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Effect of Nutritional Requirements and Feeding Regimes at First Feeding on the Survival of the Larval Olive Flounder *Paralichthys olivaceus*

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Despite the relatively high production of fingerlings of the olive flounder *Paralichthys olivaceus*, its larval rearing in terms of first feeding has not been fully analyzed. We evaluated the variations of amino acids and fatty acids of starved larvae over 96 hr after hatching. We also investigated depletion of the yolk and oil globule of starved larvae and those fed the rotifer *Brachionus plicatilis*. In addition, the optimum size of the rotifers according to the mouth size of the larvae, and the point of no return with delay of the first feeding, were also examined. The amino acids in the egg decreased abruptly during embryo development. At 48 to 72 hr after hatching, the amino acids of starved larvae decreased by 30-40% from the level in newly hatched larvae. The concentrations of fatty acids in newly hatched larvae were lower than those of floating eggs and dropped sharply at 48 hr after hatching, when the yolk disappeared. The starved larvae depleted their yolksacs and oil globules earlier than the fed larvae did. At 84 hr after hatching, rotifers were detected for the first time in the guts of the larvae, which were about 3 mm in total length. The point of no return appeared to be close to the fourth day from the first feeding. For a high survival rate of *P. olivaceus* larvae, the first feeding should occur before the third day after hatching.

Key words: Feeding regimes, First feeding, Nutritional requirement, *Paralichthys olivaceus*, Point of no return

Introduction

Paralichthys olivaceus is one of the most commercially important species of fish cultured in Korea and Japan. As is the case for other types of marine fish culture, the seed of this flounder is produced by feeding the larvae on the rotifer *Brachionus plicatilis* for about two weeks after hatching, and then later feeding them on the nauplius larvae of *Artemia* (Cabrera and Hur, 2001).

The energy of newly hatched larvae comes first from endogenous yolk and then from exogenous food supplied for them (Korsgaard, 1991). The nutritive value of the live foods depends on the diet used for them (Mercier et al., 2004), and it affects the growth and survival of the fish larvae (Hoff and Snell, 1989; Whyte and Nagata, 1990; Mercier et al., 2004). Among the factors that have been demonstrated to have strong influences on the successful rearing of marine fish larvae, the size and the nutritional value of the live food used are considered essential (Cabrera and Hur, 2001; Watanabe and Vassallo-Agius, 2003; Cabrera et al., 2005). It is important to establish a suitable size of live food for the culture of target fish because larval mouth size appears to be the limiting factor in feeding (Dabrowski and Bardega, 1984; Lee and Hur, 1997).

Moreover, it has been demonstrated that some species change their selectivity for prey size according to their larval stage, and knowledge of this feature helps to solve the problem of high mortality during the first feeding stage of the larvae (Tamaru et al., 1991; Polo et al., 1992; Shaw et al., 2003). The determination of the point of no return, which indicates the time to which 50% of newly hatched larvae can survive without exogenous food, has been useful for determining the appropriate time to feed

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the larvae (Bagarinao, 1986; Lee and Hur, 1997).

This research was carried out to elucidate the effect of nutritional requirements and feeding regimes at first feeding on the survival of early-stage larvae of *P. olivaceus* by analyzing the variation of amino acids and fatty acids of starved larvae and by comparing the relationship of the depletion of the yolk and oil globule to survival in fed and starved larvae with delay of the first feeding day.

Materials and Methods

Sampling of starved and fed larvae

Floating eggs (ca. 200,000 eggs) of *P. olivaceus* collected from a rearing tank at the Institute of Marine Science, Pukyong National University, were incubated at 18-20°C of filtered seawater in each of six 500-L tanks. After hatching, the newly hatched larvae from one batch were sampled using a 300-μm net, and starved larvae were also sampled every 24 hr for 4 days from each of four batches. The sampled larvae were washed with filtered seawater and stored at -86°C for later chemical analyses. One batch of larvae was fed from the first day after hatching with the rotifer *Brachionus plicatilis* (10 inds/mL) cultured with *Chlorella ellipsoidea* (KMCC C-20) received from the Korea Marine Microalgae Culture Center.

Larval yolk and oil globule depletion

Two hundred newly hatched larvae were reared in two 50-L containers at 20°C, separately, one without food and the other with rotifers (10 inds/mL) and *C. ellipsoidea* (2×10^4 cells/mL). Every day for 5 days, 20-30 larvae were sampled from each container and, immediately after sampling, the total length, body height, and sizes of the yolk and oil globule were measured using a micrometer installed in the microscope. The volumes of yolk and oil globule were determined according to Blaxter (1988): oil globule volume (spherical)=$0.1667\pi D^3$, yolk volume (ellipsoidal)=$0.1667\pi LH^2$, where D: diameter, L: length of maximum axis, H: height of maximum axis.

Mouth size and larval feeding

Every day for the first 5 days after hatching, 20-30 larvae were sampled from the larval rearing tank, and the total length and upper jaw length of the larvae were measured using a micrometer installed in the microscope. The number of rotifer mastaxes that remained in the gut of the larvae was determined using a microscope. The upper jaw length (UJL) was used to determine the larval mouth gape at 45° and 90° according to Strussmann and Takashima (1989); mouth gape = $\mathrm{UJL}\times\sqrt{2-Cos\alpha}$.

To determine the point of no return (PNR) for the larvae, which indicates the time at which 50% of the larvae can survive without food, survival (%) of the larvae with delay of the first feeding day was examined daily. One hundred newly hatched larvae were moved to each of six 50-L containers. The rotifers (10 inds/mL) and *C. ellipsoidea* (2×10^4 cells/mL) were first supplied to larvae in each container from the first day after hatching, and every day thereafter for 6 days, and the larvae were cultured until the ninth day after hatching. This experiment was conducted in duplicate.

Chemical analyses

For dry weight, all frozen samples were freeze-dried in a vacuum freeze dryer (VFD-100 ULVAC), and 0.5-1.0 g of each sample was dried at 110°C until a constant weight was maintained. The protein content of each non-freeze-dried sample was determined by the semimicro Kjedahl method (Joo et al., 1992). The amino acids of floating eggs and starved larvae were analyzed using an amino acid analyzer (LKB 4150 Alpha, Pharmacia Instruments, England). Total lipids of the sample were extracted by the method of Folch et al. (1957), and the fatty acids were determined using gas chromatography equipment (Model 8700, Perkin Elmer Ltd., USA) according to the method of Solver (1983).

Statistical analysis

Statistical analyses of all data were performed using the SPSS/PC statistical computer package, version 10.1.3 (SPSS Inc., Chicago, Illinois). Significant differences among mean values of the total length of starved larvae were determined by one-way analysis of variance (ANOVA) and those of yolk and oil globule volume between fed and starved larvae were also determined by the t-test at the 5% level.

Results

Chemical analyses of floating eggs and starved larvae

Although the total lengths of starved larvae increased continuously with time after hatching, the dry weight of the larvae varied until the starvation period reached 72 hr, with weights higher than those of the newly hatched larvae (25.79 μg). However, at 96 hr of starvation, the weight of larvae had decreased by about 28%, as compared to that of newly hatched larvae. The percentage of protein in starved larvae decreased continuously until 72 hr after hatching.

Table 1. Amino acid composition (dry μg/ind.) of floating egg and starved larva of *Paralichthy olivaceus* during the first 96 hr period after hatching (EAA, essential amino acids ; NEAA, non essential amino acids)

Amino acids	Floating egg	Elapsed time after hatching (hr)				
		0	24	48	72	96
Aspartic acid	0.59	0.44	0.57	0.55	0.52	0.37
Threonine	0.50	0.34	0.38	0.25	0.23	0.17
Serine	0.55	0.31	0.26	0.25	0.25	0.19
Glutamic acid	1.13	0.67	0.74	0.64	0.65	0.48
Proline	0.66	0.26	0.31	0.17	0.16	0.13
Glycine	0.38	0.26	0.29	0.20	0.23	0.18
Alanine	0.47	0.34	0.32	0.20	0.19	0.14
Cystine	0.13	0.11	0.19	0.10	0.08	0.07
Valine	0.84	0.53	0.46	0.26	0.26	0.17
Methionine	0.18	0.01	0.00	0.00	0.00	0.01
Isoleucine	0.55	0.39	0.35	0.22	0.20	0.13
Leucine	0.81	0.60	0.69	0.45	0.42	0.31
Tyrosine	0.07	0.02	0.00	0.00	0.00	0.00
Phenilalanine	0.59	0.39	0.38	0.25	0.23	0.15
Histidine	0.55	0.34	0.39	0.25	0.25	0.17
Lysine	0.59	0.45	0.51	0.36	0.35	0.28
Arginine	0.22	0.78	0.41	0.29	0.32	0.25
Total	8.81	6.24	6.27	4.44	4.35	3.18
EAA	4.82	3.83	3.59	2.33	2.26	1.62
NEAA	3.99	2.41	2.68	2.11	2.08	1.55
EAA/NEAA	1.21	1.59	1.34	1.10	1.09	1.05

An abrupt decrease of every amino acid except arginine took place during embryo development (Table 1). Methionine appeared to be the most used amino acid during this stage. At 48 to 72 hr after hatching, which was the time of the first feeding, the total amino acids and the total essential amino acids (EAA) decreased by 30-40%, in comparison with the newly hatched larvae. Arginine, valine, isoleucine, leucine, phenylalanine, and alanine, in decreasing order, were the amino acids most used during that period. No amino acid was preferentially accumulated during the first 96 hr after hatching.

The variation of total lipids and fatty acid composition of floating eggs and starved larvae is shown in Table 2. Total lipids and fatty acids of newly hatched larvae were lower than those of floating eggs and dropped sharply at 48 hr after hatching, when the yolk disappeared. However, they increased abruptly at 96 hr after hatching. The total polyunsaturated fatty acids remained almost constant for the first 24 hr, but increased suddenly at 96 hr after hatching. Until 72 hr after hatching, the 14:0, 16:1, and 18:2 fatty acids were depleted at the highest rates, while 20:0, 18:3, and 20:2 were preferentially accumulated. This result contrasted with that for amino acids, which decreased continuously until 96 hr.

Depletion of larval yolk and oil globule

The larvae began to hatch after 40 hr of incubation, and good eggs were all hatched within 60 hr. The average total length of larvae at hatching was 2.44± 0.24 mm. Each larva had a yolksac and a single oil globule and remained inactive and upside down at the surface of the water.

The average yolksac volume of the newly hatched larvae was 0.211±0.063 mm^3. In both fed and starved larvae, the size of the yolksac sharply decreased after hatching. The starved larvae depleted their yolksacs completely by the third day after hatching, while the larvae fed with rotifer and *Chlorella* did so one day later. A significant difference ($p<0.05$) in the daily variation of yolk volume between the two groups was detected on the first day after hatching (Fig. 1).

The average volume of oil globules of the newly hatched larvae was 0.0039±0.0007 mm^3. The depletion of the oil globules was slower than the yolksac depletion in both starved larvae and those fed rotifer and *Chlorella*. At the 5th day after hatching, both the fed and starved larvae had depleted their oil globules completely. Significant differences ($p<0.05$) in the daily variation of oil globule volume between the two groups were detected on the first and the second days after hatching (Fig. 2).

First feeding and mouth size of larvae

During the depletion of the yolksac and oil globule, the alimentary canal was in the process of developing and the gut was opened on the 2nd day after hatching. The eyes became gradually pigmented in the same period, and the mouth was opened by 72 hr after

Table 2. Fatty acids composition (μg/100 μg dry weight) of floating eggs and starved larvae of *Paralichthys olivaceus* during the first 96 hr period after hatching (- : not detected)

Fatty acids	Floating eggs	Elapsed time after hatching (hr)				
		0	24	48	72	96
14:0	0.24	0.14	0.10	0.06	0.04	0.44
16:0	1.07	0.72	0.55	0.37	0.33	4.69
18:0	0.17	0.18	0.18	0.12	0.13	2.14
20:0	-	0.01	-	-	0.13	-
Total saturated	1.48	1.20	0.83	0.56	0.63	7.28
14:1 n-5	-	0.01	-	-	-	-
16:1 n-7	0.28	0.19	0.14	0.08	0.05	0.39
18:1 n-7 + n-9	0.69	0.49	0.38	0.24	0.19	2.19
20:1 n-9 + n-11	0.09	0.06	0.18	0.01	0.01	0.19
22:1 n-9 + n-11	0.87	0.64	0.64	0.30	0.28	5.84
24:1 n-9	-	0.03	-	-	-	-
Total monounsaturated	1.93	1.42	0.34	0.63	0.53	8.62
18:2 n-6	-	0.13	0.09	0.06	-	-
18:3 n-3 + n-6	0.13	0.02	0.04	0.02	0.04	0.57
20:2	0.04	0.04	-	-	0.06	0.13
20:3 n-6	0.10	0.03	-	-	0.02	1.35
20:4 n-3 + n-6	-	0.05	0.12	0.01	-	-
20:5 n-3	-	0.01	-	-	-	0.25
Total polyunsaturated	0.27	0.28	0.25	0.09	0.12	2.30
Total fatty acids	3.68	2.90	2.42	1.28	1.28	18.20
Total lipid (μg/ind.)	1.47	0.75	0.80	0.33	0.33	0.35

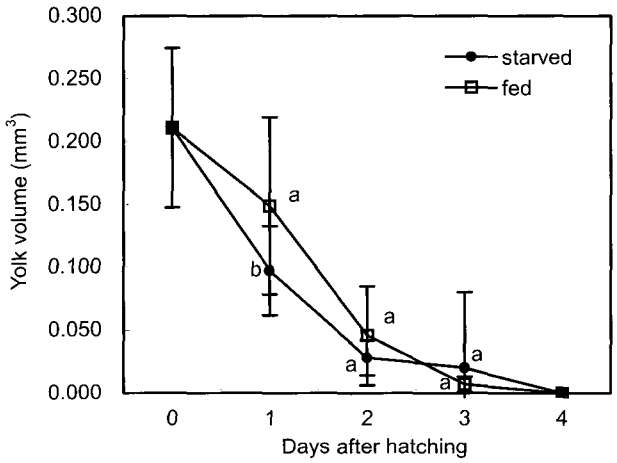

Fig. 1. Variation of yolk volume of starved and fed larvae of *Paralichthys olivaceus*. Different superscripts on each day mean significant difference at the level of 5%.

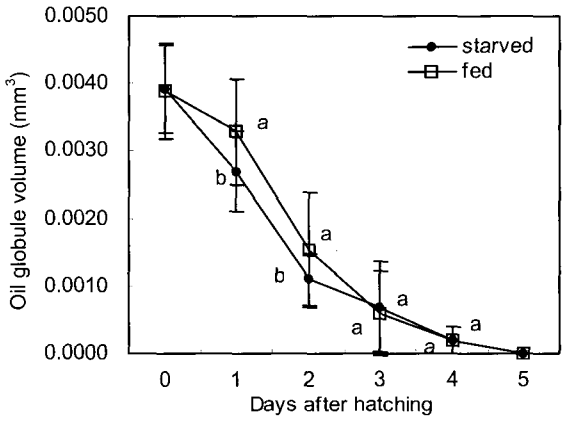

Fig. 2. Variation of oil globule volume of starved and fed larvae of *Paralichthys olivaceus*. Different superscripts on each day mean significant difference at the level of 5%.

hatching. At this time, the larva, with S-shaped flexure of the body, began to move for feeding; however, food was not detected in the gut. At 84 hr after hatching, rotifers were detected for the first time in the gut (total length-TL: 3.13 mm). The maximum number of rotifer mastaxes in the gut at this time was seven. It increased steadily to 42 in larvae of 5.27 mm by the 9th day after hatching (Fig. 3).

In accordance with relationships between total length and mouth gape at 45° and 90°, the larvae at first feeding, which were about 3 mm in total length, were able to take foodstuffs about 230 μm and 290 μm long with mouth gape at 45° and 90°, respectively. Their ability to consume larger food items increased with their total length to about 590 μm at a larval length of 5 mm and about 900 μm at 7 mm total length, with a mouth gape of 90°.

With regard to survival (%) of fed and starved larvae, 50% mortality of starved larvae occurred by the 6th day after hatching, and 100% by the 9th day. The point of no return appeared to be just before the

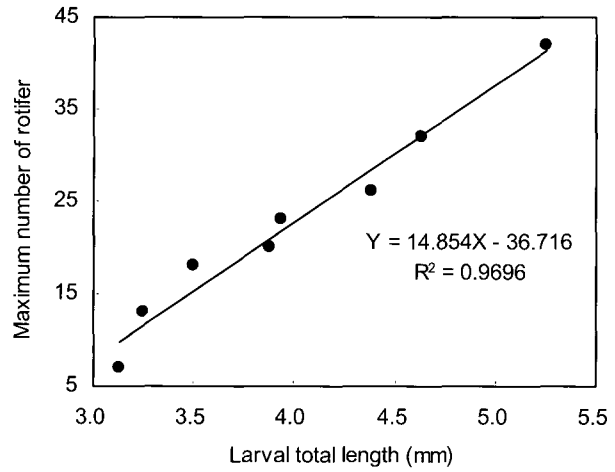

Fig. 3. Maximum number of rotifers' mastax found in the gut of *Paralichthys olivaceus* larvae.

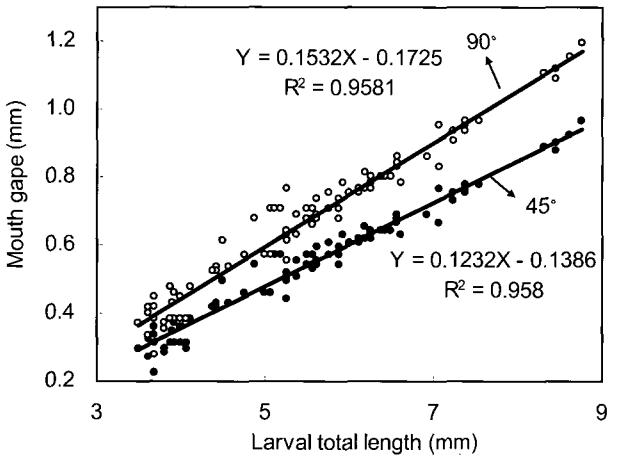

Fig. 4. Variation of the mouth gape at 45° and 90° with the different larval length of *Paralichthys olivaceus*.

4th day from the first feeding day (Fig. 5).

Discussion

The first feeding time of *P. olivaceus* larvae cultured at 19°C was on the third day after hatching (Seikai et al., 1986). The dry weight of the starved larvae dropped sharply at 96 hr in this study, which was related to the first feeding time after hatching. Protein and total amino acid contents of the starved larvae were depleted steadily during this period. The total lipids decreased until 48 hr after hatching, but after that, a relatively constant lipid content was maintained. In *Pseudopleuronectes americanus*, proteins and lipids were used before hatching, and larvae in the yolksac stage preferentially used lipids over

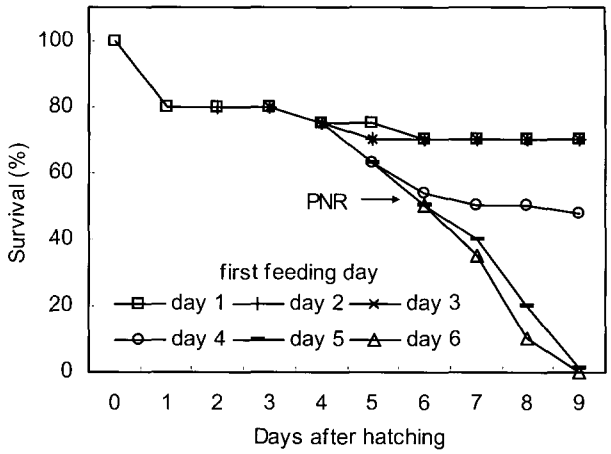

Fig. 5. Survival (%) of *Paralichthys olivaceus* larvae with delaying of first feeding day of rotifer after hatching (PNR: point of no return).

proteins (Cetta and Cappuzo, 1982). The flounder larvae in this study showed the same pattern.

The high content of 16:0 fatty acid in floating eggs and larvae during starvation indicated that this fatty acid was strongly required by *P. olivaceus* larvae. The major fatty acids (16:0, 18:1, and 22:1) detected in this experiment were also found in larvae of the same species in other studies (Fukusho et al., 1985; Izquierdo et al., 1992).

With regard to the depletion of yolk and oil globules in larval fish, the yolk is the main energy source during both the endogenous feeding period (Kamler, 1992) and the mixed feeding period or in the transition between endogenous and exogenous feeding (Korsgaard, 1991). Tsukamoto and Kajihara (1984) determined that the yolk substance in *Plecoglossus altivelis* was consumed as energy for locomotion. This finding implies that starved larvae in the tank without any food were swimming (unsuccessfully) to find food more continuously than fed larvae with enough live food would be, and exogenous feeding delayed final yolk absorption. This result was also reported in *Salmo trutta* (Kamler, 1992). Eldridge et al. (1981) postulated that the oil globule as an energy reserve was conserved when food was not abundant. Eldridge et al. (1982) reported that starved larvae of *Morone saxatilis* retained oil globules for a longer time than fed ones did. However, in this study, the depletion of yolk and oil globules was slower in the fed larvae than in the starved ones.

In the experiment on the first feeding of the larval flounder, seven mastaxes of the rotifer remained in the gut of 84-hr-old larva. The evacuation rate of rotifers from the larvae varied according to the size of

the rotifers (Polo et al., 1992; Reiriz et al., 1998). Feeding behavior of the flounder larvae was affected by many factors including water temperature, light intensity, prey density, previous prey type, and frequency of feeding (Cox and Pankhurst, 2000; Rabe and Brown, 2000). Even though these factors were not considered in this study, the maximum number of mastaxes remaining in the gut would be an acceptable index of the feeding capacity of the larval flounder. In turbot, 0 to 2 rotifers were detected in the gut of 4-day-old larvae (Witt et al., 1984). Yasunaga (1971) detected 2 to 3 rotifers in the gut of 6-day-old flounder larvae. This author considered that a larva would take maximum of 9 to 10 rotifers per day, which increased to 5 to 25 rotifers in 10-day-old larvae. In the present experiment, the maximum number of rotifers eaten by the flounder larvae was higher than that reported in previous papers. This difference may be attributable to species-specific and size-specific prey selection in the flounder larvae (Tamaru et al., 1991; Dou et al., 2003; Shaw et al., 2003).

Regarding the mouth size of larvae, the size of mouth gape determines the maximum prey size for larvae and juvenile fish (Dabrowski and Bardega, 1984). For this reason, it is important to have precise knowledge of the mouth size of the larvae (Arts and Evans, 1987); those of many marine fish larvae have been determined (Blaxter, 1988). Takahashi (1985) obtained a good fit regression between larval total length and mouth gape (90°) in flounder larvae. Since the mouth gape of the larvae at the time of feeding is not known exactly, the mouth gape of the flounder larvae was determined at 45° and 90° (Dabrowski and Bardega, 1984). The former was considered the optimum size of prey and the latter the maximum.

Although the regression lines between total length and the mouth gape of the larval flounder obtained by Takahashi (1985) and those determined in this study were similar, they appeared to be overestimated when the maximum rotifer length and width observed in the larval gut was considered. In terms of this regression lines, the relationship between the larval total length and the width of food items seems to be better than that with the length of food items. Other researchers have also recognized that the width of the live food is the critical measure for larval food intake (Detwyler and Houde, 1970; Hoff and Snell, 1989).

The point of no return (PNR) is the time to which 50% of the larvae can survive without exogenous food. The PNR in this study occurred on the 6th day after hatching. In the delayed feeding trial, 47% of the larvae fed starting on the 4th day were able to survive to the 9th day after hatching, while just 1% of those fed starting on the 5th day and none of those fed starting on the 6th day survived to the 9th day after hatching. It is assumed that the larvae lost their capacity to ingest and digest sufficient food with delayed first feeding. If the 50% threshold proposed by Strussman and Takashima (1989) is adopted, the larvae of *P. olivaceus* should be fed before the 4th day after hatching. However, the survival of larvae fed from the 4th day after hatching was 33% lower than that of larvae fed before the 3rd day after hatching.

In this study, we have shown the effects of nutritional requirements and feeding regimes at first feeding on the survival of *P. olivaceus* larvae. From these results, we can conclude that the first feeding should be performed before the third day after hatching for a high rate of survival of *P. olivaceus* larvae.

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