order to examine starvation effect on RNA/DNA ratio, experiments were conducted from 20 DAH to 27 DAH. Juveniles were divided into five groups: a fed group and groups starved 1, 2, 3, and 4 days followed by refeeding. Results showed that the RNA/DNA ratio increased in the fed group and declined continuously in the starved group. After refeeding, the recovery of the RNA/DNA ratio was observed for 1-day, 2-day, and 3-day starved treatments but was not found for the 4-day starved treatment.

Key words: Flat fishes, growth rate, RNA/DNA ratio, diel variation, starvation

1. Introduction

For flatfishes, the early developmental stages, during which fishes are extremely sensitive to external environment variables, are characterized by intensive mortality and sharp morphological transformation as well as habitat shift, and they are crucial for growth.

Traditionally, for many fish species, the increase rates of body length and body mass have been calculated to estimate growth during larval and juvenile stages (Kerambrun et al., 2012). However, due to the small size of young fish, it is very difficult to measure tiny changes in length or weight accurately. It is well known that changes in fish morphology are the external embodiment of internal biochemical composition.

Recently, nucleic acids and protein-based factors have been proved to be effective indicators of growth (De Raedemaeker et al., 2012; Zehra and Khan, 2014) because of the variation of RNA concentration and protein production as well as the stabilization of DNA concentration in a cell, and they respond quickly to changes in feeding strategies (Yamashita et al., 2003; Mercaldo-Allen et al., 2008; Kerambrun et al., 2012; Fonseca et al., 2014). Furthermore, changes in temperature or food availability are first reflected in the RNA/DNA ratio rather than growth rates (Tong et al., 2010; Zehra and Khan, 2013). As reported, variable nutritional situations are one of the factors that can influence larval survival rates and larvae with poor nutritional condition suffer higher mortality rates than those in good condition (Meyer et al., 2012; Yandi and Altinok, 2015). Recently, RNA/DNA has been widely used to estimate the nutritional condition of fish larvae, and larvae in good condition appear to have a higher RNA/DNA ratio than those in poor condition (Vidal et al., 2006; Mercaldo-Allen et al., 2008). Under a natural environment, fishes may usually suffer short or long periods of starvation, which is one of the main factors leading to high mortality rates and low growth rates for larvae and juveniles. If refeeding is available, recovery may occur, but upon reaching the point-of-no-return, recovery would be impossible (Yandi and Altinok, 2015). Therefore,
it is necessary to determine the critical point below which larvae will be classified as starving by application of the RNA/DNA ratio and the period of starvation resistance. In addition, previous reports revealed that other factors such as diel periodicity, which was investigated in a few studies, also produce a great effect on the RNA/DNA ratio of fishes, especially individuals in good nutritional condition (Rooker and Holt, 1996; Chicharo et al., 2001; Vidal et al., 2002).

The above biochemical parameters can be applied to predict growth potential and nutritional condition of fish larvae and juveniles and to observe fluctuations in food availability before measurable variations in somatic growth become detectable (El-Zaeem et al., 2014; Bandyopadhyay et al., 2015). To date, growth rates of numerous fish species have been successfully evaluated by application of nucleic acids and protein factors (Nunn et al., 2012; Paulsen et al., 2014).

Japanese flounder (Paralichthys olivaceus) is a commercially valuable teleost. Three crucial periods of dilation, weaning, and metamorphosis exist in larval and juvenile life and correspond to nutritional crisis and severe mortality, which are very critical for recruitment and control year-class strength. Therefore, it is very important to study the physiological status of larval and juvenile Japanese flounder.

The present works were conducted to establish the following aims: 1) investigating the physiological status by focusing on the ontogenetic patterns of RNA, DNA, and protein parameters and the relationships among nucleic acid-based indices and the growth rate of larval and juvenile Japanese flounder; 2) evaluating the effects of diel periodicity on the RNA/DNA ratio of fed and starved larval Japanese flounder; 3) examining the influences of starvation and refeeding on the RNA/DNA ratio of Japanese flounder juveniles.

2. Materials and methods
2.1. Larval rearing for experiment on ontogenetic changes in nucleic acid, protein contents, and growth
Larvae and juveniles were reared on a fish farm in Jiangsu Province in China. Eggs obtained from several adult Japanese flounder were stocked in circular incubation tanks with a gentle flow of seawater of 17 ± 0.5 °C after fertilization. Eggs were then transferred into three rearing tanks before hatching.

Larvae were reared in cement tanks with water temperature of 19 °C and dissolved oxygen of 8.0 mg/L as well as a natural photoperiod. Rearing density of larvae was approximately 135 larvae per liter. Larvae were fed rotifers (Brachionus plicatilis) with a density of 8000 to 14,000 individuals per liter from 5 days after hatching (DAH) to 29 DAH, Artemia nauplii with a density of 800–1500 individuals per liter from 15 DAH to 36 DAH, and compound diets from 30 DAH. The rotifers and Artemia nauplii were enriched with AlgaMac-3050 (a product of Aquafauna Bio-Marine, USA).

2.2. Larval rearing for experiments on diel variation and starvation effects on RNA/DNA ratio
Larvae and juveniles were reared on a fish farm in Jiangsu Province in China. To examine the diel variation of the RNA/DNA ratio, individuals aged 14 DAH were divided into two groups: one in the starving group and the other reared under the same conditions as above. To evaluate starvation effects on RNA/DNA ratio, experiments were designed as below:

Fed group: Food was supplied every day from 20 DAH to 27 DAH.

One-day starved group: No food was supplied at 20 DAH. Samples were reared from 21 DAH to 27 DAH.

Two-day starved group: No food was supplied from 20 DAH to 21 DAH. Samples were reared from 22 DAH to 27 DAH.

Three-day starved group: No food was supplied from 20 DAH to 22 DAH. Samples were reared from 23 DAH to 27 DAH.

Four-day starved group: No food was supplied from 20 DAH to 23 DAH. Samples were reared from 24 DAH to 27 DAH.

2.3. Sampling and preservations
To determine the ontogenetic changes in nucleic acid, protein contents, and growth, 35 individuals were collected every day before food distribution to measure total length (L_t) and body mass (M_p). Instantaneous growth rate of body mass (G_M) and total length (G_L) were calculated by the formulae

\[ G_M = \frac{\ln m_t - \ln m_0}{t} \]  

and

\[ G_L = \frac{\ln l_t - \ln l_0}{t} \]  

where \( m_t \) : body mass (mg) and total length (mm) of fish at hatching (0 DAH); \( m_0 \) : body mass (mg) and total length (mm) of fish at each sampling day; \( t \): time interval (from 0 DAH to the sampling days). Absolute growth rate of body mass \( G_{MAm} \) and total length \( G_{LAm} \) were calculated by the formulae

\[ G_{MAm} = (m_t - m_0) t^{-1} \]  

and

\[ G_{LAm} = (l_t - l_0) t^{-1} \]  

Larvae ranging from 560 to 10 individual fish were randomly collected every day from 5 DAH to 45 DAH prior to feeding, rinsed in cold distilled water, and immediately frozen in liquid nitrogen until analysis.

To examine the diel variation of the RNA/DNA ratio, the experiment was conducted over a period of 48 h, starting at 0800 hours. Fifteen individuals were sampled from the fed group and the starved group respectively at 2-h intervals, rinsed in cold distilled water, stored individually in vials, and immediately frozen in liquid nitrogen until analysis.

To evaluate starvation effects on RNA/DNA ratio, 10 individuals were sampled from each group every day at the same time, rinsed in cold distilled water, stored...
individually in vials, and frozen immediately in liquid nitrogen until analysis.

2.4. Biochemical analysis
To determine the ontogenetic changes in nucleic acid and protein contents, total length and body mass of semithawed larvae were measured before determination of biochemical indicators. Samples were then homogenized in an ice-cold fixation solution (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, pH 8.0), using a glass homogenizer placed in 0 °C ice-cold water (Tong et al., 2010). One part of the homogenization was collected to determine nucleic acids, and the other part was collected to measure protein content.

Nucleic acids were extracted and analyzed using a UV-based method according to Mercaldo-Allen et al. (2008) and Tong et al. (2010). First, free nucleotides were removed using a series of washes with cold perchloric acid (HClO₄). RNA was then hydrolyzed with potassium hydroxide and the hydrolysate was acidified with cold HClO₄ to remove RNA from DNA and protein. Then DNA was both hydrolyzed and separated from the remaining protein by the addition of hot HClO₄. RNA and DNA were estimated from the absorbance of the appropriate hydrolysate at 260 nm using the following extinction coefficient: A₂₆₀ of a 1 µg mL⁻¹ solution of hydrolyzed RNA or DNA is 0.3. Absorbance was measured using a GeneQuant pro (Biochrom Ltd., UK). Protein content was analyzed with an assay kit (a product of Jiancheng Bioengineering Institute, Nanjing, China) according to the Bradford method (Bradford, 1976).

2.5. Data analysis
One-way ANOVA and the Tukey test were used to compare variations of growth rates among different developmental periods (premetamorphic period, metamorphic period, and postmetamorphic period). Regression analysis was carried out to assess the relationships among fish size, growth rate, and biochemical parameters. Pearson correlation and the Tukey test (a method used for multiple comparisons) were used to test the relationships of nucleic acid-based indices and protein content, RNA/DNA ratio and protein/DNA ratio with $L_T$ and $M_B$ of Japanese flounder and to test the relationships of RNA:DNA ratio and protein/DNA ratio with growth rate. Significant differences were set at P < 0.05.

3. Results

3.1. Growth
The curves of $L_T$ and $M_B$ showed slow growth during metamorphosis from 20 DAH to 32 DAH and rapid growth until 40 DAH, followed by a sharp increase until 45 DAH (Figure 1). $G_M$ presented the highest value at 6 DAH and declined until 45 DAH. $G_L$ increased from 7 DAH to 15 DAH, then decreased thereafter (Figure 2A). Both $G_M$ and $G_L$ presented the maximal value in the premetamorphic period (Table 1). $G_{AM}$ had a sharp increase up to 20 DAH and a slow decrease until 30 DAH, followed by an increase thereafter (Figure 2B). $G_{AL}$ declined from 5 DAH to 13 DAH, remained at a stable level until 35 DAH, and increased thereafter. Tukey tests showed that $G_{AM}$ was significantly higher during the postmetamorphic period than the other two periods (P < 0.05) (Table 1). As for $G_{AL}$, it had the maximal value in premetamorphic period.

3.2. Biochemical indicators of nucleic acids and protein indices
Protein content presented a slow increase until 14 DAH and a sharp increase up to 24 DAH with fluctuant increase thereafter (Figure 3). Protein concentration increased to the first climax at 25 DAH, decreased rapidly until 37 days after hatching. Values are given as the mean ± SD. Each value is obtained from 35 individuals.

![Figure 1](image-url)
DAH, and fluctuated thereafter. Protein content differed significantly among the three developmental periods (P < 0.05). Protein concentration showed the maximal value in the metamorphic period.

DNA content kept a stable upward tendency up to 32 DAH and a sharp upward tendency until the end (Figure 4). DNA concentration showed two peaks around 15 DAH and 26 DAH, respectively, followed by an abrupt decrease. Tukey tests showed that DNA content differed significantly among the three developmental periods (P < 0.05), and DNA concentration varied significantly between the metamorphic period and postmetamorphic period (P < 0.05).

Figure 2. Instantaneous growth rate (A) and absolute growth rate (B) of body mass (•) and total length (v) in Japanese flounder during larval and juvenile development related to days after hatching. Values are given as the mean ± SD. Each value is obtained from 35 individuals.

Table 1. Growth rate of Japanese flounder at different stages (mean ± SD).

<table>
<thead>
<tr>
<th>Period</th>
<th>Absolute growth rate</th>
<th>Instantaneous growth rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$G_{AM}$</td>
<td>$G_{AL}$</td>
</tr>
<tr>
<td>Premetamorphic period</td>
<td>0.71 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Metamorphic period</td>
<td>0.97 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.48 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Postmetamorphic period</td>
<td>1.35 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.47 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means in the same column followed by different letters are significantly different (P < 0.05).
RNA content increased up to 28 DAH, declined until 33 DAH, and increased again until the end with a slight drop around 37 DAH (Figure 5). RNA concentration fluctuated from 5 DAH to 24 DAH and reached the maximal point around 25 DAH, followed by a decline thereafter. Both RNA content and RNA concentration produced significant differences among the three developmental periods (P < 0.05).

3.3. RNA/DNA ratio and protein/DNA ratio
Protein/DNA ratio had a downward trend from the maximal value of 61.1 ± 3.03 at 5 DAH to the minimal value of 16.3 ± 6.5 at 22 DAH followed by a fluctuant change (Figure 6). RNA/DNA ratio declined slightly up to 20 DAH and reached the peak value of 4.5 ± 0.4 around 27 DAH with an obvious decrease thereafter. Protein/ DNA ratio was significantly higher in the premetamorphic period than the other two periods and RNA/DNA ratio was significantly lower in the postmetamorphic period compared with the other two periods (P < 0.05).

3.4. Correlations of biochemical factors with morphological traits and growth rate
RNA, DNA, and protein contents and protein/DNA ratio all showed significant relations with $L_t$ and $M_b$ (P = 0.000) (Table 2). As for RNA/DNA ratio, it presented a significant
Both RNA/DNA and protein/DNA ratios had obvious relations with $G_M$ and $G_L$ during the premetamorphic period ($P < 0.05$) (Table 3). Protein/DNA ratio had a significant negative correlation with $G_M$ and $G_L$, while RNA/DNA ratio presented no significant relations with $G_M$ and $G_L$ during the metamorphic period. In addition, 63.5% of the variation in RNA/DNA ratio was explained by $G_M$ while 66.5% of the variation was explained by $G_L$. During the postmetamorphic period, RNA/DNA ratio was significantly correlated with $G_M$ and $G_L$, but protein/DNA ratio showed no significant relations with $G_M$ and $G_L$ ($P > 0.05$).

RNA/DNA and protein/DNA ratios were significantly correlated with $G_{AM}$ and $G_{AL}$ during the premetamorphic period (Table 4). Protein/DNA ratio had an obvious relation with $G_{AM}$ and $G_{AL}$ during the metamorphic period, but the RNA/DNA ratio produced no significant relations. During the postmetamorphic period, the above two ratios presented no significant correlations with growth rate ($P > 0.05$).

### 3.5. Diel variation and starvation effects on RNA/DNA ratio

As a whole, the RNA/DNA ratio in fed larvae was higher than that in starved ones (Figure 7). In the fed group, the maximal value appeared at 1400 hours on the first day and 1600 hours on the second day in the afternoon, and the

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**Figure 5.** Changes in RNA content (■) and RNA concentration (◆) of Japanese flounder larvae and juveniles. Values are given as the mean ± SD. The numbers of samples collected each day range from 5 pools of 560 fish initially to 10 individual fish.

**Figure 6.** Changes in protein/DNA (■) and RNA/DNA (◆) of Japanese flounder larvae and juveniles. Values are given as the mean ± SD. The numbers of samples collected each day range from 5 pools of 560 fish initially to 10 individual fish.
lowest values occurred at 0400 hours on the first day and 0200 hours on the second day. In addition, the average RNA/DNA ratio in the daytime was higher than that in the dark over 2 days. The starved larvae showed an RNA/DNA tendency similar to that of the fed ones.

During starvation experiments, the RNA/DNA ratio in the fed group increased from 3.3 at 20 DAH to 4.56 at 27 DAH (Figure 8A). However, in the starved groups, the RNA/DNA ratio declined continuously, i.e. starvation produced great influences on the ratio (Figures 8B–8E).

Table 2. Regression equations of the nucleic acid-based indices and protein content (µg fish⁻¹) with $L_T$ and $M_b$ of Japanese flounder. Statistically significant ($P < 0.05$) values are in bold font.

<table>
<thead>
<tr>
<th>Regression equation</th>
<th>$R^2$ value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA = 0.0005 $L_T^{4.1503}$</td>
<td>0.9644</td>
<td>0.000</td>
</tr>
<tr>
<td>RNA = 0.0085 $L_T^{3.5413}$</td>
<td>0.9343</td>
<td>0.000</td>
</tr>
<tr>
<td>Protein = 0.0563 $L_T^{4.4085}$</td>
<td>0.9401</td>
<td>0.000</td>
</tr>
<tr>
<td>RNA/DNA = –0.0038 $L_T^2$ + 0.0045 $L_T + 3.9271$</td>
<td>0.6228</td>
<td>0.030</td>
</tr>
<tr>
<td>Protein/DNA = 0.1749 $L_T^{-2} - 6.2087 L_T + 72.579$</td>
<td>0.9019</td>
<td>0.000</td>
</tr>
<tr>
<td>DNA = 0.0104 $M_b^{2} + 2.1719 M_b - 17.3$</td>
<td>0.9077</td>
<td>0.000</td>
</tr>
<tr>
<td>RNA = 0.0267 $M_b^{3} + 5.8408 M_b - 32.81$</td>
<td>0.9120</td>
<td>0.000</td>
</tr>
<tr>
<td>Protein = 0.7031 $M_b^{1.8424}$</td>
<td>0.8850</td>
<td>0.000</td>
</tr>
<tr>
<td>RNA/DNA = 0.0002 $M_b^{2} - 0.0369 M_b + 4.158$</td>
<td>0.6179</td>
<td>0.053</td>
</tr>
<tr>
<td>Protein/DNA = 0.012 $M_b^{2} - 1.4083 M_b + 52.978$</td>
<td>0.8263</td>
<td>0.000</td>
</tr>
</tbody>
</table>

$L_T$: Total length; $M_b$: body mass.

Table 3. Regression equations of RNA/DNA and protein/DNA ratios with $G_M$ and $G_L$ of larval and juvenile Japanese flounder.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Regression equation</th>
<th>$R^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premetamorphic period</td>
<td>RNA/DNA = 1.3912$G_M^{0.2844}$</td>
<td>0.5651</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = 1.756$G_M^{0.9291}$</td>
<td>0.5951</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>RNA/DNA = −0.456$G_M + 6.9958$</td>
<td>0.5445</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = −16.751$G_M + 163.58$</td>
<td>0.5997</td>
<td>0.001</td>
</tr>
<tr>
<td>Metamorphic period</td>
<td>RNA/DNA = −0.073$G_M^{2} + 2.723 G_M − 21.27$</td>
<td>0.635</td>
<td>0.957</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = −1.163$G_M + 43.03$</td>
<td>0.6644</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>RNA/DNA = −1.31$G_M^{2} + 17.054 G_M − 51.2$</td>
<td>0.6653</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = −2.51$G_M^{2} + 28.006 G_M − 53.32$</td>
<td>0.7347</td>
<td>0.000</td>
</tr>
<tr>
<td>Postmetamorphic period</td>
<td>RNA/DNA = 0.58$G_L − 5.44$</td>
<td>0.4082</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = 1.77$G_L − 4.21$</td>
<td>0.1595</td>
<td>0.176</td>
</tr>
<tr>
<td></td>
<td>RNA/DNA = 4.42$G_L − 20.43$</td>
<td>0.4146</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = 76.38$G_L^{2} − 802.16 G_L + 2125.4$</td>
<td>0.0389</td>
<td>0.871</td>
</tr>
</tbody>
</table>

$G_M$: Weight-based instantaneous growth rate; $G_L$: length-based instantaneous growth rate.
After refeeding, recovery was observed for the RNA/DNA ratio that increased significantly with 1-day, 2-day, and 3-day starvation treatments (Figures 8B, 8C, and 8D). No significant difference of RNA/DNA ratio was found between starved periods with an average of 2.16 and refeed periods with 4-day starvation treatment with an average of 2.20 (Figure 8E).

Table 4. Regression equations of RNA/DNA and protein/DNA ratios with \(G_{\text{AM}}\) and \(G_{\text{AL}}\) of larval and juvenile Japanese flounder.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Regression equation</th>
<th>(R^2)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premetamorphic period</td>
<td>RNA/DNA = –0.84(G_{\text{AM}}^2) + 0.1(G_{\text{AM}}) + 4.11</td>
<td>0.8377</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = –26.742(G_{\text{AM}}^2) – 1.411(G_{\text{AM}}) + 59.122</td>
<td>0.9259</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>RNA/DNA = –3.68(G_{\text{AL}}^2) + 6.73(G_{\text{AL}}) + 1.1874</td>
<td>0.4021</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = –73.56(G_{\text{AL}}^2) + 176.5(G_{\text{AL}}) – 29.793</td>
<td>0.489</td>
<td>0.019</td>
</tr>
<tr>
<td>Metamorphic period</td>
<td>RNA/DNA = –33.56(G_{\text{AM}}^2) + 64.489(G_{\text{AM}}) – 27.082</td>
<td>0.1775</td>
<td>0.494</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = –39.141(G_{\text{AM}}) + 59.796</td>
<td>0.7751</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>RNA/DNA = –551.51(G_{\text{AL}}^2) + 498.26(G_{\text{AL}}) – 117.09</td>
<td>0.6879</td>
<td>0.777</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = –1580.1(G_{\text{AL}}^2) + 1446(G_{\text{AL}}) – 306.14</td>
<td>0.842</td>
<td>0.000</td>
</tr>
<tr>
<td>Postmetamorphic period</td>
<td>RNA/DNA = 2.6924(G_{\text{AM}}^{-0.2904})</td>
<td>0.2699</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = 0.715(G_{\text{AL}}) + 18.944</td>
<td>0.0217</td>
<td>0.631</td>
</tr>
<tr>
<td></td>
<td>RNA/DNA = –30.38(G_{\text{AL}}^2) + 22.91(G_{\text{AL}}) – 1.48</td>
<td>0.3031</td>
<td>0.051</td>
</tr>
<tr>
<td></td>
<td>Protein/DNA = 672.18(G_{\text{AL}}^2) – 638.26(G_{\text{AL}}) + 170.33</td>
<td>0.1786</td>
<td>0.838</td>
</tr>
</tbody>
</table>

\(G_{\text{AM}}\): Weight-based absolute growth rate; \(G_{\text{AL}}\): length-based absolute growth rate.

Figure 7. Diel variation of the RNA/DNA ratio in the fed (○) and starved (■) Japanese flounder larvae during the 48-h period. Values are given as the mean ± SD. Each value is obtained from 15 individuals. The dark line means the dark time.

4. Discussion

Contents and concentrations of nucleic acid and protein as well as their ratios have been widely used in many studies as tools to predict nutritional condition and growth rate of marine fishes (Richard et al., 1991; Gwak and Tanaka, 2001; Vinagre et al., 2008).
In contrast to the observations found in turbot (Tong et al., 2010), a trend of decreasing growth rate of Japanese flounder was observed during the metamorphic period, implying that a large amount of energy may be used for reorganization of organs and tissues rather than growth. During the postmetamorphic period and settlement, a rapid rise of absolute growth rate was found, maybe due to the relatively higher water temperature increasing the metabolic level (Imsland et al., 2003; Vinagre et al., 2008). The instantaneous growth rate of Japanese flounder was found to decline on the whole throughout the experimental period, consistent with the results reported by Mercaldo-Allen et al. (2006). Many factors such as conversion of food type, transformation of body shape, and changes of livable conditions are directly related to the variability in growth rates (Phelan et al., 2000; Mercaldo-Allen et al., 2006; Nunn et al., 2012). Furthermore, rapid somatic growth of body mass and total length from 37 DAH was found, possibly due to the change in food type to an appropriate commercial feed suitable to the bottom-dwelling life (Park et al., 2008).

As shown in many documents (Mercaldo-Allen et al., 2006; Park et al., 2008; de Raedemaeker et al., 2012; Zehra and Khan, 2013), DNA content was an acute index for live body weight and cell number. In our work, the obvious increase of DNA content during the premetamorphic and postmetamorphic periods proved that a rapid increase in cell numbers occurred and indicates the occurrence of hyperplasia suitable for the development of the gut during the premetamorphic period (Tanaka et al., 1996; Tong et al., 2010) and adaptation to juvenile bottom-dwelling life, respectively. The slow increase from 25 DAH to 31 DAH may be related to the physiological status of larval Japanese flounder, which undergoes degeneration–proliferation developmental events of the skeleton and organs during this period (Gwak and Tanaka, 2002). According to previous studies on turbot (Clemmesen, 1987; Tong et al., 2010), DNA concentration increased during the premetamorphic phase and declined from midmetamorphosis in our work.

RNA content, mainly in fast-growing tissues, is very sensitive to protein synthesis (Caldarone, 2005; de Montgolfier et al., 2005; Fonseca et al., 2006). In our work, the tendency of RNA content was similar to that of protein content. As for RNA concentration, it retained at high levels until 25 DAH, similar to the results found in plaice _P. platessa_ larvae (Christensen and Korsgaard, 1999), and decreased sharply during the following days, revealing the involvement of hypertrophic and hyperplastic events.

![Figure 8. Changes in RNA/DNA ratios of Japanese flounder exposed to different starved-refeeding treatments from 20 to 27 days after hatching: (A) fed (control treatment), (B) 1-day starved, (C) 2-day starved, (D) 3-day starved, (E) 4-day starved. Values are given as the mean ± SD. Each value is obtained from 10 individuals. Dark symbols mean feeding days and empty symbols mean starved days.](image-url)
Many documents suggest that RNA/DNA ratio can sensitively respond to alterations in fish condition (Gwak et al., 2003a, 2003b; Yamashita et al., 2003; de Montgolfier et al., 2005; Mercaldo-Allen et al., 2006, 2008). As for protein/DNA, it is a very useful index for assessing cell weight or cell size (Smith and Buckley, 2003; Caldarone, 2005; Park et al., 2008). In our study, the RNA/DNA ratio varied from 1.89 to 4.49, similar to many other fishes (Gilliers et al., 2004; Vinagre et al., 2008), but it was much higher when compared to reared Solea solea (Richard et al., 1991). The different experimental procedures used may lead to the variations (Caldarone et al., 2006). The decline of RNA/DNA ratio and protein/DNA ratio before 20 DAH meant the decrease of cell size. However, the protein/DNA ratio increased obviously from 22 DAH to 27 DAH, indicating the involvement of hypertrophy, and this was followed by a decline, suggesting the occurrence of hyperplasia (Tong et al., 2010). From the above, it can be speculated that body growth of Japanese flounder is mostly hyperplastic before 20 DAH, hypertrophic from 22 DAH to 27 DAH, and hyperplastic until the end, similar to many other species (Tong et al., 2010).

The increase of protein concentration before 20 DAH may be due to consumption of nutrients such as saccharide and fat other than protein for an energy source. The increase after 20 DAH was due to the lower growth rate of body mass, as observed in blue fin tuna Thunnus orientalis (Temminck & Schlegel) (Tanaka et al., 2007). Based on previous observations (Park et al., 2008), the decline of protein concentration after 28 DAH was maybe due to a high rate of skeletal formation and cellular hydrolysis activity. Furthermore, the trend of protein content was changeable and was maybe correlated with growth potential, as reported in plaice P. platessa larvae (Christensen and Korsgaard, 1999).

In our research, nucleic acid-based indices and protein content were significantly correlated with $L_s$ and $M_s$ (P < 0.05) In addition, both RNA/DNA ratio and protein/DNA ratio were highly correlated with $G_m$ and $G_t$ during the premetamorphic period in accordance with the findings in many other fishes (Mercaldo-Allen et al., 2008; Tong et al., 2010). During the metamorphic period, the protein/DNA ratio showed a strong relationship with $G_m$ and $G_t$ in contrast to results in Atlantic cod (Gadus morhua) and haddock (Melanogrammus aeglefinus) (Peck et al., 2003), indicating that the variations of fish physiological status are not determined by fish size (Islam and Tanaka, 2005). However, when it comes to the postmetamorphic phase, RNA/DNA ratio reflected the growth rate significantly, similar to the results made in tautog (Tautoga onitis), turbot, and so on (Buckley et al., 1999; Mercaldo-Allen et al., 2006; Tong et al., 2010). From the above, the sensitive indicators of growth rate are RNA/DNA ratio and protein/DNA ratio during the premetamorphic phase, protein/DNA ratio during the metamorphic phase, and RNA/DNA ratio during the postmetamorphic phase.

As for the diel variations in RNA/DNA ratio, our results show that diel periodicity produces great influence on the RNA/DNA ratio, which was high in daylight, similar to data on loliginid squid paralarvae (Vidal et al., 2006) and red drum (Rooker and Holt, 1996), but opposite the observations in Crassostrea angulata and Ruditis decussatus (Chicharo et al., 2001) and Sardina pilchardus (Chicharo et al., 1998). Based on the foregoing reports, a circadian periodicity controlled by growth-regulating hormones in endocrine activity may be responsible for the diel variations in RNA/DNA ratio (Chicharo et al., 2001; Vidal et al., 2006). In addition, the RNA/DNA ratio in fed larvae was higher than that in starved larvae, indicating that this ratio can be applied to evaluate nutritional condition (Zehra and Khan, 2013; El-Zaeem et al., 2014; Bandyopadhyay et al., 2015).

In the starvation and refeeding treatments, our results showed that Japanese flounder juveniles exposed to 1, 2, and 3 days of starvation can recover when food is available. If the starvation period was extended to 4 days, the values of the RNA/DNA ratio at 26 and 27 DAH were lower than those at 20 and 21 DAH, which means that starvation produces great damage to the fish body and the physiological status of the fish becomes bad. Based on the changeable RNA/DNA ratios, the upper limit of starvation and the critical level of the RNA/DNA ratio may be 4 days and 1.87, respectively, for Japanese flounder juveniles. In the future, many other issues such as survival rate, growth rate, and the structure of digestive tissues should be studied to verify these speculations and test the influences of starvation on fish deeply (Meyer et al., 2012; Yandi and Altinok, 2015). Accordingly, food supply is an important regulator of physiological status for Japanese flounder juveniles. Many factors such as lower water temperature (which influences physiological processes), body size, and older age can improve the ability to recover after food shortage, and there exist certain relationships among the above factors and starvation (Clemmesen, 1996; Chicharo et al., 1998; Meyer et al., 2012; Yandi and Altinok, 2015).

Differences among rearing conditions including quality of food, density of aquiculture, and water chemistry may account for variance in growth and nucleic acid concentrations of marine fishes (Fukuda et al., 2001). Furthermore, as mentioned by Meyer et al. (2012), starvation can be compensated by catabolizing energy reserves within muscle and liver tissues at certain...
life stages in fish, which results in ambiguous changes in RNA/DNA ratio, revealing that this ratio is not usually considered an indicator of starvation. Accordingly, care should be taken when applying biochemical indicators to assess growth and nutritional status in larval and juvenile Japanese flounder. Therefore, appropriate combinations of biochemical indices and other indicators such as molecular biomarkers, lipid concentration, otolith development, life stages, environmental factors, and so on are required in future work for certain fish species to assess growth potential and nursery condition.

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