Title:

Screening of enzyme activity for assessing the condition of larvae in the seven-band grouper *Epinephelus septemfasciatus* and devil stinger *Inimicus japonicus*

Running Title:

Enzyme activity for larval quality

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Abstract:

We conducted screening tests whether enzyme activity is applicable as a biomarker for assessing the physiological condition of marine fish larvae. Two trials of rearing experiments until 5 days after hatching (DAH) of the seven-band grouper *Epinephelus septemfasciatus* and one trial until 10 DAH of devil stinger *Inimicus japonicus* were conducted using three different rearing-tank proportions (shallow tank, S; intermediate tank, I; and deep tank, D) with water volume of 100 L at an aeration rate of 50 mL/min. We determined survival, surface death, growth and enzyme activities (trypsin, esterase and alkaline phosphatase). Two species exhibited their highest survival and lowest surface death in D tank. Significant and negative correlation between survival on 5 DAH and alkaline phosphatase activity on 0 DAH was found in the seven-band grouper. The same correlation was found between survival on 10 DAH, and trypsin and alkaline phosphatase activity on 1 DAH in devil stinger. We concluded that particular enzymes activity is candidate for assessing the conditions of marine fish larvae.

Keywords:

alkaline phosphatase, devil stinger, esterase, larval quality, seven-band grouper, survival, trypsin.

INTRODUCTION

High mortality of larvae around first feeding is one of the critical obstacles in the mass seedling production of marine fish larvae. There has been a demand on suitable methods to determine the quality and/or condition of larvae in larviculture at real time in order to streamline mass seedling production costs of labor and time. Enzyme activity measurements using fluorescence technique is an advantageous method for estimating nutritional condition and stress of marine fish larvae because this technique can be done into the daily routine work in fish hatcheries and it requires a short period for determining [1-4]. Recently, Matsuo *et al*. [5] reported that enzyme activity can be applied as a real-time biomarker for evaluating the larval quality in viviparous scorpionfish *Sebastiscus marmoratus*, however, not much is known whether this method is applicable for the diagnosis of oviparous fish larvae.

In the present study, seven-band grouper *Epinephelus septemfasciatus* and devil stinger *Inimicus japonicus* larvae were chosen as model animals, because high mortality at early larval stage occur in the early phase of larviculture where the egg and larval quality are often suspected as a main cause [6, 7]. Larval rearing experiments were conducted for screening tests to determine whether enzyme activity can predict the conditions of these larvae expressed by survival rate. We set different levels of physical stress with distributing the eggs from the same batch into different rearing-tank proportions, since the rearing-tank proportion is the physical stress factor that affects larval survival of seven-band grouper and devil stinger [8]. Then we measured trypsin, esterase and alkaline phosphatase activity of both species at different sampling time, and calculated the correlation between enzyme activity at

certain age and survival rate of larvae in order to select the candidates of enzyme and sampling points. Trypsin are known as a useful indicator of digestive processes and nutritional condition in fish larvae such as herring *Clupea harengus* [2, 9], walleye pollock *Theragra chalcogramma* [10], haddock *Melanogrammus aeglefinus* [11], Atlantic cod *Gadus morhua* [11], bullseye puffer *Sphoeroides annulatus* [12] and Japanese flounder *Paralichthys olivaceus* [13]. Esterase has been reported to have some correlation with growth rate of larvae and stress of rotifer [4] while alkaline phosphatase is considered as an indicator for intensity of nutrient absorption in the intestine of larvae [14, 15].

MATERIALS AND METHODS

Materials

Three kinds of polyethylene rearing tank shape, 200 l shallow tank (S: 71×26 cm in diameter and depth, respectively), 100 l deep tank (D: 44×70 cm) and 100 l intermediate tank (I: 57×39 cm) were used in this experiment with triplicates following the former study [8]. Each rearing tank was filled with 100 l of 34 ppt artificial seawater (Marine Art Hi, Tomita Pharmaceutical Co. Ltd., Tokushima, Japan). A spherical aerator was placed at the bottom center of each rearing tank and aeration was kept at 50 ml/min using a flow meter (Kofloc, RK-1 350V, Kojima Instruments Inc., Kyoto, Japan). Specially formed ceramic beads for fish rearing

(MS0, Nora Co. Ltd., Kyoto, Japan) was placed on the bottom of each rearing tank in order to stabilize water quality during experiment.

Two rearing experiments of seven-band grouper *Epinephelus septemfasciatus* were conducted during 25-31 May 2006 and 23-28 June 2006, respectively. The range of the rearing water temperature was 20.9-21.7^{o}C and 22.8-23.6^{o}C, respectively. Artificially fertilized eggs of the seven-band grouper were transported from Nagasaki Prefectural Institute of Fisheries to Aquaculture Biology Laboratory, Faculty of Fisheries, Nagasaki University, and they were introduced into each rearing tank at a density of 10 eggs/l. The eggs hatched about 24 hours after transportation and hatching rate did not differ among rearing tanks (96.7±3.3 % and 93.8±9.5 %, respectively). After mouth opening (3 days after hatching, DAH), larvae were fed on Indonesian strain of *Brachionus plicatilis* sp. complex (SS-type rotifer) [16, 17] cultured with HUFA-enriched *Chlorella vulgaris* (Super *Chlorella* V12, Chlorella Industry Co. Ltd., Fukuoka, Japan) at a density of 10 rotifers/ml. Density of rotifer was checked once daily and the rotifer density was maintained by adding new rotifer if necessary. Super *Chlorella* V12 was added to experimental tanks to adjust the density at 5×10^{5} cells/ml once daily. Rearing experiments were carried out until 5 DAH. Surface death larvae were counted and removed every day, and the remaining larvae in each tank were counted to determine survival at the termination of the experiment. At 0, 1, 3 and 5 DAH, 15 larvae were sampled from each tank and anaesthetized with MS 222. Ten larvae were preserved at -20^{o}C for measurement of enzyme activity and five larvae were fixed in 5% formalin for measurement of growth.

Naturally spawned eggs of devil stinger *Inimicus japonicus* were obtained from Nagasaki Prefectural Institute of Fisheries and were transported to Aquaculture Biology Laboratory, Nagasaki University on 6 July 2006. These eggs were transferred into the experimental tanks by the same procedure as seven-band grouper. Water temperature during the experiment was in the range of 23.8-24.8^{o}C. These eggs hatched within 24 hours after transportation and hatching rate was same among the rearing tanks (82.5±13.2 %). After mouth opening (1 DAH), L-type rotifer (Nagasaki Makishima strain) [17, 18] cultured with Super *Chlorella* V12 was fed to the larvae at 10 rotifers/ml. Super *Chlorella* V12 was added to the experimental tanks to adjust the density to 5×10^{5} cells/ml once daily. Rearing experiments were carried out until 10 DAH with the sampling larvae on 0, 1, 5 and 10 DAH for the measurement of enzyme activities and growth.

Growth and larval movement

Growth of seven-band grouper and devil stinger larvae was expressed in terms of notochord length (NL), total length (TL) and dry weight. NL and TL of each larva were measured using a digital microscope (VH6300, Keyence, Japan). Growth rate of larvae was calculated using the following formula:

Growth rate (mm/day) = (final notochord length – initial notochord length)/time (days).

Dry weight of the larvae was measured individually using an ultra-micro balance (UMX2, Mettler Toledo, Columbus, U.S.A.) after they were dried in a hot oven at 60^{o}C for 24 hr [19].

The movement of larvae from hatching until mouth opening stage was investigated following the previous study [8]. We divided the movement of larvae in the vertical circulating flow into 2 steps; Step 1 was defined as the movement of a larva beneath the free water surface from the center of the surface toward the sidewall, and Step 2 was defined as the movement of a larva in the water column, including the total moving time from the surface of the sidewall to the bottom, from the bottom of the sidewall to the aerator, and from the aerator to the free water surface of the center of the rearing tank. Ten larvae in each rearing tank were individually observed, and then the duration (minutes) of larval movement beneath the water surface during one hour was calculated.

Enzyme activity

Enzyme activities of larvae were performed on individual larvae according to the highly sensitive fluorescence method of Ueberschär [2] with slight modifications after Matsuo *et al*. [5]. Seven band grouper and devil stinger larvae were individually homogenized in 1 ml of 35 ppt artificial seawater using a sonicator (Sonifer 150, Branson, CT, USA). The substrates used for trypsin, esterase and alkaline phosphatase activity measurements were Boc-Phe-Ser-Arg-AMC (0.26 mM, Peptide Institute Inc., Japan), 5-carboxy fluorescein diacetate, acetoxymethyl ester (0.05 mM, Molecular Probes Inc., USA) and fluorescein diphosphate, tetraammonium salt (0.05 mM, Molecular Probes Inc., USA), respectively. Two hundred μl of the homogenized samples were incubated with 10 μl of enzyme substrate at 37^{o}C in a dark

room. The reaction was terminated after 15 min by adding 20 µl of 0.5 M sodium lauryl sulfate, and the samples were then centrifuged at 10800×g for 5 min. A volume of 100 µl of the supernatant was placed in a 96-well microplate and the fluorescence was measured using a fluorescence multi-well plate reader (Cytofluor 4000, Applied Biosystems, CA, USA) at respective excitation and emission wavelengths of 360 nm and 460 nm for trypsin and 485 nm and 530 nm for esterase and alkaline phosphatase, respectively. The result was defined as the amount of hydrolysed substrate per fish per time unit (hydrolysed substrate/µg/min). For the calibration of fluorescence, 4-methyl-coumaryl-7-amind (Molecular Probes Inc., OR, USA) was used as the fluorescent standard for trypsin, and fluorescein (Molecular Probes Inc., OR, USA) was used for both the esterase and alkaline phosphatase, respectively.

Statistical analysis

The differences between means were analyzed by one-way analysis of variance (ANOVA) and the differences between samples were analyzed by pairwise comparisons of Fisher's Protected Least Significant Difference (PLSD) test using Minitab Release 13 (Minitab Inc., State College, PA, USA). Significance was determined at $P<0.05$. The linear relationship and coefficient of correlation value (r) between larval survival at the end of the rearing trial and larval enzyme activity were determined.

RESULTS

Survival rate and surface death rate

The survival rate of both trials of seven-band grouper and devil stinger larvae showed a similar results, where the highest survival rate was found in D tank and the lowest in S tank (df=2, F=41.26, P<0.05 for first trial of seven-band grouper, df=2, F=9.30, P<0.05 for second trial of seven-band grouper, and df=2, F=9.89, P<0.05 for devil stinger) (Fig. 1).

In rearing tank of all shapes, surface death of seven-band grouper and devil stinger larvae were found soon after hatching at first day and continuously occurred throughout the experimental period (Fig. 2). Both species showed significant differences in cumulative surface death among the three rearing-tank shapes. In the seven-band grouper, cumulative surface death of both trials on 5 DAH in S tank was highest (df=2, F=5.13, P<0.05 for the first trial and df=2, F=4.42, P<0.05 for the second trial of seven-band grouper). On 10 DAH of devil stinger larvae, the D tank exhibited the lowest cumulative surface death (df=2, F=5.36, P<0.05), followed by the I tank and S tank.

Growth and larval movement

Growths of seven-band grouper and devil stinger larvae were exhibited in term of NL, TL and dry weight. NL of first trial of seven-band grouper was no significant difference among three

rearing-tank shapes throughout the experimental period, whereas that value in second trial of larvae from D tank on 0-3 DAH showed higher values (df=2, F=5.65, P<0.05, df=2, F=9.36, P<0.05 and df=2, F=10.82, P<0.05 for NL on 0, 1 and 3 DAH, respectively) (Table 1). TL and dry weight of both trials of seven-band grouper exhibited similar results that there were no significant differences in among three rearing-tank shapes at the final day of experimental period, although larvae from D tank were observed to have slightly higher of those values.

For devil stinger larvae, NL and TL on 0 DAH from S tank showed the lowest values and the highest of those values was found in larvae from D tank (df=2, F=3.57, P<0.05 for NL and df=2, F=4.05, P<0.05 for TL). Afterward, there were no significant differences in NL and TL values among three rearing-tank shapes throughout the experimental period (Table 2).

On 5 DAH of seven-band grouper and 10 DAH of devil stinger, NL, TL, dry weight and growth rate of both species had no significant differences among three rearing-tank proportions.

Larval movement of both species showed the same results that the duration of larval movement under the water surface was the shortest in the D tank, followed by I and S tank (df=2, F=11.11, P<0.05 for the first trial, df=2, F=21.27, P<0.05 for the second trial of seven-band grouper, and df=2, F=24.39, P<0.05 for devil stinger). The duration of larval movement under the water surface of the first and second trials of seven-band grouper larvae in the S, I and D tank during experiment was 10.9 and 12.1, 4.0 and 9.4, and 7.4 and 4.0 min/hr, respectively. Those values of devil stinger larvae in the S, I and D tank were 7.8, 6.9 and 3.5 min/hr, respectively.

Changes in enzyme activity

Changes in enzyme activities of two trials of seven-band grouper and one trial of devil stinger larvae during reared in three rearing-tank shapes are shown in Fig. 3. In the first trial of seven band grouper, esterase activity showed a significant difference among the three rearing-tank shapes throughout the experimental period, whereas a significant difference among rearing-tank shapes was observed only on 3 DAH for trypsin activity (df=2, F=96.07, P<0.05) and 0 DAH for alkaline phosphatase activity (df=2, F=3.62, P<0.05). On 5 DAH, esterase activity of larvae in the D tank (0.15±0.01 nmol/μg/min) was lower than those of larvae in the S (0.18±0.04 nmol/μg/min) and I tank (0.28±0.02 nmol/μg/min) (df=2, F=17.57, P<0.05). In the second trial, a significant difference among the three rearing-tank shapes in trypsin activity (df=2, F=11.62, P<0.05 and df=2, F=3.70, P<0.05) and esterase activity (df=2, F=50.76, P<0.05 and df=2, F=14.81, P<0.05) were found on 0 and 1 DAH, whereas a significant among rearing-tank shapes in alkaline phosphatase activity was found on 3 (df=2, F=6.38, P<0.05) and 5 DAH (df=2, F=9.97, P<0.05). In both trials of seven-band grouper, the activity of all enzymes increased with age in days.

For devil stinger larvae, activity of trypsin and alkaline phosphatase from 0 to 5 DAH increased with age, regardless of the shape of rearing tank, and then those enzyme activities decreased until the end of the experiment. Esterase activity of devil stinger larvae in three rearing-tank shapes gradually increased in the initial stage after hatching until mouth opening,

and then decreased until the end of the experiment. There were significant differences among the three rearing-tank shapes in trypsin and alkaline phosphatase activity on 0 DAH (df=2, F=21.56, P<0.05 and df=2, F=6.98, P<0.05) and 1 DAH (df=2, F=8.24, P<0.05 and df=2, F=6.04, P<0.05), while esterase activity was not showed any significant difference among rearing-tank shapes throughout the experimental period.

The linear relationship between survival and enzyme activity of both fish species were calculated in order to determine the coefficient of correlation value (r) between survival and each enzyme activity during rearing experiment. As shown in Table 3, trypsin activity of seven-band grouper larvae in the first trial showed a significantly negative correlation with survival on mouth opening stage, 3 DAH, (n=9, r=-0.89, P<0.01) while those correlation in second trial was found on 0 DAH (n=9, r=-0.70, P<0.05). Moreover, both trials of seven-band grouper larvae were found significant negative correlations between survival and alkaline phosphatase activity on 0 DAH (Fig. 4; n=9, r=-0.66, P<0.05 for the first trial and n=9, r=-0.76, P<0.01 for the second trial). In contrast, esterase activity of both trials showed no significant correlation with survival throughout the experimental period.

The coefficient of correlation between survival on 10 DAH and enzyme activities of devil stinger larvae in different age are shown in Table 4. Survival on 10 DAH had a significant and positive correlation with trypsin activity (n=9, r=0.75, P<0.01) and a significantly negative correlation with alkaline phosphatase activity (n=9, r=0.85, P<0.01) on 1 DAH, mouth-opening stage (Fig. 4). A significant and negative correlation between survival and trypsin activity was also observed on 10 DAH (n=9, r=-0.61, P<0.05). There were no

correlations between survival on 10 DAH and activity of esterase throughout the experimental period.

DISCUSSION

In the present study, larvae of the seven-band grouper and devil stinger showed similar results where the highest survival rate, the lowest surface death and the shortest duration of larval movement under the water surface were found in larvae from D tank. We could confirm the previous study [8] that at the same working water volume and aeration rate, the decreases of water surface area with deep water depth as D tank can reduce surface death and improve survival rate of marine fish larvae such as seven-band grouper and devil stinger. Surface death of larvae is relatively common phenomenon and causes serious mortality in the process of larviculture such as groupers [20], and has been used as an index for the magnitude of larval mortality during early phase of larviculture [8, 21, 22]. Total death of the devil stinger is almost equal as the total surface death, however, cumulative surface death ranged about 30-70% of total death in case of the seven-grouper (Fig. 1, 2). We presume that some of the seven-band grouper larvae died sinking at the tank bottom, although the bottom of the tank was not surveyed whether dead fish were present due to the difficulty using the ceramic sand on the tank bottom. NL and TL on 0 DAH of second trial of seven-band grouper and devil stinger showed significant differences among three rearing-tank shapes, but those values of both species at the final day of the experimental period were not significant, indicating that

the rearing-tank shapes has no or less effects on the growth of larvae. This is also supported by the non-significant differences in growth rate among three rearing-tank shapes of both species. Since we used the eggs from same batch and hatching rate did not differ among the rearing tanks but the survival of larvae were different with the tank proportion, we could speculate that physical stress caused by water flow in different proportions of the rearing tank affected newly hatched larvae and resulted in differences in survival rate.

The detection of activity in trypsin, esterase and alkaline phosphatase in the newly hatched larvae of seven-band grouper and devil stinger indicated that a newly hatched larva can synthesize enzymes, even without diet stimulation, and absorb nutrient components such as protein, glycerol ester of fatty acids, lipids, glucose, calcium and inorganic phosphate [15]. These results were similar to the published reports in many fish larval species [9-11, 15, 23-24]. We could not compare the quantity of enzyme activities of larvae in the present study with other fish larvae in previous reports, due to the variation in methodology, measured techniques and expression of activities. Different enzymes are produced from different organs in gastrointestinal tract of fish larvae. A pancreatic enzyme as trypsin is synthesized in the pancreas while intestinal enzymes as esterase and alkaline phosphatase are synthesized in intestine. Activity of all measured enzymes in this study is mainly involved in the intensity of digestion and nutrient absorption of larvae, therefore, the increase in those activities with ages of seven-band grouper and of devil stinger may relate to the development of pancreas and an increase in the area of the intestine [11, 26-27]. This is supported by the increases in NL, TL and growth rate with ages of both larval species. For devil stinger, the decrease in enzymes

activities after 5 DAH may be illustrated by an increase of body weight of larvae (as enzyme activity is the ratio activity/dry weight/min) and does not reflect on a lowering in digestive capacity [25].

Although the first and second rearing trial of seven-band grouper in the present study were carried out by the same procedure, the results of trypsin and esterase activity showed different patterns and the content of those enzyme activities from each rearing-tank shape fluctuated (Fig. 3), presumably due to the difference in the rearing water temperature during experiment. These results corresponded well with the report of Pedersen *et al.* [9] who suggested the oscillations in enzyme activity content during the first weeks of larval life may be a common phenomenon for marine fish larvae, regardless of food offering. Therefore, the difference in the pattern of enzyme activities of seven-band grouper and devil stinger would be supported by these reasons.

A number of studies reported the use of enzyme activity as a indicator for detecting the digestive ability and specific nutritional requirement of several fish larvae in order to develop a formulated compound diet for those larvae [9-11, 15, 23-28]. Additionally, some studies used the enzyme activity as an indicator for assessing the fitness and survival potential of fish larvae [2, 5]. Supporting evidence was found by Ueberschär [2] who reported a tryptic activity measurement is useful in detecting unfavorable health condition of herring larvae. Matsuo *et al*. [5] also reported enzyme activity, esterase and alkaline phosphatase, can be a real-time index for evaluating the quality of viviparous scorpionfish larvae. Although Araujo *et al.* [4] did not find correlation between enzyme activity and survival in Japanese flounder

larvae, they observed whenever esterase and phospholipase fluorescence intensities were low, survival and growth of larvae were also low. As described in the results, significant correlations between survival and enzyme activities of seven-band grouper and devil stinger were observed. These findings indicate that both species in the different rearing-tank shapes may demonstrate their health condition in terms of enzyme activities which resulted in survival rate. Therefore, measurements of specific enzyme activity of marine fish larvae in the present can be a possible candidate in order to asses the magnitude of stress in larvae and to predict the survival of larvae.

In the present study, both trials of seven-band grouper and devil stinger showed similar results that survival have significant negative correlation with alkaline phosphatase activity during mouth opening stage, considering that the measurement of this enzyme activity may be more suitable than activity of trypsin and esterase for assessing the condition and predictive survival rate of larvae in these species. The reason for the relation between alkaline phosphatase activity and larval condition is unclear, but the lower level of alkaline phosphatase activity in the initial stage exhibited higher survival rate of seven-band grouper and devil stinger larvae. Kolkovski [28] illustrated that the development of each enzyme activity during ontogenesis of larvae is independently with variation related to fish species. His evidence may interpret the reason why there was a species difference in the significant negative correlations between survival and alkaline phosphate activity in this study. It was observed only on 0 DAH in seven-band grouper and 1 DAH in devil stinger, respectively. It was assumed that physical stress was critical during the yolk absorption stage (until 3 DAH)

in the seven-band grouper whereas sensitive stage to the physical stress may be at the first feeding stage in the devil stinger (1 DAH).

In summary, our results showed that the measurement of enzyme activity as alkaline phosphatase at the initial stage of marine fish larvae is a candidate for assessing the physical stress and the potential of survival. Further experiments should be conducted to determine whether the enzyme activity measurement can be a real-time biomarker for assessing the condition and/or quality of seven-band grouper and devil stinger using different batches at the same rearing conditions as revealed in the viviparous scorpion fish [5].

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Figure legends

Fig. 1 Survival rate of two trials of seven-band grouper and one trial of devil stinger larvae in three differently shaped rearing tanks. Column and bar indicate average and standard deviation, respectively (n=3). Columns with different letters above them show significant differences between the samples (Fisher's PLSD test, $P<0.05$)

Fig. 2 Cumulative surface death of two trials of seven-band grouper and one trial of devil stinger larvae in three differently shaped rearing tanks. Plot and bar indicate average and standard deviation, respectively (n=3). Plots with different letters show significant differences between samples in the same age group (Fisher's PLSD test, $P<0.05$)

Fig. 3 Changes in the enzyme activities of two trials of seven-band grouper and one trial of devil stinger larvae from rearing tanks of all three shapes. Plot and bar indicate average and standard deviation, respectively (n=3). Plots with different letters show significant differences between samples in the same age group (Fisher's PLSD test, $P<0.05$)

Fig. 4 Linear relationship between survival and enzyme activities of two trials of seven-band grouper and one trial of devil stinger larvae

Fig. 1

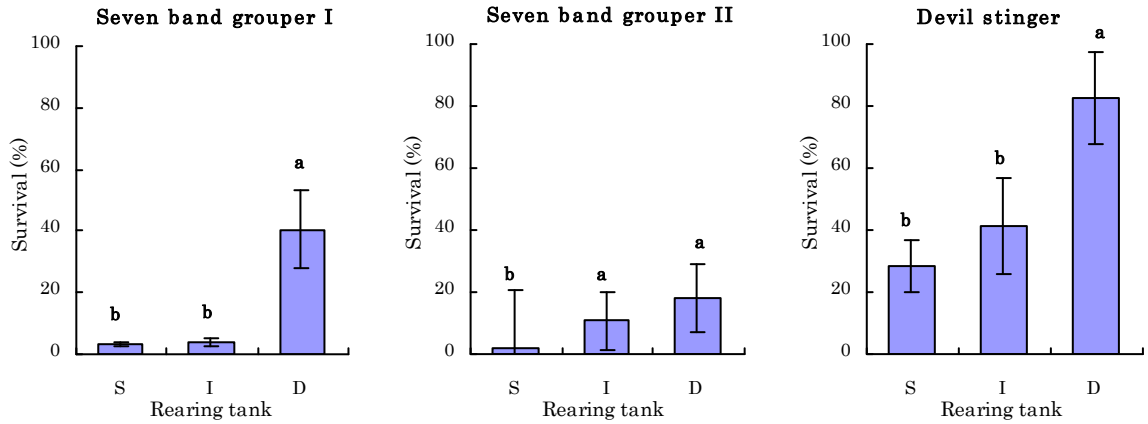
Seven band grouper I
Seven band grouper II
Devil stinger
Survival (%)
Rearing tank
S
I
D
a
b

Fig. 2

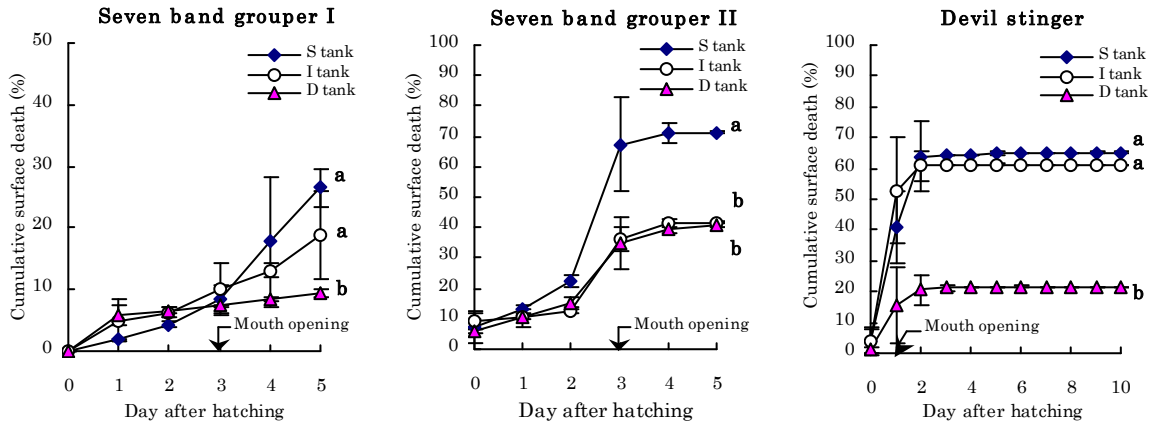
Seven band grouper I
Seven band grouper II
Devil stinger
Cumulative surface death (%)
Day after hatching
S tank
I tank
D tank
Mouth opening
a
b

Fig. 3

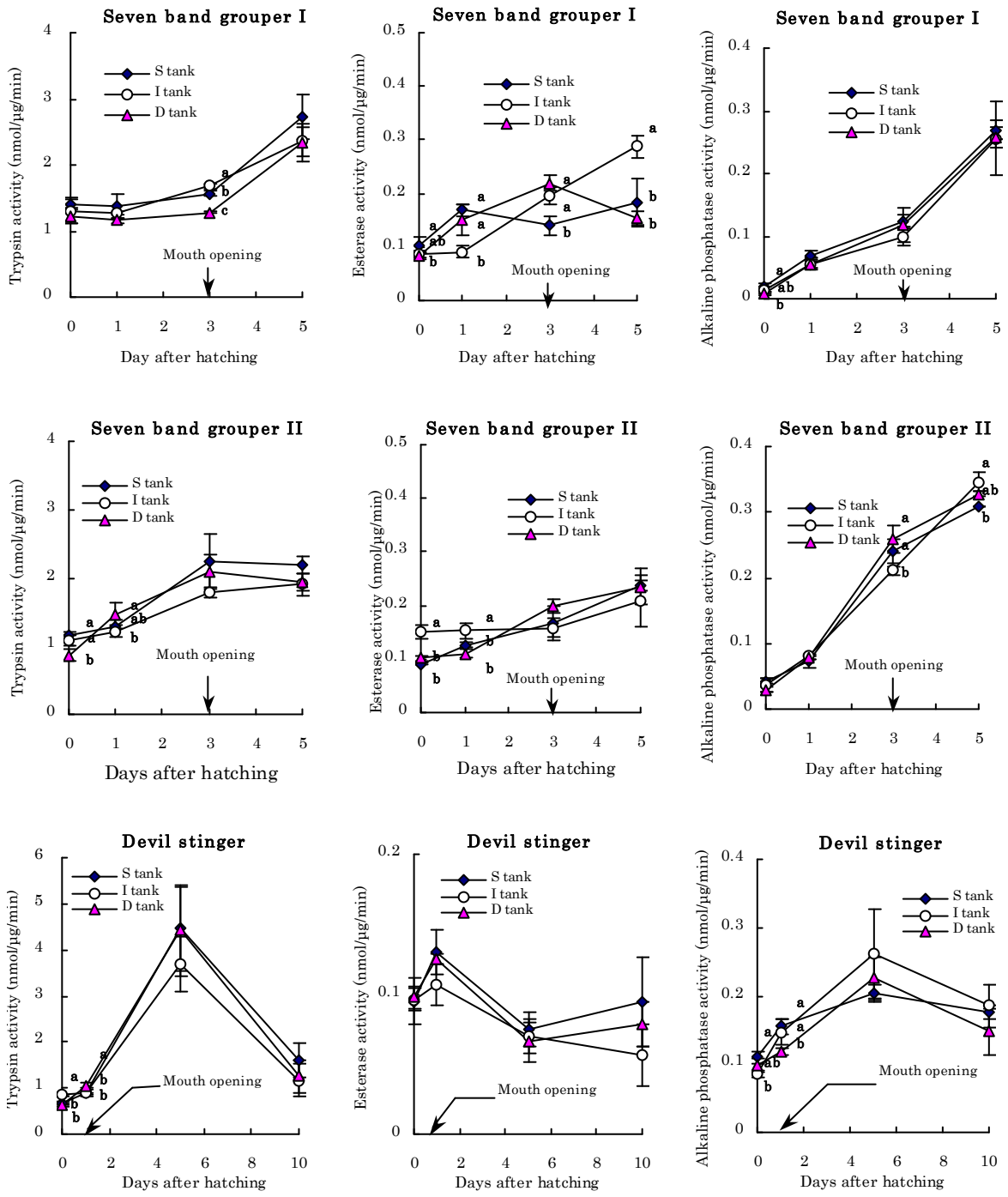
Seven band grouper I
Trypsin activity (nmol/μg/min)
Esterase activity (nmol/μg/min)
Alkaline phosphatase activity (nmol/μg/min)
S tank
I tank
D tank
Mouth opening
Day after hatching
Seven band grouper II
Days after hatching
Devil stinger

Fig. 4

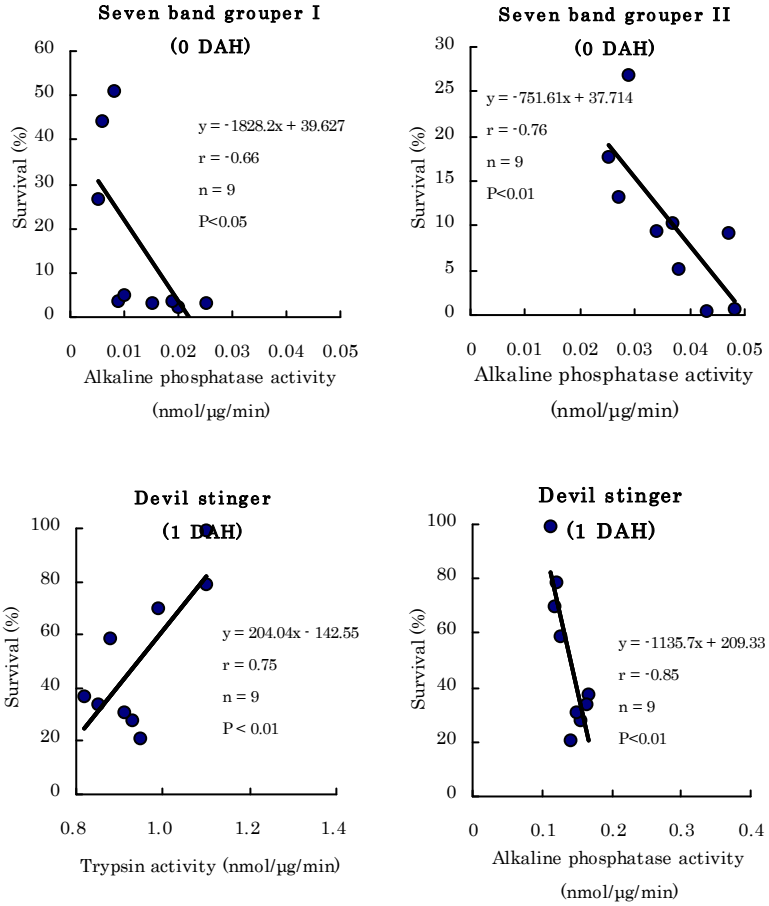
Seven band grouper I
(0 DAH)
y = -1828.2x + 39.627
r = -0.66
n = 9
P<0.05
Survival (%)
Alkaline phosphatase activity
(nmol/μg/min)
Seven band grouper II
(0 DAH)
y = -751.61x + 37.714
r = -0.76
n = 9
P<0.01
Survival (%)
Alkaline phosphatase activity
(nmol/μg/min)
Devil stinger
(1 DAH)
y = 204.04x - 142.55
r = 0.75
n = 9
P < 0.01
Survival (%)
Trypsin activity (nmol/μg/min)
Devil stinger
(1 DAH)
y = -1135.7x + 209.33
r = -0.85
n = 9
P<0.01
Survival (%)
Alkaline phosphatase activity
(nmol/μg/min)

Table 1 Growth of seven-band grouper larvae reared in rearing tanks of all three shapes from 0 to 5 days after hatching (DAH)

		Trial I			Trial II		
	DAH	S Tank	I Tank	D Tank	S Tank	I Tank	D Tank
Notochord length (mm)	0	1.71 ± 0.01	1.71 ± 0.01	1.71 ± 0.01	1.91 ± 0.06 [ab]	1.78 ± 0.11 [b]	2.00 ± 0.04 [a]
	1	2.07 ± 0.06	2.05 ± 0.07	2.14 ± 0.02	2.21 ± 0.02 [a]	2.12 ± 0.04 [b]	2.21 ± 0.02 [a]
	3	2.20 ± 0.03	2.16 ± 0.04	2.22 ± 0.03	2.22 ± 0.02 [b]	2.28 ± 0.01 [a]	2.28 ± 0.03 [a]
	5	2.32 ± 0.05	2.28 ± 0.05	2.31 ± 0.01	2.27 ± 0.05	2.33 ± 0.01	2.31 ± 0.04
Total length (mm)	0	1.81 ± 0.01	1.81 ± 0.01	1.81 ± 0.01	2.04 ± 0.08 [ab]	1.90 ± 0.11 [b]	2.12 ± 0.04 [a]
	1	2.20 ± 0.06	2.17 ± 0.08	2.27 ± 0.02	2.35 ± 0.02 [a]	2.26 ± 0.03 [b]	2.37 ± 0.02 [a]
	3	2.35 ± 0.02 [ab]	2.30 ± 0.05 [b]	2.37 ± 0.03 [a]	2.37 ± 0.03	2.44 ± 0.01	2.38 ± 0.09
	5	2.48 ± 0.05	2.44 ± 0.06	2.46 ± 0.02	2.45 ± 0.05	2.49 ± 0.01	2.47 ± 0.04
Dry weight (μg)	0	14.6 ± 0.2	14.6 ± 0.2	14.6 ± 0.2	16.1 ± 0.6	15.9 ± 0.4	16.9 ± 0.1
	1	14.2 ± 0.2	14.1 ± 0.1	14.4 ± 0.2	15.5 ± 0.2 [ab]	15.1 ± 0.1 [b]	16.1 ± 0.5 [a]
	3	12.7 ± 0.5 [ab]	12.4 ± 0.3 [b]	13.1 ± 0.1 [a]	13.5 ± 0.8	13.8 ± 0.6	14.1 ± 0.6
	5	12.5 ± 1.2	12.6 ± 0.3	13.0 ± 0.3	12.7 ± 0.4	11.8 ± 0.6	11.8 ± 0.6
Growth rate (mm/day)	5	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01

Results are mean values ± SD of 3 replicates for each tank proportion. The sample size from each tank was 5 larvae (a>b, Fisher's PLSD, $P<0.05$)

Table 2 Growth of devil stinger larvae reared in rearing tanks of three different shapes from 0 to 10 days after hatching (DAH)

	DAH	Tank		
		S Tank	I Tank	D Tank
Notochord length (mm)	0	2.78 ± 0.12 [b]	2.84 ± 0.08 [ab]	2.98 ± 0.06 [a]
	1	3.48 ± 0.05	3.54 ± 0.10	3.56 ± 0.10
	5	4.56 ± 0.17	4.64 ± 0.05	4.52 ± 0.29
	10	5.23 ± 0.25	5.25 ± 0.23	5.36 ± 0.13
Total length (mm)	0	2.99 ± 0.12 [b]	3.06 ± 0.04 [ab]	3.18 ± 0.06 [a]
	1	3.72 ± 0.06	3.79 ± 0.10	3.81 ± 0.10
	5	4.91 ± 0.15	4.97 ± 0.08	4.86 ± 0.26
	10	6.24 ± 0.33	6.35 ± 0.16	6.49 ± 0.33
Dry weight (μg)	0	45.9 ± 0.6	44.9 ± 0.4	45.9 ± 0.6
	1	43.9 ± 0.3 [b]	45.9 ± 1.3 [a]	44.6 ± 0.9 [ab]
	5	124.0 ± 5.6 [a]	104.4 ± 6.2 [b]	116.2 ± 3.8 [a]
	10	431.7 ± 46.1	473.1 ± 40.9	459.5 ± 49.6
Growth rate (mm/day)	10	0.24 ± 0.02	0.24 ± 0.03	0.24 ± 0.01

Results are mean values ± SD of 3 replicates for each tank proportion. The sample size from each tank was 5 larvae. a>b, Fisher's PLSD, $P<0.05$

Table 3 Coefficient of correlation between survival at 5 DAH and enzyme activities at 0, 1, 3 and 5 DAH of seven-band grouper larvae during rearing experiment

Enzyme activity	Trial I (DAH)				Trial II (DAH)			
	0	1	3	5	0	1	3	5
Trypsin	-0.57	-0.59	-0.89**	-0.48	-0.70*	0.36	-0.48	-0.32
Esterase	-0.57	0.19	0.60	-0.57	0.10	-0.39	-0.43	-0.32
Alkaline phosphatase	-0.66*	-0.20	-0.09	-0.41	-0.76**	0.44	-0.05	0.34

n = 9, * = $P< 0.05$, ** = $P<0.01$

Table 4 Coefficient of correlation between survival at 10 DAH and enzyme activities at 0, 1, 5 and 10 DAH of devil stinger larvae during rearing experiment

Enzyme activity	DAH			
	0	1	5	10
Trypsin	-0.37	0.75**	-0.07	-0.61*
Esterase	0.14	-0.03	-0.46	0.07
Alkaline phosphatase	-0.07	-0.85**	-0.13	0.48

n = 9, * = $P< 0.05$, ** = $P<0.01$