

# Ontogenetic shifts in diel feeding pattern and digestive tract morphology in larval and early juvenile barfin flounder *Verasper moseri*

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<https://doi.org/10.67784/1010>

**Abstract.** To establish efficient feeding schedules for seed production of hatchery-reared barfin flounder *Verasper moseri*, we examined ontogenetic shifts in diel feeding pattern and digestive tract morphology during the larval and early juvenile stages. Under culture conditions (lights on 08:00, off 18:00), the number of prey items in the digestive tract (individuals fish<sup>-1</sup>) showed a unimodal diel pattern, peaking at 17:00-19:00 during the initial feeding (9 days after hatching; DAH) and pelagic (15 DAH) stages, whereas it showed a bimodal pattern, with peaks at 11:00-12:00 and 17:00-19:00 during the pre-settlement (40 DAH) and settlement (62 DAH) stages. Daily food consumption increased markedly from 0.18 mg day<sup>-1</sup> in the initial feeding stage to 14.59 mg day<sup>-1</sup> in the pre-settlement stage and 17.56 mg day<sup>-1</sup> in the settlement stage. The corresponding daily food consumption rate (% body weight) was 42%, 90%, and 42%, respectively. The digestive tract gradually acquired an adult-like morphology through the expansion of the foregut into a sac-like structure and differentiation of the pyloric caeca during the pre-settlement stage. These results indicate that feeding characteristics change in association with the development of feeding and digestive capacities, particularly immediately before the onset of eye migration. Continuous feeding throughout the light period during the rotifer-feeding (initial feeding and pelagic) stage and twice-daily feeding in the morning and evening during the *Artemia*-feeding (pre-settlement and settlement) stage are therefore recommended.

**Keywords:** barfin flounder, digestive system, early life stage, feeding characteristic, feeding schedule, ontogeny.

**Introduction.** Feeding success during the larval and juvenile stages strongly influences survival and growth in fish, both in aquaculture settings and in the wild (Pepin, 2024; Treanor et al., 2024). During the larval and early juvenile stages, fish are small and have limited swimming ability, which constrains prey capture. Concurrently, they have high energetic demands because of their rapid morphological and physiological development associated with metamorphosis. As a result, even minor reductions in feeding success have been shown to lead to substantial mortality in various species (China & Holzman, 2014).

Against this background, the feeding characteristics of larvae and juveniles of aquaculture species have been extensively studied. Many fish species exhibit a diel feeding rhythm in response to the light-dark cycles, under natural (Huusko & Sutela, 1998; Shoji et al., 2001; Tomiyama et al., 2016) or rearing conditions (Dou et al., 2000; Iwasaki et al., 2015; Navarro-Guillén et al., 2015). Kotani & Fushimi, (2011) pointed out that the timing of peak feeding activity, the number of feeding peaks, and the daily food consumption (DFC; Table 1) vary among species and developmental stages. Therefore, establishment of an appropriate feeding schedule requires an accurate understanding of the species-specific feeding characteristics across ontogeny.

Table 1

## List of abbreviations used in this study

| Abbreviation | Full term                                   | Unit                     | Description                                                              |
|--------------|---------------------------------------------|--------------------------|--------------------------------------------------------------------------|
| AGR in BW    | Absolute growth rate in wet body weight     | mg day <sup>-1</sup>     | Daily increase in larval body weight                                     |
| AGR in TL    | Absolute growth rate in total length        | mm day <sup>-1</sup>     | Daily increase in larval body length                                     |
| BW           | Wet body weight                             | mg                       | Wet body weight of larvae and juveniles                                  |
| DAH          | Days after hatching                         | —                        | Number of days since larvae hatched                                      |
| DFC          | Daily food consumption                      | mg day <sup>-1</sup>     | Prey weight per day per fish                                             |
| DFR          | Daily food consumption rate                 | %                        | DFC per body weight of larvae                                            |
| DNP          | Daily number of prey items consumed         | inds. day <sup>-1</sup>  | Number of prey items per day per fish                                    |
| NPI          | Number of prey items in the digestive tract | inds. fish <sup>-1</sup> | Number of prey items found in the digestive tract of an individual larva |
| SD           | Standard deviation                          | —                        | Statistical dispersion                                                   |
| SE           | Standard error                              | —                        | Statistical dispersion                                                   |
| TL           | Total length                                | mm                       | Total length of larvae and juveniles                                     |

Barfin flounder *Verasper moseri* is a large pleuronectid flatfish which is distributed in the northwestern Pacific, ranging from northern Japan to the Yellow Sea, East China Sea, Korean Peninsula, southern Sea of Okhotsk, Sakhalin, and the Kuril Islands (Matsuda, 2003; Zolotov & Buslov, 2025). In Japanese waters, it occurs along the Pacific coast north of Ibaraki Prefecture, the Sea of Japan coast north of Wakasa Bay, around Hokkaido, the southern Kuril Islands, and the southern Sea of Okhotsk, and forms an important target of commercial fisheries and aquaculture in Japan (Matsuda, 2003). Annual catches of this species in Hokkaido ranged from approximately 20 to 50 t between 1965 and 1975, but declined sharply after the late 1970s, and had been negligible since the 1980s (Wada et al., 2011). Following the initiation of a large-scale stock enhancement program in 2006, in which more than one million juveniles had been released annually, the stock has shown signs of recovery, and in recent years annual catches have exceeded 150 t. However, most of the currently harvested individuals are of hatchery origin (Kayaba, 2013). In recent years, there have been some reports of juveniles that were naturally spawned and recruited, but such records remain limited (First record of a wild juvenile barfin flounder collected in the Pacific coastal west of Erimo (in Japanese): <https://www.hro.or.jp/upload/41349/ima851.pdf> "Accessed 28 Mar. 2026"; Sakanoue, 2025). This finding indicates that the current fisheries for barfin flounder are highly dependent on artificially produced juveniles.

In preparation for the large-scale seed production program, several aspects of the species' biology and culture techniques have been investigated, such as the process of sexual maturation in broodstock (Kayaba, 2005), rearing methods in tank conditions (Kayaba et al., 2002, 2003; Kayaba, 2005), morphological changes associated with larval and juvenile development (Aritaki et al., 2000), and the relationships between feeding activity and environmental factors (light intensity, prey density, and water temperature) in early developmental stage larvae (Kayaba, 2005). However, it remains unknown how the feeding characteristics of larval and juvenile barfin flounder, including diel feeding patterns and DFC change during ontogeny, and also how the digestive system, which is closely associated with feeding capacity, develops through early life.

In this study, we examined the diel feeding patterns, DFC, and digestive tract morphology of barfin flounder at different ontogenetic stages (initial feeding stage 9 DAH, pelagic stage 15 DAH, pre-settlement stage 40 DAH, and settlement stage 62 DAH) to develop an appropriate feeding regime for hatchery seed production of this species.

## Material and Method

**Rearing of experimental fish.** Fertilized eggs used in the experiment were obtained by artificial fertilization of broodstock barfin flounder reared at the Date Station of Hokkaido Aquaculture Promotion Corporation on April 2, 2024. The fertilized eggs were transported to Hakodate Research Center for Fisheries and Oceans, where they were stocked in 100 L hatching tanks and maintained under a continuous flow of pumped, UV-sterilized seawater until hatching (water temperature: 8.0-8.9°C; salinity: 31-32 ppt; water exchange rate: 400% day<sup>-1</sup>). Hatching was observed 10 days after stocking, with a normal hatching rate of 54%; the remaining 46% either died before hatching or hatched with morphological abnormalities.

Newly hatched larvae (0 days after hatching; DAH) were transferred to three 200 L circular tanks at 1400 individuals per tank and reared until 63 DAH (the end of the experiment). UV-sterilized seawater (salinity: 31-32 ppt) was supplied continuously at a water exchange rate of 120% day<sup>-1</sup>. The rearing temperature regime was established following Matsuda, (2023). Water temperature was set to 10.0°C at 0 DAH and subsequently increased at 0.3°C day<sup>-1</sup>. It was maintained at 14.7-15.0°C from 15 to 23 DAH, then lowered to 13.6-14.1°C between 24 and 56 DAH to prevent skewed sex ratios (Goto et al., 1999). Thereafter, it was gradually increased and maintained at 14.7-15.2°C from 57 DAH onward. Illumination was provided by incandescent lamps under a 10L:14D photoperiod (lights on at 08:00 and off at 18:00). The maximum light intensity during the light period was 700 lx, and that during the dark period was 0.63 lx.

Larvae were fed L-type rotifers *Brachionus plicatilis* from 8 to 26 DAH, and *Artemia* sp. nauplii (Institute for Marine Aquaculture College of Agriculture Can Tho University, Can Tho, Vietnam) from 27 to 63 DAH. Both live feeds were enriched with Super Capsule A1 powder (Chlorella Industry Co., Ltd., Tokyo, Japan) at a concentration of 0.1 g L<sup>-1</sup> for 6-14 h prior to feeding. Supplemental feeding was conducted twice daily (08:00 and 15:00), and the ration was adjusted according to the residual prey density in the tanks. *Nannochloropsis* (Frozen Chlorella; SAN-U Fish Farm, Osaka, Japan) was added to the rearing water at a concentration of 1 × 10<sup>6</sup> cells mL<sup>-1</sup> to enhance larval feeding activity during the rotifer-feeding period.

The wet weight of individual prey items used in this study was estimated as in the following method. First, the prey density (individuals; inds. mL<sup>-1</sup>) was determined by counting under a stereomicroscope (SMZ1500; Nikon Solutions Co., Ltd., Tokyo, Japan). The samples were then blotted dry with filter paper and weighed using an analytical balance (AE200; Mettler-Toledo, Greifensee, Switzerland). As a result, the wet weight per individual was estimated at 2±0.2 µg for rotifers and 19±0.3 µg for *Artemia* (mean±SD, *n* = 3). These mean values were used to estimate the DFC of larvae and juveniles.

**Experiment for estimating the diel feeding pattern.** To investigate the relationship between the ontogenetic stage and the diel feeding pattern, a series of experimental trials was conducted during the larval and juvenile stages. The experimental dates, materials, and conditions are summarized in Table 2.

Table 2  
Developmental stages of barfin flounder *Verasper moseri* larvae and juveniles and rearing conditions used in each experimental trial

| Date     | DAH | Ontogenetic stages    | Developmental stage* | Prey items             | Prey density threshold (inds. mL <sup>-1</sup> ) | Water temperature (°C) |
|----------|-----|-----------------------|----------------------|------------------------|--------------------------------------------------|------------------------|
| '24.4.21 | 9   | Initial feeding stage | C                    | Rotifer                | 3-5                                              | 12.6                   |
| '24.4.27 | 15  | Pelagic stage         | D                    | Rotifer                | 3-5                                              | 14.9                   |
| '24.5.22 | 40  | Pre-settlement stage  | E, F                 | <i>Artemia</i> nauplii | 0.5-1.2                                          | 13.9                   |
| '24.6.13 | 62  | Settlement stage      | G, H, I              | <i>Artemia</i> nauplii | 0.5-1.2                                          | 15.0                   |

Note: DAH = days after hatching; \* Aritaki et al., (2000).

Experimental trials were performed four times according to ontogenetic stage: at the initial feeding stage (9 DAH), pelagic stage (15 DAH), pre-settlement stage (40 DAH), and settlement stage (62 DAH). According to the developmental stage classification of Aritaki et al. (2000), these correspond to stage C at 9 DAH, stage D at 15 DAH, stages E-F at 40 DAH, and stages G-I at 62 DAH (Figure 1).

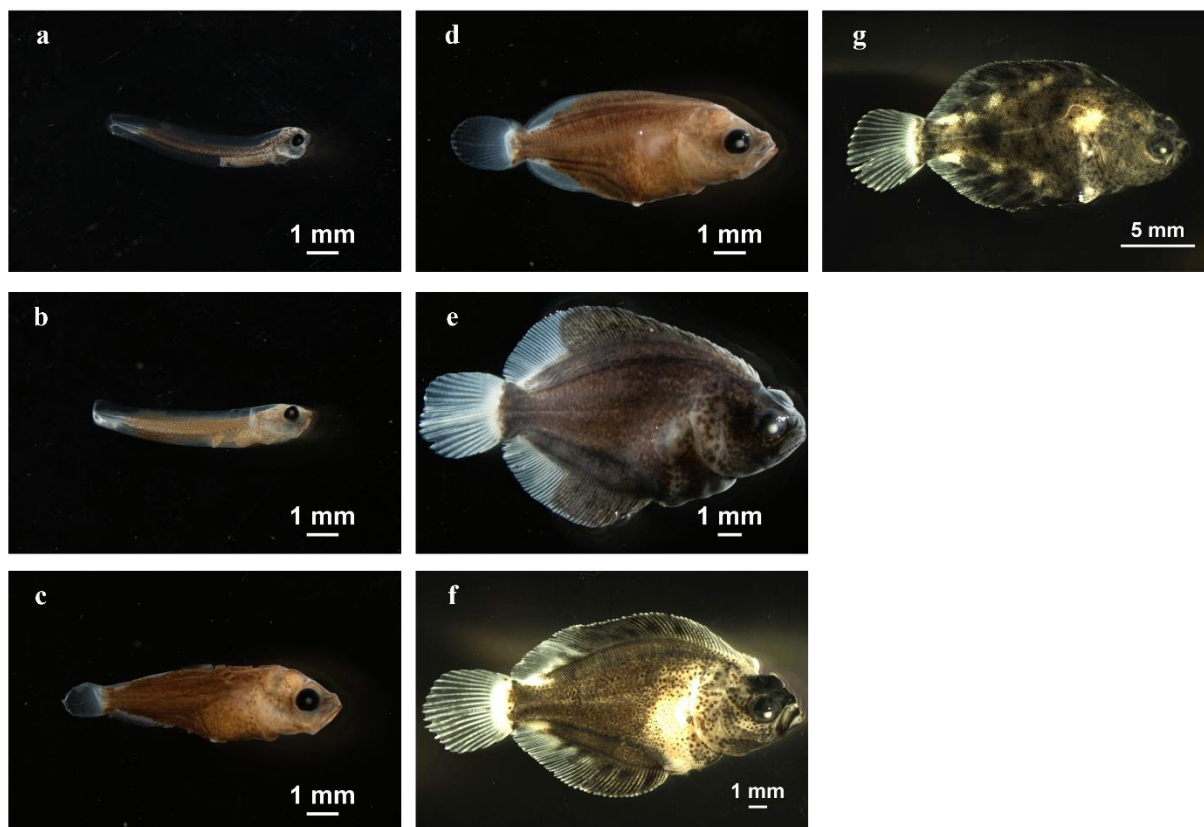


Figure 1. Development of barfin flounder larvae and juveniles during the experiments. The classification of developmental stages follows as defined by Aritaki et al., (2000). (a) Stage C: initial feeding stage, 9 days after hatching (DAH), (b) Stage D: pelagic stage, 15 DAH, (c) Stage E: pre-settlement stage, 40 DAH, (d) Stage F: pre-settlement stage (onset of metamorphosis), 40 DAH, (e) Stage G: settlement stage (early metamorphosis), 62 DAH, (f) Stage H: settlement stage (late metamorphosis), 62 DAH, (g) Stage I: settlement stage (juvenile), 62 DAH.

On each of the four experimental days, 20 larvae or juveniles were randomly sampled from the rearing tanks at 09:00, 11:00, 13:00, 15:00, 17:00, 21:00, and at 01:00, 05:00, and 09:00 on the following day. The sampled individuals were preserved in 90% ethanol. Total length (TL; to the nearest 0.01 mm) and wet body weight (BW; to the nearest 0.1 mg) were measured using a stereomicroscope ( $< 10.00$  mm) or digital calipers ( $\geq 10.00$  mm) and an analytical balance. After measurement, the abdominal region of each specimen was dissected under a stereomicroscope, and the number of prey items in the digestive tract (NPI; inds. fish<sup>-1</sup>) counted.

To maintain prey density within the target range during the experimental trials, prey density in the tanks was monitored at each sampling time (Table 3). During the rotifer-feeding period, supplemental rotifers were added whenever the prey density fell below 3 inds. mL<sup>-1</sup>, restoring the density to 5 inds. mL<sup>-1</sup>. During the *Artemia* feeding period, supplemental *Artemia* nauplii were provided whenever the prey density decreased to  $\leq 0.5$  inds. mL<sup>-1</sup>, increasing the density to 1.2 inds. mL<sup>-1</sup>.

Table 3

Prey densities in tanks at each time during experiments

| <i>Ontogenetic stages</i> | <i>Prey items</i>      | <i>Mean±SD of prey density in tanks at each time (inds. mL<sup>-1</sup>)</i> |              |              |              |              |              |              |              |              |
|---------------------------|------------------------|------------------------------------------------------------------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
|                           |                        | <i>09:00</i>                                                                 | <i>11:00</i> | <i>13:00</i> | <i>15:00</i> | <i>17:00</i> | <i>21:00</i> | <i>01:00</i> | <i>05:00</i> | <i>09:00</i> |
| Initial feeding stage     | Rotifer                | 2.3±0.74                                                                     | 4.6±1.02     | 3.3±0.53     | 1.7±1.25     | 4.4±0.94     | 3.7±0.72     | 2.4±0.42     | 2.7±1.44     | 1.8±1.37     |
| Pelagic stage             | Rotifer                | 2.7±0.74                                                                     | 2.3±1.25     | 3.3±0.98     | 4.3±1.15     | 2.9±1.66     | 3.1±1.50     | 3.7±1.47     | 2.1±0.96     | 1.7±0.94     |
| Pre-settlement stage      | <i>Artemia</i> nauplii | 0.1±0.07                                                                     | 0.3±0.07     | 0.6±0.1      | 0.3±0.07     | 0.4±0.08     | 0.2±0.05     | 0.3±0.13     | 0.4±0.22     | 0.8±0.17     |
| Settlement stage          | <i>Artemia</i> nauplii | 0.5±0.16                                                                     | 0.7±0.25     | 0.5±0.12     | 0.4±0.05     | 0.6±0.21     | 0.6±0.14     | 0.5±0.22     | 0.5±0.12     | 0.7±0.09     |

**Experiment to estimate the digestion time.** To estimate DFC of larvae and juveniles, the temporal decrease in NPI was examined under starvation conditions to determine digestion time. In this experiment, approximately 150 larvae or juveniles were transferred from the rearing tanks to a 20 L circular tank and starved for 24 h. Subsequently, rotifers or *Artemia* nauplii were provided. After feeding activity had largely ceased (two hours after prey were added), all individuals were transferred to another 20 L circular tank (a non-feeding environment) to prevent further feeding. A total of 15-20 individuals were sampled at 1 h intervals from immediately after transfer (0 h) to 6 h post-transfer and preserved in 90% ethanol. The collected samples were dissected under a stereomicroscope, and the number of prey items remaining in the digestive tract was counted. For each DAH, ontogenetic stage, prey type, prey density, and water temperature used in this experiment were matched to those used in the diel feeding pattern experiment (Table 2). Digestion time was defined as the time required for NPI to decline to one-tenth of its value at the cessation of feeding. It was estimated from the exponential regression describing the temporal decline in NPI (see below).

**Estimation of DFC.** To estimate DFC ( $\text{mg day}^{-1}$ ) at each ontogenetic stage, the consumption model developed by Elliott & Persson, (1978) was applied to the number of prey items instead of the prey mass to calculate the daily number of prey items consumed (DNP; inds.  $\text{day}^{-1}$ ), defined as the number of prey organisms consumed per fish per day. This method has previously been employed by Yamamoto et al., (2005a) and Iwasaki et al., (2015). First, the temporal decrease in the number of prey items in the digestive tract under no supplemental feeding conditions was described as:

$$S_t = S_0 \cdot \exp(-R \cdot t) \quad (1)$$

where  $S_t$  is the number of prey items in the digestive tract at time  $t$ ,  $S_0$  is the number at  $t = 0$ , and  $R$  is the gut evacuation rate. The number of prey items consumed during each time interval was estimated as:

$$C_i = ([S_{i+1} - S_i \cdot \exp(-R \cdot \Delta t_i)] \cdot R \cdot \Delta t_i) / (1 - \exp(-R \cdot \Delta t_i)) \quad (2)$$

where  $C_i$  is the estimated number of prey items consumed during interval  $i$ ,  $S_i$  and  $S_{i+1}$  are the mean number of prey items in the digestive tract at the beginning and end of interval  $i$ , respectively, and  $\Delta t_i$  is the duration of interval  $i$ . DNP (inds.  $\text{day}^{-1}$ ) was calculated by summing the estimated prey consumption over all time intervals within a 24 h period:

$$\text{DNP} = \sum_{i=1}^m C_i \quad (3)$$

where  $m$  is the number of time intervals within a 24 h period. DFC ( $\text{mg day}^{-1}$ ) and daily food consumption rate (DFR; %) then estimated as:

$$\text{DFC} = \text{DNP} \cdot W \quad (4)$$

$$\text{DFR} = \text{DFC} \cdot 100/B \quad (5)$$

where  $W$  is the mean wet weight of an individual prey item,  $B$  is the wet body weight of larvae or juveniles.

**Observation and measurement of digestive tract.** To elucidate digestive system development, the digestive tract morphology and volume were examined at each of the four ontogenetic stages. Larvae and juveniles with empty digestive tracts preserved in 90% ethanol at initial feeding stage, pelagic stage, pre-settlement stage, and settlement stage ( $n = 15$  for each stage) were used for these analyses.

After dissection of the abdominal region, the entire digestive tract was excised and sketched. Digestive tract volume was estimated from the projected area and thickness of each segment (foregut, midgut, and hindgut). The project area ( $A$ ) was measured from lateral images using an image analysis system (NIS-Elements D; Nikon Solutions Co., Ltd., Tokyo, Japan) based on images captured with a video camera attached to a stereomicroscope (SMZ25; Nikon Solutions Co., Ltd., Tokyo, Japan). The thickness of the

digestive tract along the z-axis (z) was measured after rotating the specimen by 90°. Assuming an elliptical cross-section, digestive tract volume was calculated as:

$$V = \sum_i \left( \frac{\pi}{4} \cdot A_i \cdot z_i \right) \quad (6)$$

where  $V$  is the digestive tract volume (mm<sup>3</sup>),  $A_i$  is the projected lateral area of segment  $i$  (mm<sup>2</sup>), and  $z_i$  is the lengths of the z-axes of segment  $i$ .

**Statistical analysis.** To evaluate diel variation in NPI, generalized additive models (GAMs) were applied. Because the data contained zeros (empty digestive tracts), a hurdle-type modeling approach was adopted, separately modeling (1) feeding occurrence (presence or absence of prey items in the digestive tract) and (2) prey counts conditional on feeding. Feeding occurrence was modeled using a binomial GAM, and positive prey counts were modeled using a negative binomial GAM. Time of day was included as a smooth term using cyclic cubic regression splines to account for the 24 h periodicity. Peak times were estimated from the fitted GAM predictions. Predictions were generated at 500 equally spaced time points, and local maxima were identified using numerical differentiation. Peaks were identified as local maxima where the first derivative changed sign from positive to negative.

For comparisons among ontogenetic stages, data normality was assessed using the Shapiro–Wilk test. When the normality assumption was met, one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) test was used; otherwise, Kruskal–Wallis test followed by Dunn’s post hoc test was used. Significance level was set at  $p = 0.05$ . All statistical analyses were conducted using R software (version 4.5.2; <https://www.r-project.org> “Accessed 9 Jun. 2026”).

## Results

**Growth of larvae and juveniles.** Larval total length (TL) and body weight (BW) increased more rapidly in the later developmental stages compared to the early stages (Table 4; Figure 2). The absolute growth rate (AGR) in TL or BW were 0.162 mm day<sup>−1</sup> and 0.002 mg day<sup>−1</sup> between the newly hatched and initial feeding stage (0–9 DAH); 0.112 mm day<sup>−1</sup> and 0.095 mg day<sup>−1</sup> between the initial feeding and pelagic stage (9–15 DAH); 0.158 mm day<sup>−1</sup> and 0.606 mg day<sup>−1</sup> between the pelagic and pre-settlement stage (15–40 DAH); and 0.408 mm day<sup>−1</sup> and 1.191 mg day<sup>−1</sup> between the pre-settlement and settlement stage (40–62 DAH).

Table 4

Growth of barfin flounder *Verasper moseri* larvae and juveniles

| Ontogenetic stage     | DAH | Mean±SD of TL<br>(mm) | Mean±SD of BW<br>(mg) | AGR in TL<br>(mm d <sup>−1</sup> ) | AGR in BW<br>(mg d <sup>−1</sup> ) |
|-----------------------|-----|-----------------------|-----------------------|------------------------------------|------------------------------------|
| Newly hatched stage   | 0   | 5.2±0.03              | 0.4±0.03              | —                                  | —                                  |
| Initial feeding stage | 9   | 6.7±0.24              | 0.4±0.11              | 0.162                              | 0.002                              |
| Pelagic stage         | 15  | 7.4±0.36              | 1.0±0.25              | 0.112                              | 0.095                              |
| Pre-settlement stage  | 40  | 11.3±1.24             | 16.1±7.20             | 0.158                              | 0.606                              |
| Settlement stage      | 62  | 20.3±1.98             | 42.4±11.98            | 0.408                              | 1.191                              |

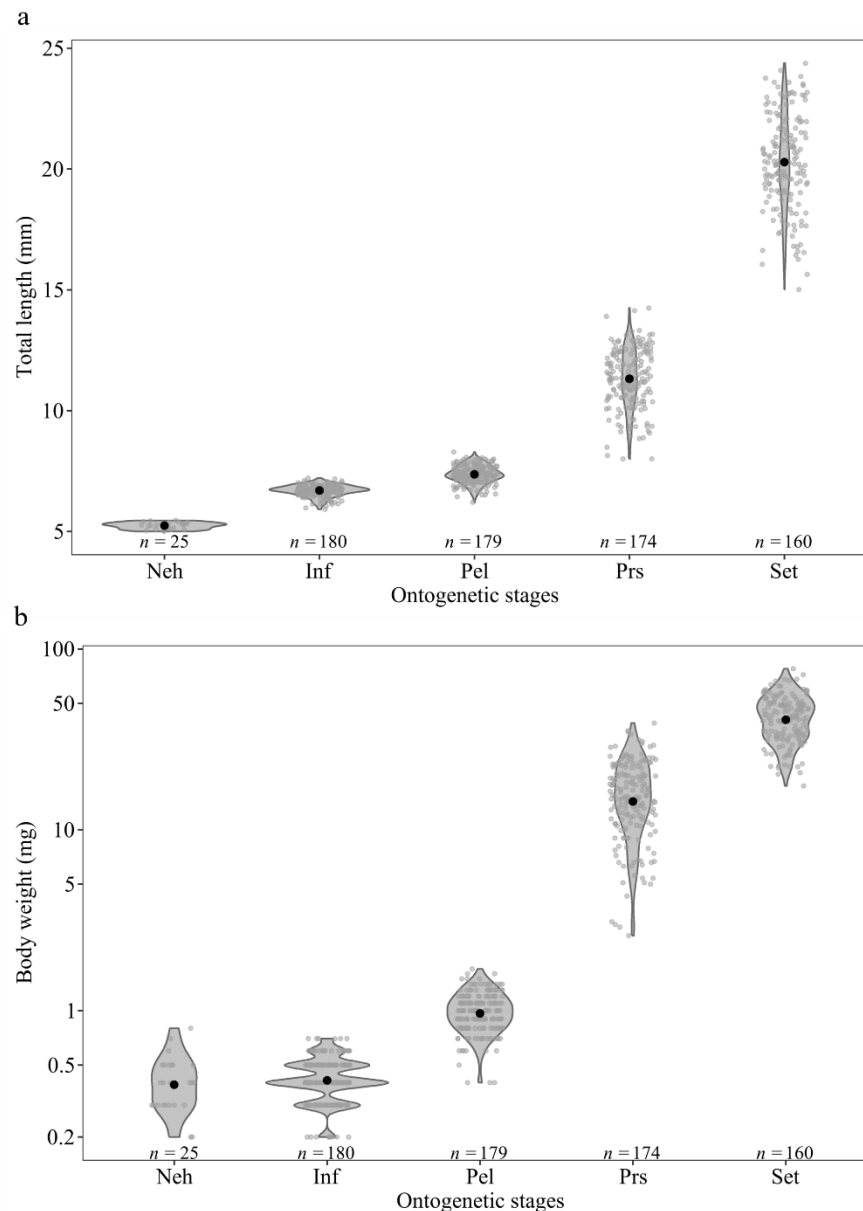


Figure 2. Growth in total length (TL; a) and wet body weight (BW; b) of barfin flounder larvae and juveniles. The violin plots show the distribution of measured values in each ontogenetic stage (Neh: newly hatched stage, Inf: initial feeding stage, Pel: pelagic stage, Prs: pre-settlement stage, and Set: settlement stage), and the mean values are indicated by black dots.

**Diel feeding pattern.** In all ontogenetic stages examined in this study, prey items were present in the digestive tract at 09:00, and larval NPI decreased after the transition to the dark period (Figure 3; Table 5). For the initial feeding stage, mean NPI generally increased throughout the light period (09:00-17:00), and GAM analysis showed a single peak around 19:00 (Figure 3b). In the pelagic stage, a GAM analysis indicated a similar pattern, with a single peak at 17:00 (Figure 3c). The decline during the dark period was more rapid than that found for the initial feeding stage, and digestion was nearly complete by 05:00, with only small amounts of rotifers remaining in the hindgut. In contrast, diel variation in NPI in the pre-settlement stage exhibited two distinct peaks, one around 12:00 and another between 18:00 and 19:00, as identified by GAM analysis (Figure 3d). In the settlement stage, similarly two peaks were observed at 11:00-12:00 and 17:00-18:00 (Figure 3e). Mean NPI in pre-settlement and settlement stages remained relatively stable or decreased slightly between 11:00 and 15:00, followed by a marked increase and reaching a maximum

at 17:00. The decline during the dark period was rapid and all individuals had completely emptied their digestive tracts by 05:00.

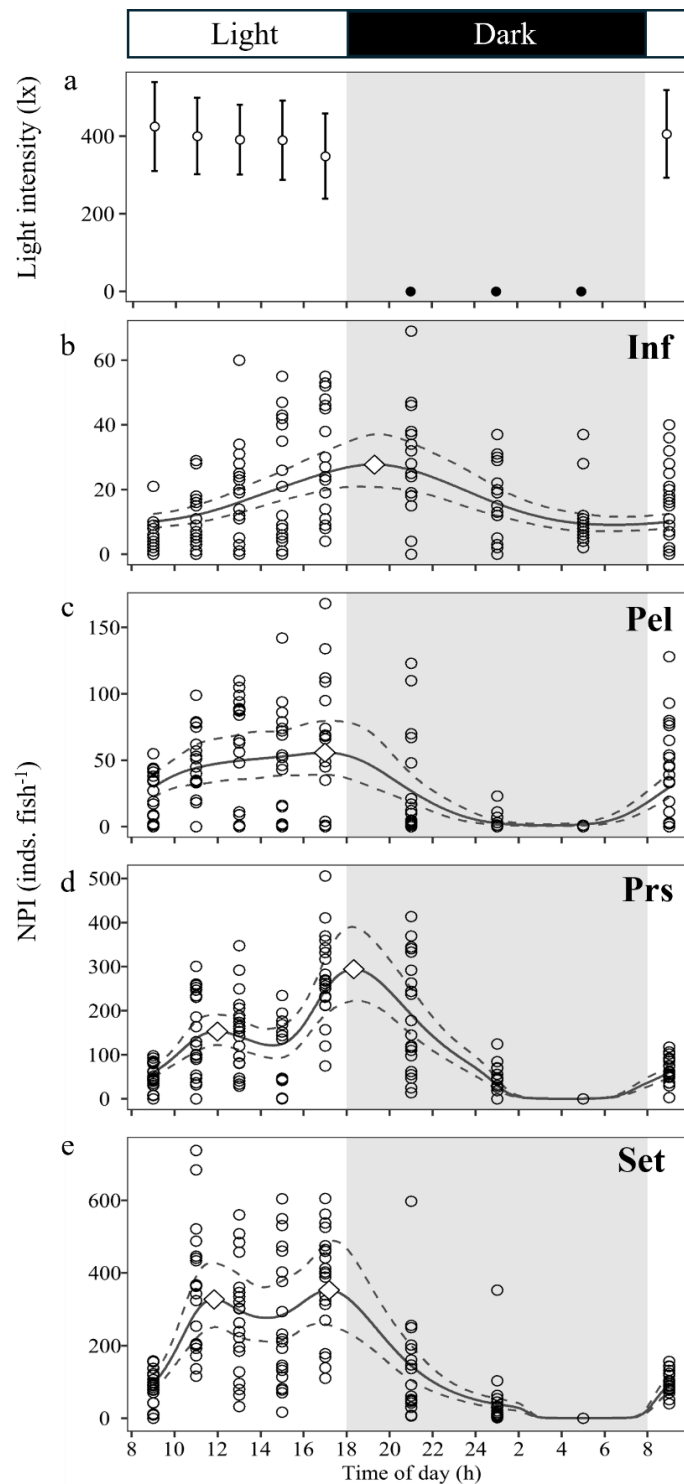


Figure 3. Diel change in light intensity at the water surface of breeding tanks (a) and NPI of barfin flounder larvae and juveniles (b-e). Values of (a) are expressed as mean (open in the light period, solid in the dark period) and standard error (error bar). The line in (b) to (e) represents the predicted curve obtained by applying GAM, and the diamond symbols indicate the local maxima of the predicted curve. Dashed lines indicate the 95% confidence interval. Rotifers were used as food organisms in the initial feeding stage (b: Inf) and pelagic stage (c: Pel) experimental trials, and *Artemia* sp. nauplii in the pre-settlement stage (d: Prs) and settlement stage (e: Set) experimental trials.

Table 5

Light intensity and NPI of barfin flounder *Verasper moseri* larvae and juveniles at each time

| Time  | Mean±SE of light intensity (lx) | Mean±SE of NPI (inds. fish <sup>-1</sup> ) |               |                      |                  |
|-------|---------------------------------|--------------------------------------------|---------------|----------------------|------------------|
|       |                                 | Initial feeding stage                      | Pelagic stage | Pre-settlement stage | Settlement stage |
| 09:00 | 425.0±114.5                     | 4.5±1.08                                   | 18.8±4.13     | 52.6±6.18            | 84.2±9.89        |
| 11:00 | 400.7±98.7                      | 9.1±1.92                                   | 39.0±6.66     | 147.1±20.23          | 350.0±37.70      |
| 13:00 | 391.3±90.0                      | 18.5±3.29                                  | 53.6±9.00     | 150.4±18.96          | 274.3±33.47      |
| 15:00 | 389.7±101.9                     | 20.6±4.07                                  | 42.4±8.97     | 92.3±20.58           | 266.4±45.89      |
| 17:00 | 348.5±109.7                     | 27.9±3.67                                  | 61.9±11.34    | 273.6±21.70          | 376.2±31.49      |
| 21:00 | 0.1±0.01                        | 25.4±4.08                                  | 28.8±8.36     | 190.6±27.89          | 143.6±29.15      |
| 01:00 | 0.5±0.06                        | 16.5±2.41                                  | 2.3±1.23      | 31.1±7.90            | 38.6±17.13       |
| 05:00 | 0.6±0.05                        | 9.4±1.86                                   | 0.2±0.08      | 0.0±0.00             | 0.0±0.00         |
| 09:00 | 405.7±112.8                     | 15.3±2.70                                  | 48.3±7.20     | 65.8±6.19            | 94.4±7.38        |

Note: The first and last measurements at 09:00 represent the beginning and end of the 24 h observation period, respectively.

**Estimation of the gut evacuation rate (*R*) and digestion time.** After transfer to the non-feeding environment, NPI showed a rapid decrease and followed an exponential decline over time (Figure 4).

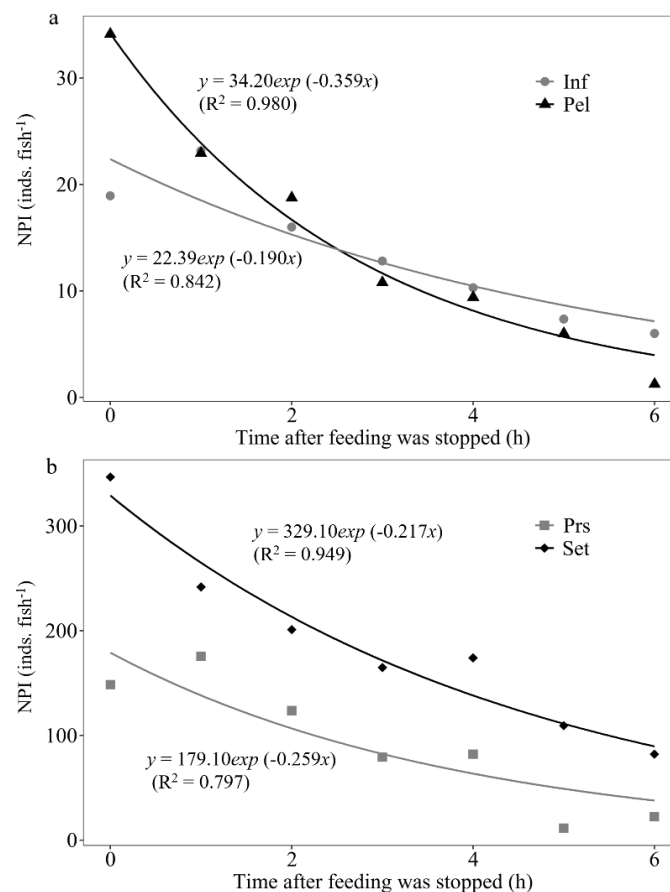


Figure 4. Time-course decline of contents in the digestive tract of barfin flounder larvae and juveniles after transfer to a non-feeding environment. (a) shows initial feeding (Inf) and pelagic stage (Pel) experiments, and (b) shows pre-settlement (Prs) and settlement stage (Set) experiments. Points (different symbols) represent mean values, and the lines represent the fitted regression curves. The gut evacuation rate (*R*) was estimated from the parameter *b* of  $y = a \cdot \exp(-b \cdot x)$  following Elliott & Persson, (1978).

Exponential regression analysis yielded gut evacuation rates ( $R$ ) of  $0.190 \pm 0.042$  (mean  $\pm$  SE;  $p < 0.01$ ) in the initial feeding stage,  $0.359 \pm 0.029$  ( $p < 0.001$ ) in the pelagic stage,  $0.259 \pm 0.073$  ( $p < 0.05$ ) in the pre-settlement stage, and  $0.217 \pm 0.025$  ( $p < 0.001$ ) in the settlement stage. All estimates were significantly different from zero. Estimated digestion times varied among ontogenetic stage, being 12.1 h for the initial feeding stage, 6.4 h for the pelagic stage, 8.9 h for the pre-settlement stage, and 10.6 h for the settlement stage.

**DFC and DFR.** DNP (inds. day<sup>-1</sup>), DFC (mg day<sup>-1</sup>) and DFR (%) were calculated for each ontogenetic stage following the results of diel feeding pattern and gut evacuation rate (Table 6). DNP ranged from 90.3 to 924.1 inds. day<sup>-1</sup> from the initial feeding stage to the settlement stage. DFC was estimated to be 0.18 mg day<sup>-1</sup> in the initial feeding stage and 0.58 mg day<sup>-1</sup> in the pelagic stage when rotifers were provided. After the diet was shifted to *Artemia*, DFC increased to 14.59 mg day<sup>-1</sup> in the pre-settlement stage and 17.56 mg day<sup>-1</sup> in the settlement stage. DFR increased from 42% at the initial feeding stage to 90% at the pre-settlement stage, and subsequently decreased to 42% at the settlement phase.

Table 6

Parameters of daily feeding in each experimental trial

| DAH | Ontogenetic stages    | $R^*$ | DNP (inds. day <sup>-1</sup> ) | DFC (mg day <sup>-1</sup> ) | DFR (%) |
|-----|-----------------------|-------|--------------------------------|-----------------------------|---------|
| 9   | Initial feeding stage | 0.190 | 90.3                           | 0.18                        | 42      |
| 15  | Pelagic stage         | 0.359 | 287.9                          | 0.58                        | 58      |
| 40  | Pre-settlement stage  | 0.259 | 768.0                          | 14.59                       | 90      |
| 62  | Settlement stage      | 0.217 | 924.1                          | 17.56                       | 42      |

Note: DAH = days after hatching;  $R$  = gut evacuation rate; DNP = Daily number of prey items consumed; DFC = Daily food consumption; DFR = Daily food consumption rate; \* Elliott & Persson, (1978).

**Changes in the digestive tract morphology and volume.** The digestive tract was divided into the foregut (oesophagus and stomach in later stage), midgut (intestine), and hindgut (rectum) (Figure 5).

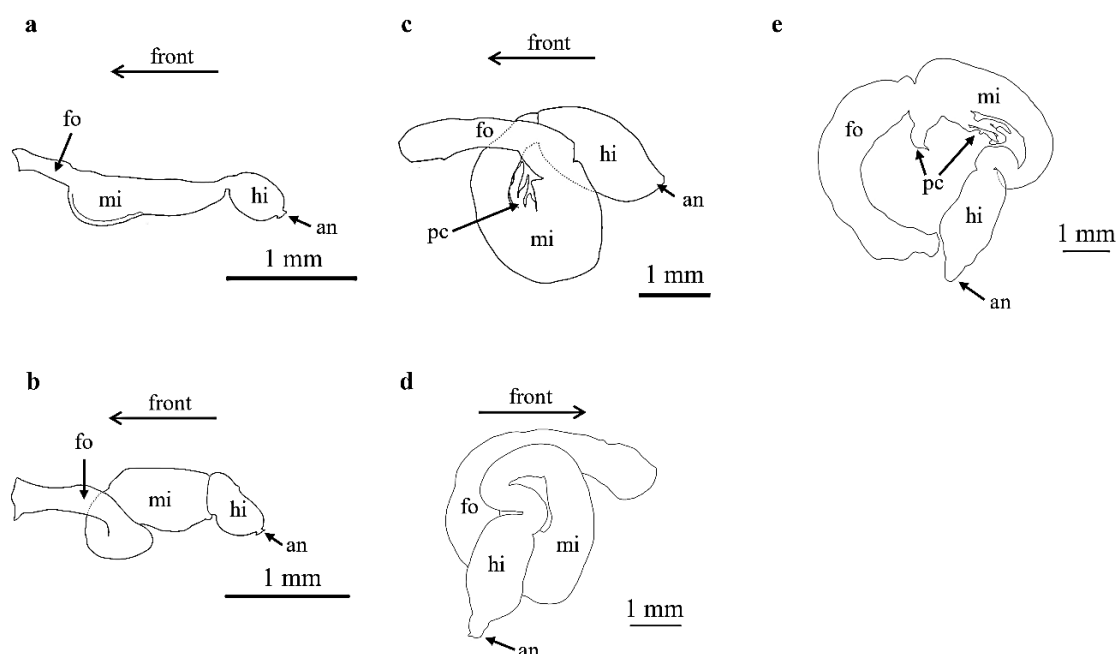


Figure 5. Sketches of the digestive tract of larval and juvenile barfin flounder representative ontogenetic stages: (a) initial feeding stage, (b) pelagic stage, (c) pre-settlement stage, (d) settlement stage (ocular side view), (e) settlement stage (after extension). Arrows (front) show the anterior direction of the head (mouth). Abbreviations: fo: foregut; mi: midgut; hi: hindgut; pc: pyloric caeca; an: anus.

In the initial feeding stage, the tract was linear from the foregut to the anus, and the foregut was narrow and short (Figure 5a). The midgut and hindgut were already clearly distinguishable during this stage. In the pelagic stage, the foregut became elongated, and coiling of the anterior part of the midgut began (Figure 5b). In the pre-settlement stage, the posterior part of the foregut developed into a sac-like structure resembling a stomach (Figure 5c). Pyloric caeca began to develop from the anterior part of the midgut at the same time, and intestinal coiling became more pronounced than in the pelagic phase. In the settlement stage, the posterior part of the midgut became folded into an S-shaped configuration, resulting in increased structural complexity of the digestive tract (Figure 5d). The pyloric caeca increased in both number and size (Figure 5e). Digestive tract volume increased significantly with ontogenetic development (Dunn's post hoc test,  $p < 0.01$ ; Figure 6). Estimated digestive tract volume (mean $\pm$ SE) was  $0.07\pm0.002\text{ mm}^3$  for the initial feeding stage,  $0.18\pm0.012\text{ mm}^3$  for the pelagic stage,  $2.88\pm0.176\text{ mm}^3$  for the pre-settlement stage, and  $4.38\pm0.353\text{ mm}^3$  for the settlement stage.

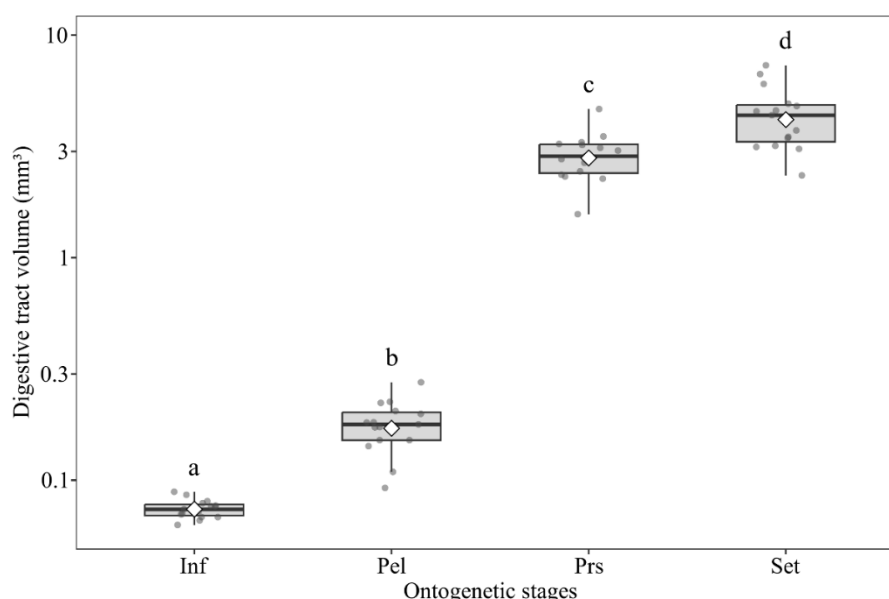


Figure 6. Digestive tract volume of barfin flounder larvae and juveniles in each ontogenetic stage (Inf: initial feeding stage, Pel: pelagic stage, Prs: pre-settlement stage, and Set: settlement stage). The boxplots show the distribution of digestive tract volume; individual observations are indicated by dots, and the mean values are shown as diamonds. Different letters indicate significant differences according to Dunn's post hoc test ( $p < 0.05$ ).

**Discussion.** Larval and juvenile barfin flounder exhibited a clear diel feeding pattern throughout ontogeny and were identified as predominantly diurnal visual predators. This interpretation is supported by the absence of any increase in NPI during the dark period for all ontogenetic stages, indicating that feeding activity is restricted to the light period. Furthermore, the highest NPI peak consistently occurred before lights-off (17:00) throughout development. Many marine fish larvae and early juveniles are known to depend on light for successful prey detection and capture (Pereira-Davison & Callan, 2018; Kiyono, 2025), primarily because feeding relies heavily on visual cues (Lim et al., 2021). A feeding peak immediately before dark has also been reported in larvae of other fish species under both natural and rearing conditions (Yamada et al., 1989; Shoji et al., 2001). This phenomenon has been attributed to synchronization between the circadian rhythms and feeding activity (Rønnestad et al., 2013) and may represent compensatory feeding prior to the nocturnal fasting period, during which visually oriented larvae are unable to feed efficiently.

The diel pattern of NPI during the light period changed markedly with ontogeny. During the initial feeding and pelagic stages, NPI showed a unimodal pattern with a single peak around lights-off. In contrast, in the pre-settlement and settlement stages, a bimodal

pattern was observed, with two peaks occurring at 11:00-12:00 and around lights-off. Similar ontogenetic shifts from a unimodal to a bimodal feeding pattern have been reported in the larval redspotted grouper *Epinephelus akaara* (Kayano, 2009) and the longtooth grouper *Epinephelus bruneus* (Iwasaki et al., 2015) and have been attributed to the development of the digestive system (Kato et al., 2004). In barfin flounder, the timing of this shift coincided with a marked enlargement of the digestive tract (see below), as well as rapid relative growth of the eyes and mouth and completion of the fin-ray development (Aritaki et al., 2000). These findings suggest that improvements in sensory perception, swimming performance, and digestive capacity collectively enhanced feeding ability, enabling larvae to consume a large amount of prey after lights-on when the digestive tract was nearly empty. After this initial period of consumption, the following period of digestion lead to a reduced level of prey intake, and then a subsequent resumption of feeding later in the afternoon. The bimodal feeding pattern observed after the pre-settlement stage therefore likely reflects a transition to a more intermittent feeding pattern. Continuous feeding may shorten prey retention time in the digestive tract through displacement by newly ingested prey, thereby reducing nutrient absorption efficiency (Harboe et al., 2009; Rønnestad et al., 2013). The intermittent feeding pattern may represent an adaptive strategy that temporarily suppresses feeding through satiety feedback, improving feeding efficiency.

Although feeding activity remained restricted to the light period in the present study, with no feeding observed at 0.09-0.63 lx, feeding ability under dark conditions may improve through the development of sensory systems during ontogeny. Kayaba, (2005) reported that feeding activity ceased below 160 lx in the pelagic stage (stage D; 21 DAH), whereas individuals in the settlement stage (stage G; 47 DAH) were capable of feeding even at 20 lx. However, it remains unclear whether this ontogenetic change is attributable to the development of the visual system or to the maturation of other sensory organs involved in detecting chemical or mechanical stimuli, such as the olfactory system and lateral line. In several pleuronectid species, larvae and juveniles have been reported to feed under extremely low light conditions. For example, larvae of *Pleuronectes platessa* have been reported to feed at light intensities of 0.01-1 lx (Blaxter, 1968). The marbled flounder *Pseudopleuronectes yokohamae* (Yamamoto et al., 2005b) and soleids such as *Solea senegalensis* (Cañavate et al., 2006) and *Solea solea* (Bonvini et al., 2016) acquire the ability to feed under dark conditions around the metamorphic stage. The ability to feed under low-light conditions may extend the time available for feeding and reduce resource competition with other species.

The most pronounced morphological changes in the digestive tract of barfin flounder larvae occurred during the pre-settlement stage, when the posterior region of the foregut expanded into a sac-like structure and differentiation of the pyloric caeca began. Concomitantly, digestive tract volume increased to approximately 16 times that observed during the pelagic stage, and DFC increased by more than 25-fold. In teleosts, the digestive tract undergoes two major phases of differentiation, one at the onset of exogenous feeding and the other during metamorphosis, when the stomach, gastric glands, and pyloric caeca develop to establish an adult-type digestive system (Zambonino-Infante et al., 2008; Nazemroaya et al., 2020). The present results suggest that barfin flounder similarly acquires a storage-oriented digestive system during the pre-settlement stage, allowing larvae to continue feeding before previously ingested food has been completely evacuated from the digestive tract. This morphological transition likely contributed to the marked increase in DFC observed during this ontogenetic stage.

Although digestive tract differentiation around metamorphosis is common among pleuronectid species, the timing of this process differs among species. In barfin flounder (the present study) and the congeneric spotted halibut *Verasper variegatus* (Hotta et al., 2001), differentiation of the pyloric caeca begins immediately before the onset of eye migration. In the marbled flounder (Fukuhara, 1988) and the olive flounder *Paralichthys olivaceus* (Yasunaga, 1972), differentiation occurs after the eye migration has begun. Such interspecific differences may have adaptive significance. Aritaki, (2013) suggested that, in several pleuronectid species, the developmental stage immediately preceding metamorphosis is the period of greatest susceptibility to factors causing post-metamorphic

morphological abnormalities, and that conditions experienced during this stage strongly influence post-metamorphic morphology. Minami, (1995) proposed that metamorphosis constitutes a critical period because the transition to a benthic lifestyle constrains swimming behavior, potentially resulting in reduced feeding activity and increased mortality from starvation and predation. These findings suggest that the period from immediately before the onset of metamorphosis to its completion is of considerable ecological importance for survival in pleuronectid species. Accelerated development of the digestive system and the associated enhancement of feeding capacity before metamorphosis may therefore represent an adaptive response that enables larvae to cope with this vulnerable developmental period.

DFC continued to increase from the pre-settlement to the settlement stage, and DFR, which represents the relative DFC with respect to body weight, declined. During the same period, AGR in BW reached its maximum. These findings suggest that, based on the digestive system that had undergone rapid development by the pre-settlement stage, nutrients acquired during the settlement stage were utilized primarily for rapid somatic growth. Such accelerated growth would facilitate the transition from a pelagic to a benthic lifestyle and may shorten the duration of the critical period associated with metamorphosis.

Overall, the ecological findings obtained in this study improve our understanding of feeding and growth strategies during the early life history of barfin flounder and providing a basis for predicting appropriate feeding schedules and feeding rations during seed production. From the initial feeding to the pelagic stage, feeding activity was sustained throughout the light period, indicating that prey density should be maintained above a certain level during the daytime. Accordingly, a feeding regime similar to that proposed by Russo et al., (2017), in which nutritionally enriched rotifers are supplied continuously in small amounts throughout the light period, is likely to be effective. In contrast, from the pre-settlement stage onward, feeding activity temporarily declined during the daytime. As discussed above, this developmental period is ecologically important and requires adequate nutritional support for normal development. However, excessive feeding may lead to a deterioration of the water quality and increase production costs. If *Artemia* nauplii are supplied continuously throughout the light period, prey introduced during periods of low feeding activity may remain uneaten, thereby contributing to these negative effects. Furthermore, because the nutritional value of *Artemia* nauplii has been shown to decline with time (Navarro et al., 1999), larvae may consume a greater proportion of nutritionally depleted prey remaining in the rearing tank. Considering these factors, providing *Artemia* twice daily - once in the morning and evening - appears to be the most efficient feeding strategy.

**Conclusions.** Diel feeding patterns of larval and juvenile barfin flounder shifted from a unimodal to a bimodal pattern between the pelagic and pre-settlement stages. This transition coincided with pronounced morphological development of the digestive tract, accompanied by marked increases in digestive tract volume and DFC. These results suggest that barfin flounder enhances its feeding capacity immediately before the onset of eye migration, representing an adaptive strategy for surviving the metamorphosis period. Based on these ecological findings, we propose that a feeding regime should be shifted ontogenetically from the continuous feeding to the intermittent (twice daily) feeding to maximize feeding efficiency in seed production. Further studies should investigate the development of sensory organs and ontogenetic changes in behavior and prey responsiveness during metamorphosis to achieve a more comprehensive understanding of the early life history of this commercially important species.

**Acknowledgements.** We would like to express our gratitude to Shoichi Akaike of the Stock Enhancement Promotion Department, Hokkaido Aquaculture Promotion Corporation, for providing the fertilized eggs used in this study. We are also grateful to Mitsuhito Kon and the staff of the Date Station of the Hokkaido Aquaculture Promotion Corporation for their valuable advice on fish rearing. We sincerely thank Dr. Jun Yamamoto of Hokkaido University for his assistance with the rearing experiments. We are also grateful to Dr. Chris

Norman of Japan Scientific Texts for his careful English editing and valuable comments on the manuscript.

**Authors Contributions.** Conceptualization: YE, MN, TT. Methodology: YE, MN, CG, TM, TT. Formal analysis: YE, MN, TT. Investigation: YE, MN, CG, TM, TT. Data curation: YE, MN, TT. Writing–Original Draft: YE, MN, TT. Writing–Review & Editing: YE, MN, CG, TT. Supervision: MN, TT.

**Conflict of Interest.** The authors declare that there is no conflict of interest.

**Data Availability.** The data supporting the findings of this study are available from the corresponding author upon reasonable request.

**Ethical Approval.** All experimental procedures involving animals were conducted in accordance with the Guidelines for Animal Experimentation of Hokkaido University.

**Funding.** This work was supported by JST SPRING, Grant Number JPMJSP2119.

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Received: 15 July 2026. Accepted: 30 August 2026. Published online: 21 October 2026.

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#### How to cite this article:

Eno, Y., Nakaya, M., Goto, C., Matsuda, T., & Takatsu, T. (2026). Ontogenetic shifts in diel feeding pattern and digestive tract morphology in larval and early juvenile barfin flounder *Verasper moseri*. *Aquaculture, Aquarium, Conservation & Legislation*, 19(5), 2479-2496. <https://doi.org/10.67784/1010>