INTRODUCTION

Aggressive behavior, including cannibalistic behavior, has a significant impact on the early life history of fishes not only in the rearing conditions but also in the wild. The Japanese flounder (Paralichthys olivaceus), which is one of the major target species for stock enhancement in Japan, is not an exception. In the seedling production of Japanese flounder, aggressive behavior and cannibalism cause high mortality. Although more than 20 million juveniles are produced artificially and released every year, many cases do not show an increase in the flounder population around the coastal waters because of the high mortality of seedlings after release. It is reported that predation, including cannibalism by wild stocks, is one of the major reasons for this unsatisfactory output from the stock enhancement of this species, and that predation of newly settled juveniles by the earlier settled ones may occur. Therefore, the study of the aggressive behavior of this species can provide not only practical information to improve the seedling production methods, but also biological information on the ecological reasons for this behavior in both released and newly settled wild flounder juveniles.

There have been many studies on the early life stages of this species, such as morphological development, environmental effects on growth, physiological development, nutritional requirements, feeding behavior, settlement and diel rhythms in spacing and swimming behaviors. Recently, Dou et al. reported environmental effects, such as fish density, starvation and fish size on cannibalistic behavior using fully grown juveniles (over 20 mm in body size). However, there has been no study on the ontogenetic changes of aggressive behavior of this species, which would give us basic biological information regarding this behavior. Therefore, we conducted detailed behavioral observations in metamorphosing to juvenile stages and aimed to determine the ontogenetic changes of aggressive behavior concerning settlement, Ohm (Ω)-posture, which is regarded as feeding behavior, and diel rhythms.
MATERIALS AND METHODS

Materials

Two batches (Batches 1 and 2) of Japanese flounder were used. Fertilized eggs of Batch 1 were transferred from Miyako-station of Japan Sea Farming Association (JASFA) to the Fisheries Experimental Station of Kyoto University. About 7000 newly hatched larvae (day 0) were obtained on 10 July 1999. Larvae were kept in a 500-L transparent rearing tank with a filter system using specially formed ceramic beads for fish rearing (Micros Ceramic M10; Norra Inc., Kyoto, Japan). Enriched rotifers (Brachionus plicatilis) were fed from day 3 to day 18 and then enriched Artemia nauplii were fed from day 12 to day 48. Artificial diets (A-400 and B-700, Kyowa-hakko, Tokyo, Japan) were fed from day 27. Water temperature was kept at 18–20°C and light condition was natural.

Fertilized eggs of Batch 2 were transferred from Obama-station of JASFA to the Nagasaki Prefectural Fisheries Experimental Station. About 20 000 newly hatched larvae (day 0) were obtained on 26 May 2000. Larvae were kept in the same circulating system as Batch 1. Enriched rotifers (Brachionus rotundiformis) were fed from day 3 to 23 and then enriched Artemia nauplii were fed from day 18 to day 24. Water temperature was kept at 18°C and light condition was natural.

Behavioral indexes

In order to quantify the behavioral observations, we defined four behavioral indexes: (i) aggressive behavior (Nip): a fish attacks and bites at the tail or body of another fish; (ii) Ω-posture: a specific behavior during which fish hold a position, hovering with pectoral fins and bending the body strongly in an Ω or reversed Ω shape; (iii) feeding: attacking and ingesting rotifers or Artemia; and (iv) settlement: a fish stays and adheres to the bottom or side of the tank. We define Nip as an index of aggressive behavior because we could clearly observe that the fish showed aggression towards another fish. However, we could not observe swallowing, which Dou et al. included in the aggressive behavior of this species, because the size difference of fish was less than 50%, which causes cannibalism in this species. We also realized that there is a settlement-like behavior named temporary settlement in the metamorphosing stage, in which flounder lie on the bottom with the left side up. In the present study, we included this temporary settlement into the category of Settlement for the convenience of the behavioral observation.

Observation of ontogeny of aggressive behavior

Fish from Batch 1 were randomly sampled from the rearing tank with a 2-L white plastic beaker. Six white experimental tanks (ø 30 cm), each containing 5 L of seawater (7 cm depth) were kept in a water bath at 18°C. A total of 30 fish were used for observation in one age group. Five fish (one fish/L) were gently introduced in each experimental tank at 8.00 h using a glass pipette. Fish were acclimatized for 2 h before observations. After acclimatization, behavior of fish in each tank was observed from above and frequency of index behaviors was counted for 5 min at 4 h intervals until 8.00 h. At the dusk and dawn we conducted additional observations. Light condition was natural and the light intensity at the water surface was measured with an illumination-meter (IM-2D; TOPCON, Tokyo, Japan). During the night, a flash-light covered with an infrared filter (IR76; Fuji Film Inc. Tokyo, Japan), transmit wave length (= 740 nm) was used for observation. Fish were fed rotifers at a density of five individuals/mL until day 20, and Artemia at the density of one individual/mL thereafter, three times a day (at 9.00 h, 16.00 h and dawn). Experiments were conducted nine times, on days 13, 16, 19, 22, 25, 29, 35, 39 and 46, respectively. After observations, all fish were immediately anesthetized with MS222 (Tricaine; Sigma Chemical Co., St Louis, MO, USA) and were fixed in 5% formalin solutions. Standard length (SL, mm) of the fish was measured with a microscope or caliper. Developmental stages of the fish were determined following the definition by Minami.

Frequencies of defined index behavior were pooled and mean value (per minute per fish) was obtained at each observation time and age group (six tanks). For the comparison of behavioral units, data of the 8.00 h observation time (24 h after introducing the fish to the experimental tanks) were used. Differences in the frequencies of Nip and Ω-posture between age groups and between observation times in the same age group were examined by the Mann–Whitney U-test. Proportions of settled fish number (Settlement, %) were calculated from each experimental tank. For the comparison of Settlement (%) and SL between age groups and between observation times, a one-way analysis of variance (ANOVA) was applied. In cases where significant differences were found among
the means by ANOVA \((P<0.05)\), Fisher’s PLSD test was applied for comparison among groups.

\(\Omega\)-Posture and feeding behavior

This experiment was conducted on 21 June 2000 using the larvae from Batch 2. Larvae were unfed from 7.00 h and were taken from the rearing tank with a 13-L bucket at 10.00 h (3 h starvation). A total of 42 fish were used for observation and were selected from the bucket sample to minimize size variation.

Fourteen white plastic beakers (\(\varnothing 12\) cm) each containing 1 L of filtered seawater at 18\(^\circ\)C (about 8 cm in depth) were placed in a temperature-controlled room (18\(^\circ\)C) as experimental tanks. Light condition was set at the same light intensity as the surface of the rearing pond at 10.00 h (200 lx). These experimental tanks were divided into two groups with seven tanks for each. One group (Unfed) was kept unfed and another group (Fed) was fed Artemia at the density of one Artemia/mL in each tank. Three fish were gently introduced by a glass pipette into each experimental tank and were acclimatized for 10 min. Frequency of \(\Omega\)-posture and feeding in each experimental tank were recorded from above for 5 min. In addition, because a flounder bends its body to the left and right sides frequently when in the sequence of \(\Omega\)-posture, we recorded the duration of bending to the left and right sides in a sequence of \(\Omega\)-posture as many times as possible.

After observations, all fish were immediately anesthetized with MS222 and were fixed in 5% formalin solutions. SL of the fish was measured with a microscope or caliper. Developmental stages of the fish were determined following the definition by Minami.\(^8\)

Frequencies of \(\Omega\)-posture were pooled and mean value (per minute*fish) was obtained for two groups. Mann–Whitney \(U\)-test was applied to examine the difference in the frequency of \(\Omega\)-posture between Fed and Unfed groups, and the difference in the bending duration between left and right side bending during \(\Omega\)-posture. Student’s \(t\)-test was applied for the comparison of SL between two groups.

RESULTS

Growth

Fish grew relatively slowly between day 13 (SL: mean \(\pm\) standard deviation = 6.1 \(\pm\) 0.5 mm) and day 35 (9.7 \(\pm\) 0.7 mm) and rapidly after day 39 (13.2 \(\pm\) 1.9 mm, Fig. 1). SL of days 16, 25, 35, 39 and 46 were significantly higher than those of the immediately preceding ages (i.e. SL of day 16 was significantly higher than that of day 13, SL of day 25 was significantly higher than that of day 22 etc.; \(F=318.178\), Fisher’s PLSD, \(P<0.05\); Fig. 1). The coefficient of variation (CV,%) of SL increased from 5 to 10% until day 35 (7.1%) and increased rapidly from day 39 (14.3%) and became stable thereafter (Fig. 1).

Fig. 1 Growth of Japanese flounder. Top figure: \(\varnothing\) and (vertical) bar indicate the mean standard length \(\pm\) SD of fish used for the behavioral analysis (\(n=30\) for each age group). ● indicates coefficient variation of standard length calculated from each age group. An asterisk indicates a significant difference from the previous point (Fisher’s PLSD, \(P<0.05\)). Bottom figure: Composition of developmental stages of experimental fish (\(n=30\) for each age group) determined by the definition of Minami\(^8\). D- and E-stage are the preflexion, E- and F-stage are the beginning of metamorphosis, G- and H-stage are the climax phase (eye-migration) of metamorphosis and I-stage is juvenile.
All fish completed the transition from larva to juvenile (I-stage) on day 39 (Fig. 1). The significant increases of SL between days 13 and 16, and between days 25 and 39 coincided with the beginning of the metamorphosing stage (E- and F-stage) and the climax of metamorphosis (G- and H-stage), respectively (Fig. 1).

**Behavioral developments**

Nip was observed from day 39 and increased thereafter in the experimental tanks (Fig. 2) coinciding with the complete transition to the juvenile stage (Fig. 1).

Ω-Posture was already observed from day 13 (SL 6.1±0.5 mm) and its frequency showed a peak on day 19 (SL 7.6±0.5 mm; Mann–Whitney U-test, P<0.05; Fig. 2). This peak coincided with the developmental F-stage (Fig. 1), which is the early metamorphosing stage. Frequency of Ω-posture decreased significantly from day 22 (SL 8.1±0.6 mm; Mann–Whitney U-test, P<0.05) and disappeared from day 35 (Fig. 2).

Settlement was first observed at 12.00 h (3.3±7.5%, 620 lx) and 19.00 h (26.7±9.4%) of day 19. The rate of Settlement increased significantly until day 39, on which day all individuals became juveniles and were settled (F=97.717, Fisher’s PLSD, P<0.05; Fig. 2). From day 39, about 10% of fish adhered to the side of the experimental tank (F=2.188, Fisher’s PLSD, P<0.05; Fig. 2). These fish were subordinates that were frequently attacked by the other fish. The increase of Settlement was accompanied by an increasing number of larvae at the climax phase in metamorphosis (G- and H-stage). The occurrence of Settlement to the side of the experimental tank coincided with the juvenile (I-) stage and the onset of aggressive behavior.

**Diel rhythm in behavior**

Nip, Ω-posture and feeding behavior were observed only during the day at over 0.4 lx throughout the experimental period (Fig. 3).

Between days 13 and 35, frequency of Ω-posture and feeding increased from dawn to 8.00 h and decreased from dusk to 20.00 h (U-test, P<0.05; Fig. 3). Settlement was not observed on day 13 and was first observed at a very low rate (less than 30%) on day 19 between dawn and dusk (Fig. 3). During the night, fish that did not show Settlement stopped swimming and floated beneath the water surface. From day 22, 20–30% fish settled all day long (F=1.066, P=0.4029) and Settlement was 60–70% during the night on days 25 and 29 and it decreased significantly during the daytime (day 25, F=4.032, Fisher’s PLSD, P<0.05; day 29, F=5.899, Fisher’s PLSD, P<0.05; Fig. 3). On day 35, Ω-posture was observed at very low frequency (approx. 0.04/min per fish) and Settlement constantly showed about 80% throughout the observation time (F=0.224, P=0.977).

On days 39 and 46, aggressive behavior increased significantly from dawn to 8.00 h (U-test, P<0.05), and showed a decreasing trend towards dusk (Fig. 3). Feeding behavior also increased from dawn to 8.00 h (U-test, P<0.05), was stable during the day and decreased rapidly from dusk (U-test, P<0.05; Fig. 3). All individuals settled, but about 10% of individuals (one fish per experimental tank)
Fig. 3  Diel changes in the frequency of (△) aggressive behavior; (●) Ω-posture; (○) feeding behavior; (■) ratio (%) of settlement for 30 fish in each experimental tank; and (×) the fish stuck on the side of the experimental tanks in early life stages of Japanese flounder (upper, day 19, early metamorphosing phase; middle, day 29, climax phase of metamorphosis; bottom, day 46, juvenile stage). Vertical bars represent standard deviations (n=6). An asterisk indicates a significant difference (P<0.05) from the previous point by Mann–Whitney U-test for left figures or Fisher’s PLSD for right figures. Shaded areas represent night-time (<0.4 lx).
stuck to the side of the experimental tank only during the day (Fig. 3).

Ω-Posture and feeding behavior

Average SL of the Fed group was 7.4 ± 0.4 mm \((n=21)\) and that of the Unfed group was 7.6 ± 0.6 mm \((n=21)\), which were not different statistically \((t=-1.005, P=0.321)\). About 60% of the fish were in F-stage and the others were in D- or E-stage in both groups. The frequency of Ω-posture \((\text{count/min per fish})\) was not different statistically between Fed \((1.0 \pm 0.2)\) and Unfed \((0.9 \pm 0.4)\) groups, while feeding behavior was observed only in the Fed group \((3 \text{ times/min per fish})\).

We were able to record the duration of the sequence of Ω-posture completely for 12 fish. The average duration of one Ω-posture was 54.1 ± 44.3 s ranging from 10 to 163 s. The rate of duration for left-sided bending in one sequence of Ω-posture \((65.8 \pm 29.3\%)\) was significantly longer than right-sided bending \((34.2 \pm 29.3\%, U\text{-test}, P<0.05)\).

DISCUSSION

Ontogeny of aggressive behavior and other behaviors

The onset of aggressive behavior in Japanese flounder occurred after the transition from the larval to the juvenile stage and developed thereafter. The same ontogenetic changes in aggressive behavior have been confirmed in the yellowtail Seriola quinqueradiata.\(^{23}\) Nutritional studies on behavioral and brain developments in the yellowtail revealed that docosahexaenoic acid (DHA), which is the essential fatty acid for marine fishes, induces the development of the CNS, particularly the cerebellum, in the early life stages of Japanese flounder, similar to the yellowtail. Thus, we speculate that the development of the CNS in the Japanese flounder, which undergoes more drastic metamorphosis than yellowtail, is essential for the onset of aggressive behavior.

Settlement was frequently observed from the climax phase of metamorphosis (G-stage) as in other reports of this species \((\text{Noichi}^{28})\). After fish became juvenile, about 10% of fish, which were subordinates, stuck to the side of the experimental tanks. This phenomenon was caused by agonistic interactions among settled individuals and can be interpreted as each individual occupying a specific space for feeding and/or hiding.

Ω-Posture was first described by Fukuhara\(^{10}\) and was interpreted as one form of feeding behavior, as it looks like an S-spine posture, which is a common attacking posture of food items in fish larvae. Dou et al.\(^{19}\) also used Ω-posture as a magnitude of feeding activity due to the positive correlation between the frequency of Ω-posture and the number of food items consumed within a certain duration. In our study, however, Ω-posture was observed in the absence of food at the same frequency as though there was food present. By contrast, no feeding behavior was observed when there was no food. Moreover, during the beginning of the metamorphosing stage (F-stage), the duration of left-sided bending was significantly longer than right-sided Ω-posture. It is noteworthy that flounder spent a longer time for left-sided Ω-posture, and this sidedness is the eye-migrating side after the metamorphosis. The left-sided bending of Ω-posture and these behavioral changes may indicate that Ω-posture is possibly related to the metamorphosis of this species. However, at present, whether Ω-posture is induced by the limitation of body bending because of the eye-migration or it is specific behavior in the metamorphosing process is unclear. Synthesizing our results, it is reasonable to define that Ω-posture is not a feeding behavior.

In the transition from the larval to the juvenile stage in yellowtail, a Ω-posture-like behavior named J-posture is frequently observed and it disappears after the onset of aggressive behavior when yellowtail became juvenile. We have confirmed that J-posture is a precursor behavior of aggressive behavior, because a larva that showed J-posture strongly became aggressive and dominant in a school at the juvenile stage.\(^{27}\) Considering that the ontogenetic changes of Ω-posture and aggressive behavior in the Japanese flounder is similar to that of yellowtail, we predict that Ω-posture is the precursor behavior of aggressive behavior of this
species. Further study on the relationship between Ω-posture and aggressive behavior in the Japanese flounder is needed.

**Diel rhythm**

Since aggressive behavior, Ω-posture and feeding were only observed during the day (Fig. 3), Japanese flounder larvae and juveniles are diurnal under 20 mm SL. In the daytime, subordinates stuck to the side of the experimental tanks instead of settling down to the bottom, but during the night all individuals settled on the bottom (Fig. 3). This suggests that aggressive behavior is diurnal in this species. The former finding that yellowtail shows diurnal activity in aggressive behavior, J-posture and feeding supports the assumption that the developmental pattern of aggressive behavior in the Japanese flounder is similar to that of yellowtail and that Ω-posture is the possible precursor behavior of aggressive behavior. In the metamorphosing stage, flounder larvae settled mainly during the night, when they stop active swimming (Fig. 3). Since calcifications of bones begin in the metamorphosing stage in the Japanese flounder, it may lead to an increase of specific gravity and settlement during the night.

Miyazaki et al. reported nocturnal activity of off-bottom swimming (off-bottom swimming is the behavior in which individuals swim up from the bottom and swim in the water column) in the flounder juvenile (66 mm in total length) under laboratory conditions and Takahashi et al. reported nocturnal feeding in wild flounder juveniles (40–100 mm in body length). As juveniles did not show nocturnal behavior at approximately 20 mm SL (Fig. 3), the shift in behavior from diurnal to nocturnal or that flounder begin to become active during the night, may occur between 20 and 40 mm in body length in this species. The number of settled individuals (11–15 mm SL) decreases rapidly in the wild. It may be related to the predation pressure during the night when flounder do not show active swimming, but instead settle.

**Meaning of aggressive behavior**

Size variations in SL increased slowly in the metamorphosing stage and remained relatively unchanged after the onset of aggressive behavior (CV < 15%, Fig. 1). Yellowtail also shows the same ontogenetic changes in CV. In the wild, juvenile yellowtail schools consisting of similar ages aggregate to drifting seaweed in current rips. It is assumed that aggressive behavior and social rank in schools of yellowtail function to make the body size of school members uniform both in wild and artificial rearing conditions in order to minimize individual predation risk by predator confusion. However, this is not the case in Japanese flounder, which does not show schooling behavior. Metamorphosing Japanese flounder larvae appear in a high population density at inshore regions (over 10 fish/m²) and they settle on sandy nursery grounds shallower than 10 m in depth foraging mysids. Juveniles spend about 2 months in these shallow nursery grounds and then migrate offshore when they change their food preference from mysids to crustaceans and fishes at about 100 mm in body length. High population density of juvenile flounders in these nursery grounds will induce offshore migration, because competition for food and space becomes higher with growth and, in fact, both population density and starvation accelerate cannibalistic behavior of the juvenile flounder in experimental conditions. Thus, we presume that aggressive behavior in the early juvenile stage may have a function for spacing at settlement and offshore migration thereafter.

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