Title:

Improvement of the survival in the seven-band grouper, *Epinephelus septemfasciatus*, larvae by optimizing aeration and water inlet in the mass-scale rearing tank

Running title:

Flow field control of the larval rearing

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Abstract

The water flow in larval rearing tanks has been indicated to cause mass mortality of the seven-band grouper (*Epinephelus septemfasciatus*) larva. Therefore, we tested a new aerating method in an actual scale of intensive rearing tank (8.0 m in diameter, 1.87 m of water depth, 100 m^3 of volume), in which an aerator was set at the center of the rearing tank surrounding cylindrical drain (1.2 m in diameter) to generate the flow filed, and 7 larval rearing trials were performed. Then, we compared the survival rate with the former aeration methods, in which several aerators are located in the rearing tank. The survival rate at 10 days after hatching in the new aeration method (61.5 ± 5.1 %, n=7) was about 3 times higher than the former methods (21.2 ± 13.7 %, n=6). We also examined the flow environment of rearing tanks by quantifying the flow field and discussed the relationship between the flow field in the rearing tank, behavior of larvae and survival. We confirmed that the vertical circulating flow was observed in rearing tanks, and determined effectively the survival and the behavior of grouper larvae in patchiness.

Key words: behavior, early mortality, flow field, larviculture

Introduction

Recently, groupers (Subfamily Ephinephelinae) have been recognized as new candidates for aquaculture and stock enhancement in Japan because of their high commercial values.[1)] However, mass mortality of the grouper larvae is catastrophic shortly after hatching.[2-6)] Many studies have reported mass mortality of grouper larvae due to physical factors in environment, such as water temperature,[4)] salinity,[5)] light illumination [3-5,7)] and aeration rate,[3,5,6)] in addition to the early feeding activities.[2,8-10)] The flow field in a rearing tank, which is often termed as turbulence, is commonly generated by aerators, and is very important to prevent stratification, to insure oxygenation and to disperse live and artificial foods.[11)] On the other hand, the water flow field in rearing tanks can be an important physical stress factor for fish larvae.[12)] However, so little attention has been paid to quantify the flow field in rearing tanks for fish larvae.[13-15)]

Our previous study [13)] has reported systematic measurements of flow in rearing tanks and have concluded that rearing for the seven-band grouper (*Epinephelus septemfasciatus*) larvae can be significantly affected by flow. Measurements of flow in rearing tanks were carried out in a 1 m^3 polyethylene tank. After examining the relation between the mean velocity of flow in the tank and the aeration rate from a spherical air-stone set on the central bottom in the tank, experiments on larval survival and growth were made under four aeration rates; no aeration, 50 mL/min, 200 mL/min and 1000 mL/min. The aeration rate of 200 mL/min provided the highest survival and growth of *E. septemfasciatus* larvae. The flow in the larval rearing tank formed a remarkable vertical flow. The horizontal circulating flow was very weak compared to the vertical circulating flow. These results strongly indicate that measurements of flow

velocity can visualize the flow field in the rearing tank and this information provides us not only the actual image of larval distribution in the rearing tank but also the practical idea to design an optimal flow field for larviculture. However, since a considerable amount of time was required to measure the flow in the rearing tank, we developed the computation model for estimating the flow field in the tank [16] and this computation model enables us to estimate the flow field in various scales of rearing tanks and to utilize the estimated flow to plan the rearing conditions for larviculture.

In this study, we expanded this hydrodynamic approach [13,16] to the actual mass-culture scale tank used in the Nagasaki Prefectural Fisheries Experimental Station, Japan and investigated the optimal flow field for the seven-band grouper larviculture. In many hatcheries using such mass-scale rearing tank for larviculture, several aerators are located in the rearing tank and this was the case in the previous culture method for *E. septemfasciatus* where the early survival of larvae fluctuated. Therefore, we hypothesized that setting the multiple aerators in the mass-scale rearing tank causes the several upwelling in the rearing tank and it will cause the physical damage to larvae more frequently. Also we hypothesized that the optimum flow field by adjusting aerator and water inlet for larviculture of the seven-band grouper in the mass-scale rearing tank can be the same level as 1 m^3 tank whose vertical upwelling was at about 8 cm/sec determining optimal flow field.[13] Thus, we conducted rearing trials for the seven-band grouper larvae with the improved aeration method following the optimal flow field in the small-scale tank [13] and compared the early survival with former method. Furthermore, the flow velocity in the rearing tank was measured and the flow field was visualized, and then the effect of flow in rearing tank on the survival of groper larvae was discussed.

Materials and Methods

We conducted rearing trials of the seven-band grouper larvae and measurements of flow velocity in a rearing tank. The rearing tank is a cylindrical water tank with a diameter of 8.0 m, a depth of 2.12 m, a water depth of 1.87 m and a capacity of 100 m^3 currently used in the Nagasaki Prefectural Fisheries Experimental Station, Japan (Fig. 1). It has a cylindrical pole 1.2 m in diameter with two slits installed in the center of the rearing tank. Two 10 cm diameter pipes provides fresh seawater (6.9 L/min, Fig. 1a). Water is discharged from a hole at the center of the slit on the bottom to a connecter pipeline, so the water depth of larval tank is kept constant. Aeration method was changed from year 2002. Prior to 2002, several tubes with an outer diameter of 25 mm and spherical aerators were set at several positions on the bottom of tank and weak aeration at about 1000 mL/sec in total was provided (Fig. 1b,c). After we changed the aeration in 2002, the air bubbles were generated from tubes with an outer diameter of 25 mm surrounding the cylindrical pole at the center of the rearing tank. In order to create the similar upwelling flow (about 8 cm/sec) as the optimal flow rate in the 1 m^3 rearing tank for *E. septemfasciatus* larval rearing, [13] flow rate was adjusted for 630 mL/sec. We assumed that air bubbles from the tube aerators and the water inlet from water pipes generate the water flow in the larval rearing tank.

Thirteen rearing trials were conducted in total, 3 trials for each year 2000 and 2001 and 7 trials for 2002, respectively. Except for the aeration method described above, the rearing conditions were kept comparable. Broodstock, which were about 7 years old in year 2000 and 2.7–8.5 kg of body weight (Table 1), were reared in a floating net cage (5×5×5 m) and were used for 3 years rearing trials. The fish were fed to satiation with

moist pellets (mackerel : squid : raw krill : fish meal at a ratio of 1:1:1:3) 3 times a week. From May to July in these 3 years, females at late vitellogenic stage (oocyte diameter >420 μm) were selected and subjected to the hormonal treatment described by Shein *et al*.[17] Fertilized eggs were obtained from each female by artificial insemination using cryopreserved sperm.[18] Then, floating and sinking eggs was separated by laying eggs and seawater in measuring cylinders, and embryonic development of 30 floating eggs from each female was observed in order to determine the fertilization rate using a profile projector (Nikon, Tokyo, Japan). Thirty floating eggs from each female were also transferred into a 500 mL beaker containing 500 mL of seawater, and were incubated in a temperature-controlled chamber (20°C) until hatching to determine hatching rate. Floating eggs were transferred into the rearing tank at a density of 10-20 eggs/L. Surface film was formed from hatching by addition of oil at 0.2 mL/m^2 water surface (Riken Feed Oil Omega, Riken Vitamin Co. Ltd., Tokyo, Japan) once daily to prevent surface tension-related death of larvae.[3,4] After the mouth of larvae opened (3 days after hatching, day 3), enriched *Brachionus rotundiformis* (SS-type, Indonesian strain)[19] fed at a density of 15 rotifers/mL, and HUFA enriched *Chlorella vulgaris* (Super *Chlorella* V12, *Chlorella* Industry Co. Ltd., Fukuoka, Japan) was added to the rearing tank at a density of 3×10^5 cells/mL once daily. UV-disinfected sand-filtered seawater (25°C) was supplied at the water exchange rate of 10-20 %. We kept light constant at 1000-3000 lx from mercury-vapor lamp in the ceiling and adjustment of natural sunlight. In addition, we used five fluorescent lights for illumination on the open water surface in the larval rearing tank during 4:00 to 16:00 hours. On day 5 and 10 for each trial, we collected samples from the water column from surface to the bottom of the rearing tank using the long polyvinyl chloride pipe 10 to 15 times in the night (dark

condition) and measured water volume and the fish numbers to estimate fish numbers in the rearing tank. Differences in average body weight of broodstock, floating egg rate, fertilization rate and hatching rate among 3 years were compared using one-way ANOVA. We also compared arcsine transformed survival rate at each age group using one-way ANOVA. When significant difference was detected ($P<0.05$), the data was further analyzed using Fisher's PLSD test ($P<0.05$).

Grouper larvae form a patch of high density near the open surface of the water in the daytime. To examine the behavior of grouper larvae, we observed the distribution of patch of larvae from day 4 to 20 at about 5 days intervals. We counted larvae in the patch using the method of Masuda *et al.* [20] We put an L-shape white board (10×10 cm) above the water surface projected the shade of the board over a water volume of approximately 1 L. We counted the number of fish in the projected water volume. This counting was held at 10-27 points in the rearing tank at each age and 200 mL of water at the same observation point were collected to check the rotifer density. We measured the light intensity on the open water surface as our usual observations to test for any relation.

We used an acoustic doppler velocity meter (Nortek Company, Sweden), and measured velocity distribution of flow in a vertical section on a radius of the rearing tank. We carried out the measurements of flow under three conditions. We measured two conditions of maximum aeration only (21240 mL/min) and maximum water supply only (about 250 L/min) to compare the effects of water and aeration against flow. As our third condition, we measured the flow of water (6.9 L/min) and aeration (630 mL/min) combined as typical in the rearing conditions for the seven-band grouper larvae in 2002. The number of grid points for measurements was 23×18 in the (r, z) directions, and the

grid spacing was at 4-15 cm. In the actual rearing condition, additional 23 measurements from the free surface water to 5 cm depth at 5 mm intervals was carried out for the transect from the center of the tank to the side wall of the tank. The flow in the rearing tank was measured at each position in the half plane of vertical section included the center of water tank. The mean velocities of three components (u, v, w) of flow in the rearing tank were obtained from sampling data. The time for sampling the flow was 0.1 sec, and the sample continued for 50 sec.

Results

The body weight of broodstock increased significantly by year (ANOVA, df=2, P=0.0008, F = 8.501), while there were no significant differences in floating egg rate, fertilization rate and hatching rate among 3 years (Table 1). The survival of the seven-band grouper larvae in the rearing tank for day 5 and 10 were significantly different before and after we changed the aeration method (ANOVA, df=2, P<0.0001; F = 45.627 for day 5 and F = 27.084 for day 10, respectively; Fig. 2). Survival on day 10 was low in year 2000 (14.6±3.5 %, n=3) and 2001 (27.8±14.7 %, n=3) before we changed the aeration method, and average survival for these 2 years was 21.2 % (10.0-47.0 %). However, the average larval survival in 7 trials was 61.5 % (53.0-69.2 %) in year 2002 after we changed the aeration method.

The changes in distribution of the patch of larvae under the open water surface in the larval rearing tank until day 20 and the distribution of light intensity on the open water surface are shown in Fig. 3. The larval patches of high density (about 20-200 larvae/L) were formed under the open water surface near the sidewall of the rearing tank until day 15. However, the patch of larvae at day 20 formed a toroidal pattern

surrounding the center of the rearing tank. There was no correlation between larval density under the open water surface and the light intensity on the water surface until day 15 (n=11-27, r=-0.259 – 0.024, P>0.05). On day 20, there was a positive relationship between larval density and light intensity (n=27, r=0.473, P=0.0117). The average rotifer density under the open water surface was 14.6 individuals/mL (11-18 individuals/mL) indicating that rotifers were distributed equally in the water column of the rearing tank.

We measured the flow generated by the maximum aeration (21240 mL/min). Fig. 4(a) and (b) show the flow velocity distributions of *u*-*w* and *v*-*w* components of the vertical measured section in the larval rearing tank. The abscissa x is the horizontal direction of the diameter of the cylindrical larval rearing tank and the vertical axis z is the upward vertical direction. In the flow velocity distribution *u*-*w*, we observed a strong upward flow from the aerators at the center of larval rearing tank, a radial horizontal flow under the open surface, a downward flow near the side wall, and a horizontal flow toward the center of the tank near the bottom. The flow in the measured vertical section was a remarkable circulating current. On the other hand, the velocity distribution v-w was not regular and the strength of flow was very weak as a whole.

The flow generated by maximum pour water of about 250 L/min is measured. Fig. 5(a) and (b) show flow velocity distributions of *u*-*w* and *v*-*w* components on measured section in larval rearing tank. In *u*-*w* velocity distribution, the strength of flow is very weak as a whole as compared with the result in Fig. 4(a) and it is not observed the remarkable vertical circulating flow. Also, in *v*-*w* velocity distribution, the flow is not regular and become weaker as well as Fig. 5 (a).

The flow generated by an aeration rate of 630 mL/min and an inlet water rate

of 6.9 L/min on the conditions for rearing grouper larvae is shown in Fig. 6. We adjusted the volume of aeration and water based on the professional experience in this station. Figure 6(a) and (b) show the flow velocity distributions of the *u*-*w* and *v*-*w* components in the larval rearing tank. In the *u*-*w* velocity distribution, flow in the measured vertical section forms a relatively small circulating cell as well as in Fig. 4(a). On the other hand, the flow v-w velocity distribution is not regular and the flow was very weak as a whole.

Figure 7 shows the mean flow velocity distribution at a depth of 5 cm under the open surface of water. In the figure, total *V* (closed circles) indicates the strength of flow (*vu*2+*v*2+*w*2). It decreases gradually in the radial direction from the center of the larval rearing tank. The *u* component of flow velocity is similar to values of the total *V*, and the *v* component of flow is very small compared to values of the other components. The large *w* component of flow near the center of the larval rearing tank is due to the aeration.

Figure 8 shows the velocity distribution of flow near the open water surface at the three positions in the larval rearing tank. Fig. 8 (a) is at the position near the center of the rearing tank, (b) is at the middle position and (c) is at the position near the side wall. The flow velocity distribution shows a logarithmic profile in Fig. 8 (a) and a parabolic one in Fig. 8 (b). The flow velocity near the open surface of the water becomes greater than that of the inner region of the rearing tank. However, the flow velocity in the Fig. 8 (c) is reduced at the front of the side wall of rearing tank.

Discussion

The physical conditions in larval rearing tanks other than the aeration method

are almost the same before and after we changed the aeration in 2002. However, the percent survival of the seven-band grouper larvae in 2002 was about 3 times higher than in earlier years. We realize that the rearing experiments were conducted in different years and it is possible that conditions of broodstock and egg quality can be different each year, even though the same batch of broodstock and rearing conditions were used. However, parameters of egg quality were not different among 3 years (Table 1), indicating that the egg quality of *E. septemfasciatus* can be comparable in this study. Therefore, it seems that the physical environment of water flow caused by aeration in larval rearing tank is improved by the aeration.

We had conducted preliminary observations on the flow field in the rearing tank with several spherical aerators similar to the year 2000 and 2001 in this study. However, the flow velocity was very fluctuated at each observation point and we could not visualize those flow field in the rearing tank as a whole. We conclude that several spherical aerators set at random positions on the bottom in the rearing tank do not produce uniform flow such as vertical circulation and that they generate large partial variations of flow velocity around the aerators. On the other hand, tube aerators surrounding the cylindrical pole at the center of the tank in the year 2002 produce a smooth, vertical circulating flow. Furthermore, in the case of the latter, since the flow velocity in inner region from the center to the half distance radius of the rearing tank is very weak and irregular, it decreases the chances that seven-band grouper larvae are transported into this region and encounter direct physical damage caused by air bubble from the aerators. Also, considering that the rotifer density was kept at constant level under the free surface water, this aeration method was presumably effective for increasing the encounter rate of live feed to the larvae.

Masuda and Tsukamoto [21] reported that the formation of patches by striped jack (*Pseudocaranx dentex*) larvae depends on the distribution of illumination on the open water surface of larval rearing tank. However, in our experiments the distribution of larval patches is not always coincident with the positions of the highest illumination. It seems that larvae until day 15 have poor ability of swimming, but follow vertical circulating flow. Thus, larvae are carried near the open surface by upward flow from aeration or their inherent buoyancy, are moved in a radial direction by relatively fast flow under the open water surface, are held by reduced flow under the open water surface near the side wall, and the larval patch finally forms in the region near the side wall. However, as grouper larvae in day 20 have relatively active motion as a result of their growth, they move toward the center of the rearing tank against the direction of flow near the open water surface, but they are not able to reach the center of the rearing tank to encounter the strong flow from the aeration. Finally, grouper larvae form the toroidal patch surrounding the center of the rearing tank. Thus, the relation between forming of the patch of larvae and the flow field in rearing tank is a very important factor in addition to light.

The fact that the flow in the 100 m^3 larval rearing tank is similar to the vertical circulating flow in the 1 m^3 tank [13] is remarkable, especially because the aeration rate (630 mL/min) in the former tank is relatively small compared to that (200 mL/min [13]) in the latter because of the volume ratio of the rearing tanks. As result, air bubbles from aeration are dominant in the flow field in larval rearing tanks and produce environments with smooth vertical circulating flow for rearing larvae. Though the inlet water from two pipes on the bottom in the larval rearing tank affects the water flow in the immediate region of that position, effects on the whole flow field in the larval rearing

tank are very small compared to the aeration. This is due to the fact that whole air bubbles from aerators on the bottom in the rearing tank are able to cause upward flow from the bottom through the open surface of the water; the flow from inlet water affects only a narrow flow field. Therefore, effects on the flow field by small water inlets can be neglected in the case of rearing seven-band grouper larvae.

As the relatively strong flow and the partial gradient of flow velocity in the vertical direction near the open water surface seem to provide a physical stress to grouper larvae, we conclude that reductions of high flow velocity and the vertical flow velocity gradient should produce more favorable circumstances for grouper larvae. Our results also indicate that quantification and visualization of the flow field in rearing tanks for larviculture can provide useful information on improvement of rearing environment not only for small-scale tanks [13,22] but also actual mass-scale rearing tanks.

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Table 1. Brood stock and egg quality of *E. septemfasciatus* used in this study

Year	n	Female broodstock	Egg quality		
		body weight (kg)	floating egg rate (%)	fertilization rate (%)	hatching rate (%)
2000	13	4.9 ± 1.0 [a]	76.7 ± 25.2	82.8 ± 17.1	78.4 ± 19.5
2001	17	6.4 ± 1.2 [b]	82.6 ± 21.4	81.8 ± 11.0	74.2 ± 13.0
2002	15	6.7 ± 1.5 [b]	89.5 ± 10.8	85.8 ± 12.2	73.2 ± 17.7

Data are represented as mean ± standard deviation (a<b, Fisher's PLSD test, $P<0.05$).

Figure legends

Fig.1 Diagram of the rearing tank (8 m in diameter and 1.87 m in water depth) in 2002(a), and horizontal view of arrangement of aerators in 2000 (b) and in 2001 (c).

Fig.2 Changes in percent survival of *E. septemfasciatus* larvae in 2000 (n=3), 2002 (n=3) and 2002 (n=7). Points and vertical bars indicate average survival and standard deviation, respectively. Alphabets on the point indicate significant difference in each age (a>b, Fisher's PLSD test, $P<0.05$).

Fig.3 Ontogenetic changes in patch formation of *E. septemfasciatus* larvae. Each figure in the left column indicates the light intensity (lux) at the surface, and figures and shaded area in the right column indicate the density of larvae (fish/L) from the surface to a depth of 10 cm and patch distribution, respectively.

Fig.4 Flow velocity distribution at maximum aeration rate (21,240 mL/min) in the rearing tank. (a) velocity distribution (*u*-*w*) and (b) velocity distribution (*v*-*w*).

Fig.5 Flow velocity distribution at maximum water inlet (250 L/min) to the rearing tank. (a) velocity distribution (*u*-*w*) and (b) velocity distribution (*v*-*w*).

Fig.6 Flow velocity distribution during grouper larvae rearing in the rearing tank (630 mL/min for aeration and 6.9 L/min for water inlet). (a) velocity distribution (*u*-*w*) and (b) velocity distribution (*v*-*w*).

Fig.7 Mean flow velocity under the open water surface during grouper larvae rearing in the rearing tank (630 mL/min for aeration and 6.9 L/min for water inlet).

Fig.8 Flow velocity distribution under free surface during grouper larvae rearing in the rearing tank (630 mL/min for aeration and 6.9 L/min for water inlet). (a) center position, (b) middle position, and (c) side wall position.

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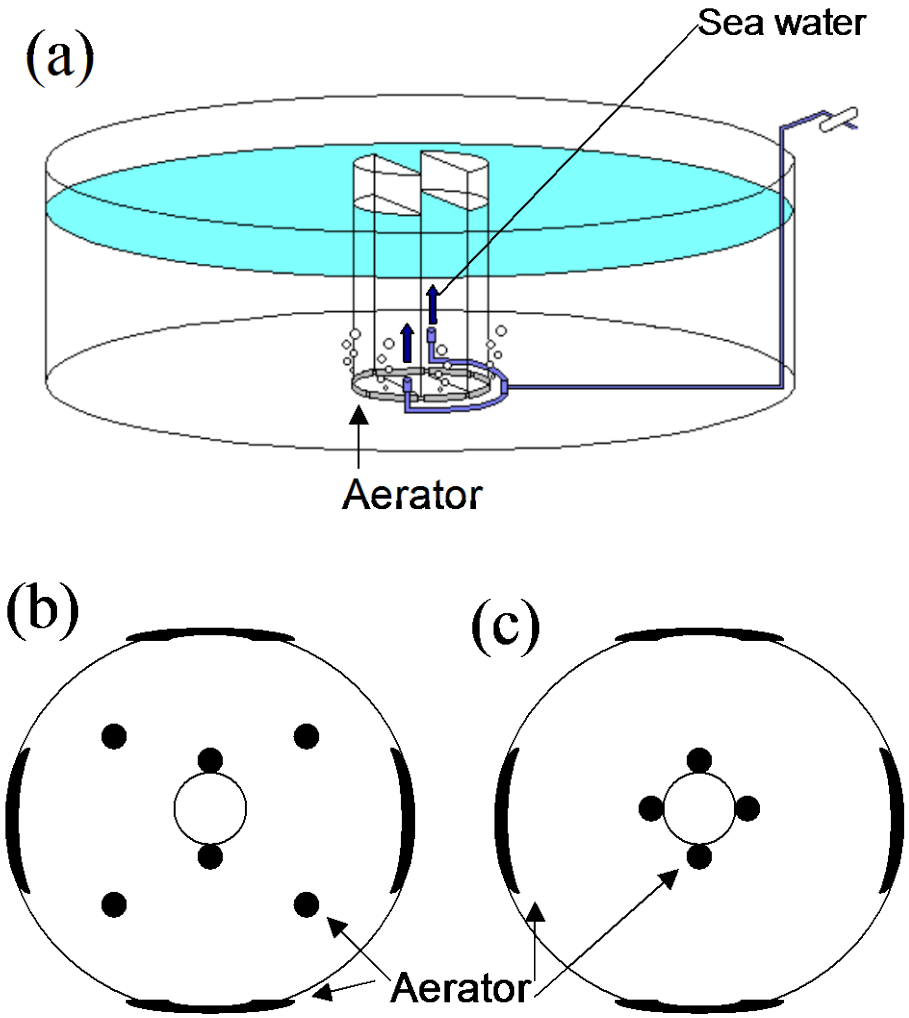

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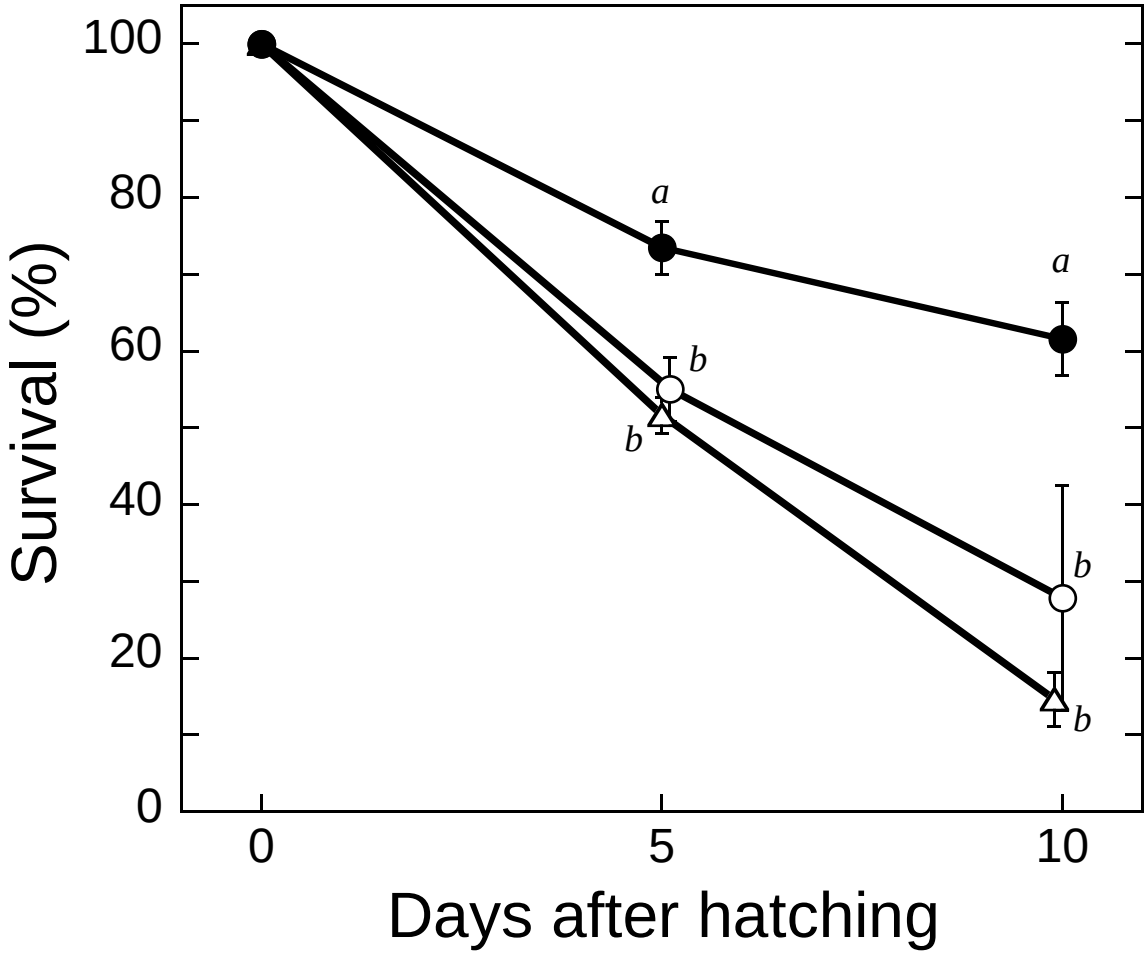

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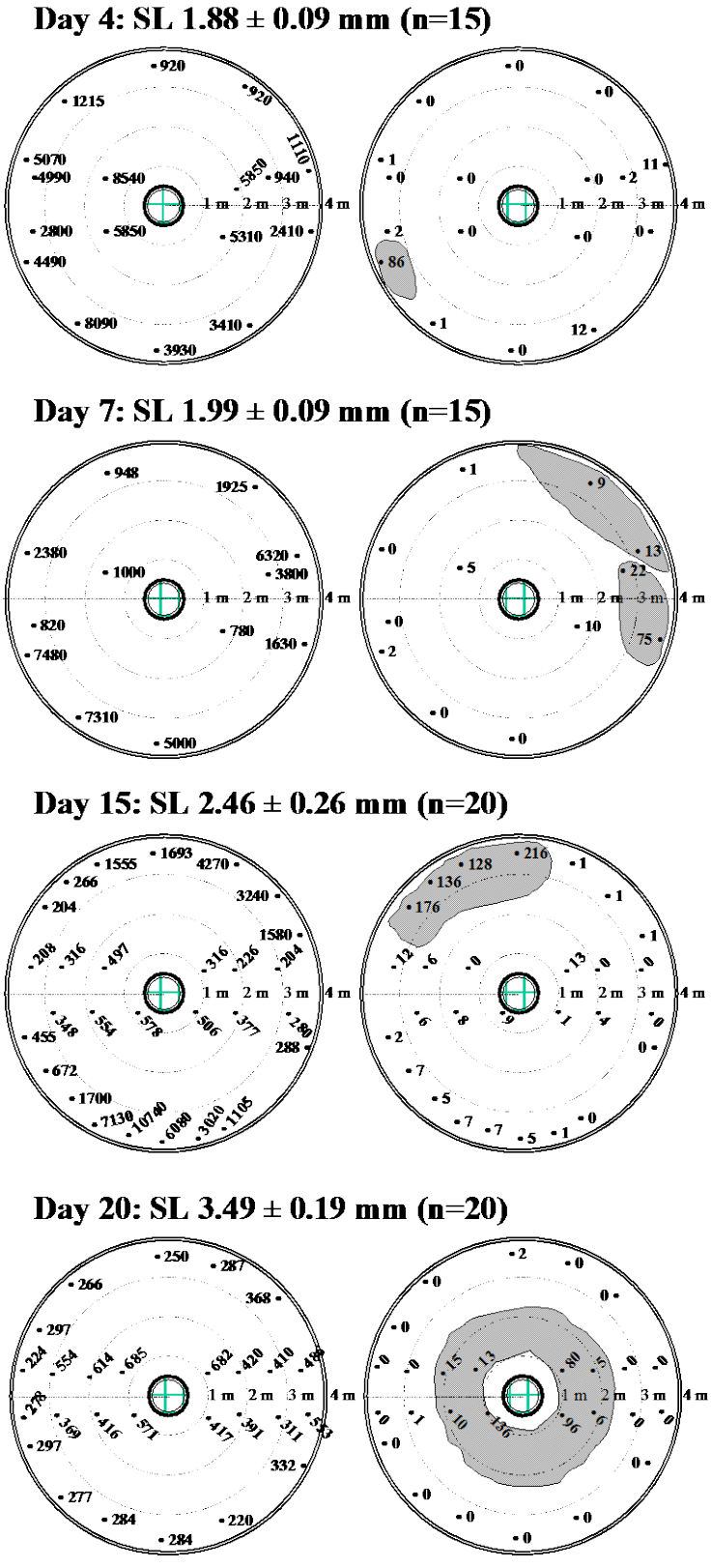

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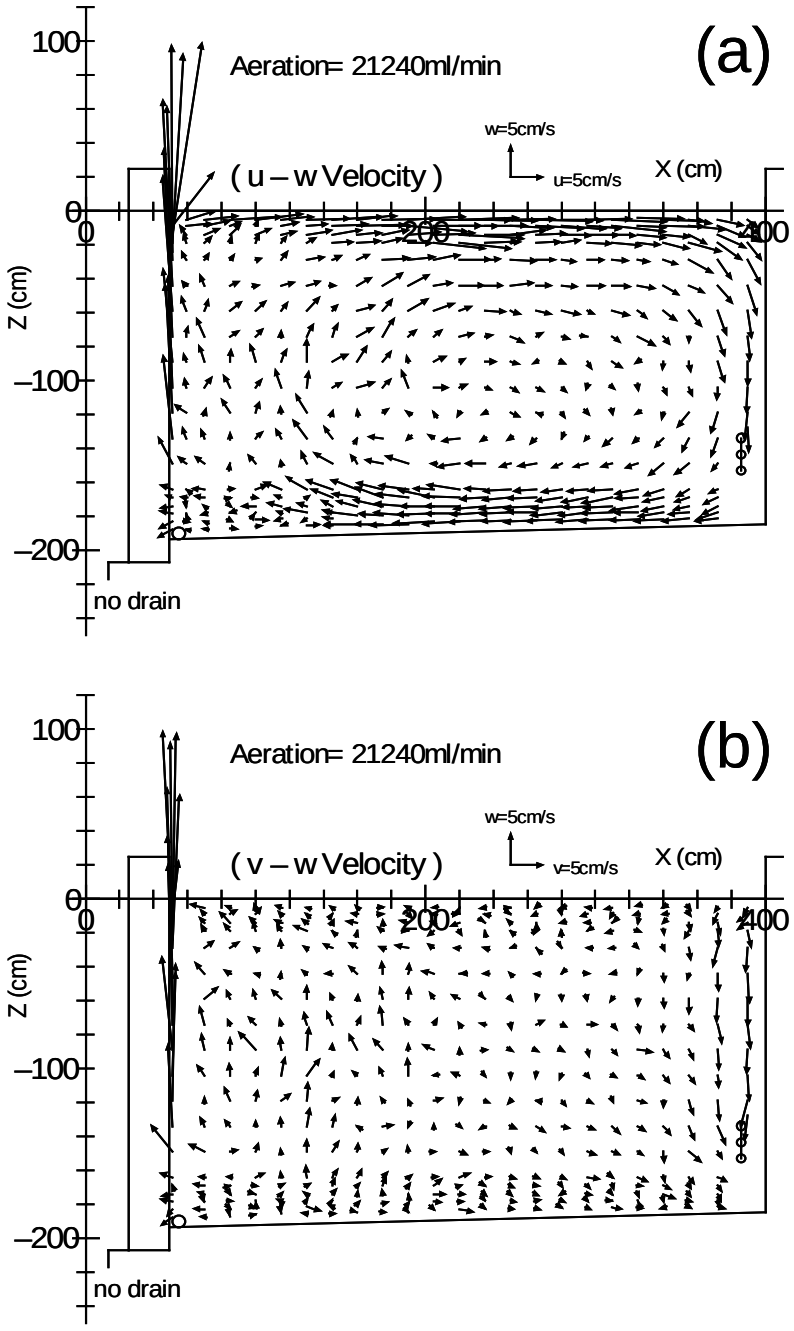

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Fig.5 Sakakura et al. (2006)

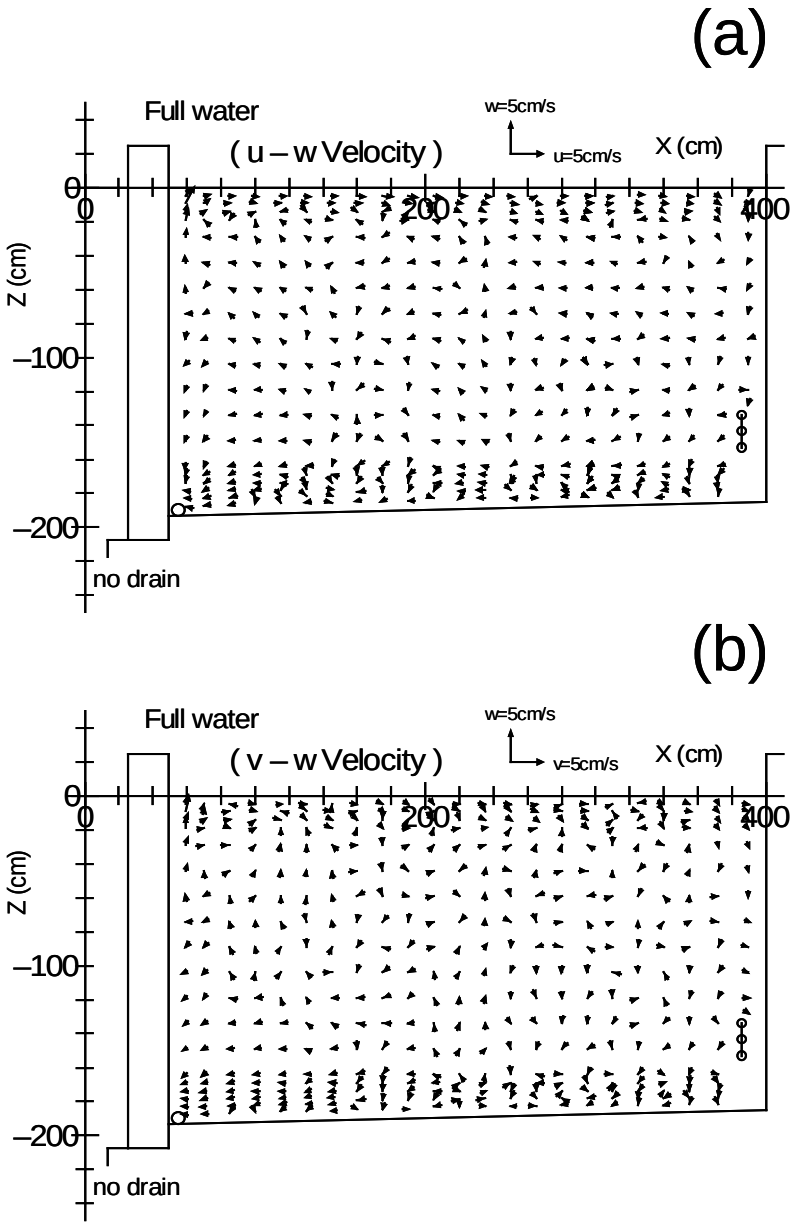

Fig.6 Sakakura et al. (2006)

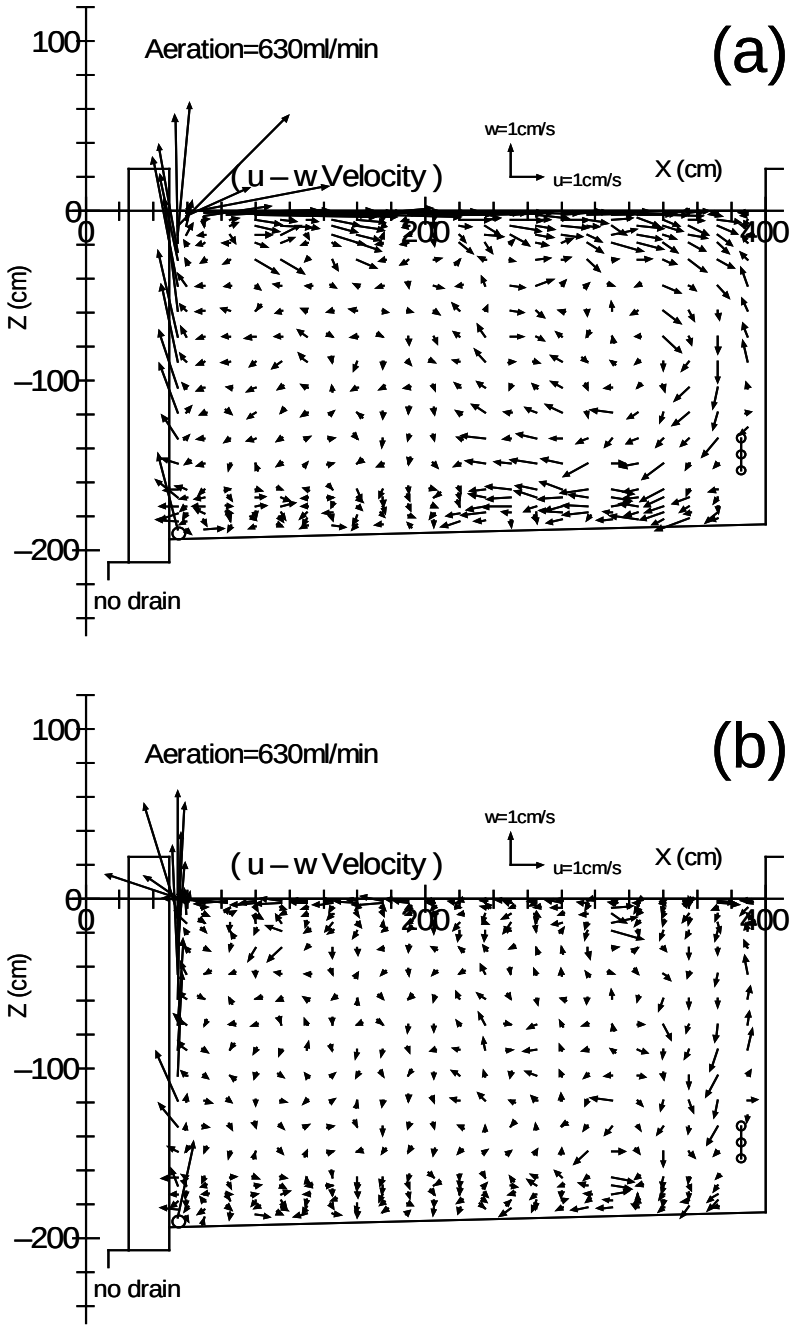

Fig.7 Sakakura et al. (2006)

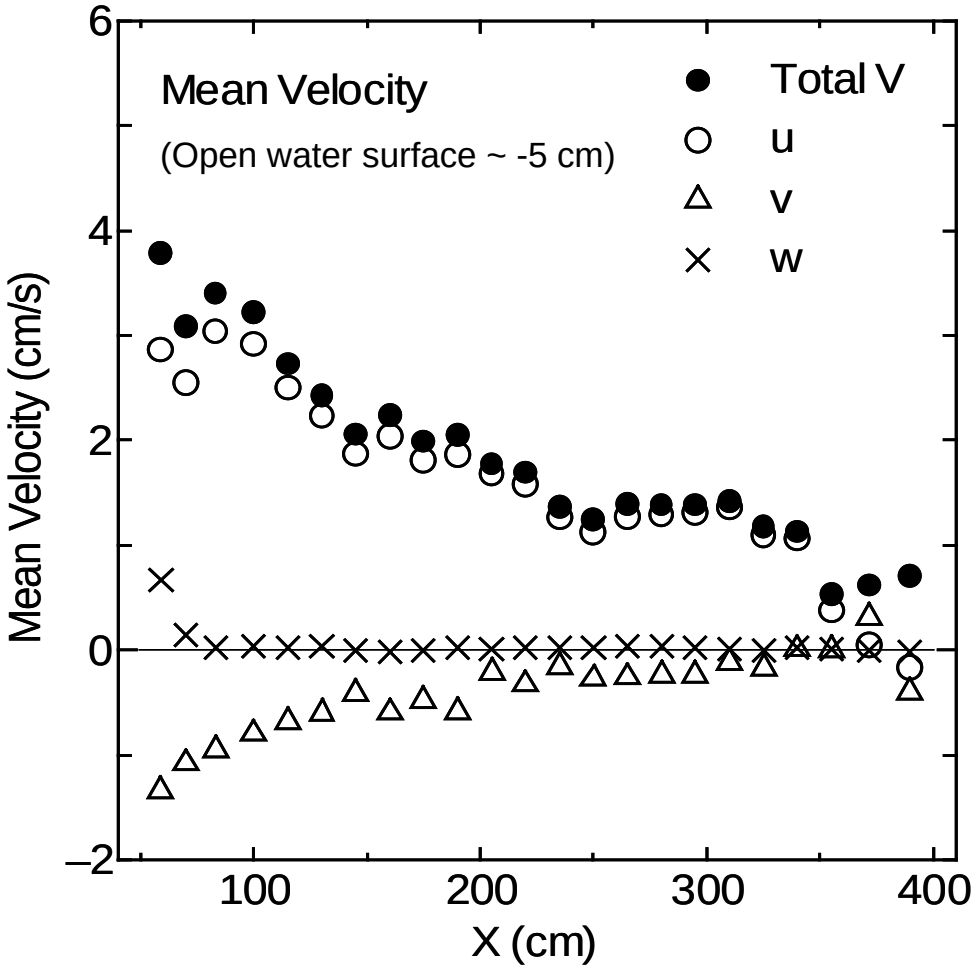

Fig.8 Sakakura et al. (2006)