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Study on prevention of sinking death in the fingerling production of Pacific bluefin tuna

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クロマグロ仔魚に多発する沈降死の防除に関する研究

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Contents

General introduction

Three species of bluefin tuna, *Thunnus thynnus*, *Thunnus orientalis*, *Thunnus maccoyii*, are pelagic scombroid species distributed in the Atlantic, Indian, and Pacific Oceans (Collette and Nauen 1983). Expanding demand for these high economic value tunas has increased their natural resource utilization, which has led to the rapid reduction of their resources (Ottolenghi 2008; Kumai 2012). Recently, strengthened measures have been enacted to restrict the capture of both Atlantic (ICCAT 2009) and southern bluefin tuna (CCSBT 2009) by the international tuna resource conservation committees.

To stabilize and control tuna supply, bluefin tuna aquaculture has been developed an economically important industry in Mediterranean countries for *Thunnus thynnus*, Australia for *Thunnus maccoyii*, Mexico and Japan for *Thunnus orientalis* targeting mainly the Japanese market (Hidaka 2008; Ono 2012). Despite the importance in industry, disputes have arisen with respect to natural resource management due to the reliance of tuna aquaculture on wild-caught fish for its seedling fish supply (Ottolenghi 2008). Therefore, development of full-life cycle aquaculture technology of bluefin tunas, which does not rely on their natural resources, is necessary for their sustainable aquaculture (Miyashita 2002; Sawada et al. 2005; Kumai 2012). Attempts at developing such technology have actively been made in the area of tuna bloodstock management and larviculture technique in recent years in some countries (Kinki University 2009; Anonymous 2010; Mylonas et al. 2010).

The fingerling production technology for Pacific bluefin tuna (PBT), *Thunnus orientalis* (Temminck and Schlegel), has been developed for a long period, since the

first natural spawning of the brood stock cultivated at the Fisheries Laboratories, Kinki University (FLKU) in 1979 (Harada et al., 1971; Kumai 1997, Miyashita 2002; Ishibashi 2012). In 2002, the FLKU have achieved the landmark to complete PBT full-life cycle under aquaculture conditions (Sawada et al. 2005; Ottolenghi 2008). Thereafter, the FLKU have developed particularly important and practical fingerling production technologies which enabled to start supplying mass-produced PBT fingerlings to the Japanese tuna aquaculture industry in 2008 (FLKU 2008; Normile 2009). In 2009, FLKU successfully produced over 40,000 fingerlings for domestic tuna aquaculture industry (Kumai 2012).

Although the technologies developed at the FLKU is promising, the efficiency in PBT fingerling production is still low (Kumai 2012; Sawada 2012; Ishibashi 2012). The survival in mass fingerling production was 2.35% during hatchery phase, 21.3% during intervening culture, and consequently 0.5% from egg to the shipment in the 2009 year group, whereas the survival from eggs to juveniles of other species with well-developed technologies has the efficacy of several tens of percent (Kumai 2012; Ishibashi 2012).

Poor PBT survival in their fingerling production is considered to be due to various causes, including surface and sinking death of early larvae during the first 10 days-post-hatch (dph), aggressive behavior and cannibalism after approximately 10 dph, and trauma due to contact or collision with rearing tank wall or sea cage netting after approximately 30 dph, heavy damage due to skin injuries during transport from hatchery to sea net cages at approximately 30 dph and iridoviral infection, blood fluke infection in sea cages (Miyashita et al. 2000, Munday et al., 2003; Sawada et al., 2005, Ishibashi et al., 2008; Ishibashi 2012; Shirakashi et al. 2012). Among these, mass

mortality by both surface death and sinking death during the early larval stage has been serious since the early stage of PBT fingerling production, and it still largely affects the present PBT aquaculture operations (Sawada et al. 2005; Takashi et al. 2006; Tanaka et al. 2009; Nakagawa et al. 2011; Kumai 2012).

Surface death, which has been reported in several other marine fishes, is a phenomenon where larvae become trapped by surface tension of the rearing water and the trapped larvae consequently die (Battaglene et al. 1994; Sawada et al. 1999; Yamaoka et al. 2000; Kaji et al. 2003; Fui et al. 2012). Concerning its mechanism, it has been suggested that mucus secretion from larval skin is promoted by the contact with water surface, and the mucus functions as a glue to trap larvae at the water surface in kelp grouper, *Epinephelus bruneus* (Bloch), red spotted grouper, *Epinephelus akaara* (Temminck and Schlegel) and striped bonito, *Sarda orientalis* (Temminck and Schlegel) (Sawada et al. 1999; Yamaoka et al. 2000; Kaji et al. 2003). It has been reported that oil film, which is supposed to inhibit contact by larvae with the water surface and/or reduces surface tension, prevents surface death in red spotted grouper and striped bonito (Yamaoka et al. 2000; Kaji et al. 2003). In the PBT larviculture, surface death has been reported to occur between 1 and 4 dph (Takashi et al. 2006), and making oil film on the water surface is also carried out in practical PBT larviculture to prevent surface death on an empirical basis (Munday et al. 2003).

Sinking death is a phenomenon where larvae sink to the bottom of rearing tanks mainly during the nighttime when larvae cease swimming. Consequently, larvae die due to the damage suffered by the contact with tank bottom. This has been identified as a particularly serious problem causing mass mortality in the PBT larviculture (Sakamoto et al. 2005; Miyashita 2006; Tanaka et al. 2009). PBT larval body density is

greater than that of rearing water, even when larvae possess an inflated swimbladder, and this larval greater body density is considered to be the primary cause of sinking death during the night-time on ceasing swimming (Takashi et al. 2006). Sinking death of larvae have also been reported in various other marine fish species; amberjack, *Seriola dumerili* (Risso), barfin flounder, seven-band grouper, kelp grouper, where it has also been suggested that sinking has had a relationship with larval body density or sinking velocity (Teruya et al. 2009; Kayaba et al. 2003; Shiotani et al. 2003; Sakakura et al. 2006; Hirata et al. 2009; Fui et al. 2012).

Flow control of rearing water by aeration has been reported to prevent larval sinking death effectively in other fishes, for barfin flounder, and seven-band grouper, kelp grouper (Kayaba et al. 2003; Shiotani et al. 2003; Sakakura et al. 2006; Fui et al. 2012). Flow control in larval rearing tanks by overnight aeration has also been applied in PBT mass-scale larviculture in the hatchery of Kinki University to prevent larval sinking death on an empirical basis; however, it has not yet been examined in detail its enhancement effect of survival in PBT.

Regarding fish body density, swimbladder is an important organ which controls buoyancy by reducing fish body density relative to that of surrounding water (Magnuson 1978; Itazawa 1991; Alexander 1993). In the larviculture of some fishes, it has been reported that swimbladder inflation failure results not only in poor survival, but also in growth and/or vertebral deformity, such as lordosis from larval to juvenile stage (Spectorova and Doroshev 1976; Kitajima et al. 1981, 1994; Chatain 1989; Chatain and Dewavrin 1989; Goolish and Okutake 1999; Jacquemond 2004; Trotter et al. 2005a). Therefore, swimbladder inflation success during larval stage and subsequent normal swimbladder development are considered to be important biological

factors capable of contributing to larval survival improvement by prevention of sinking death via control of body density. Moreover, the poor growth and vertebral deformity, such as lordosis due to swimbladder inflation failure often negatively affect the production efficiency in other aquaculture fish species; therefore, also in PBT, it has the possibility to reduce production efficiency. However, the definite relationships not only between swimbladder inflation failure and larval survival but also between swimbladder inflation failure and growth, vertebral deformity have not yet been investigated in PBT.

Physoclistous-swimbladders in fishes are transiently connected to the digestive tract via a pneumatic duct during early larval stages (Itazawa 1991; Alexander, 1993). During this period, air gulping at the water surface is required in order to achieve initial swimbladder inflation (ISI; Kitajima et al. 1981; Chatain 1989b; Chatain and Ounais-Guschemann 1990; Kitajima et al. 1994). Presence of a film on the water surface composed of oil, protein and other materials originating from live feeds, larval feces, and other organic substances (surface film) inhibits larval air gulping and subsequent ISI, and removal of this film using cleaning devices, such as surface skimmer, can effectively promote larval ISI (Kitajima et al. 1981, 1994; Chatain and Ounais-Guschemann 1990; Trotter et al. 2005a). Moreover, the period for air gulping to achieve ISI, i.e., the period when effective ISI promotion is possible (so-called “window”), is limited and species specific (Bailey and Doroshov 1995; Friedmann and Shutty 1999; Trotter et al. 2005a; Kitajima et al. 1981). The PBT belonging to the Scombridae is also a physoclistous fish, and they have a physostomous swimbladder in the early larval stage (Itazawa 1991; Alexander 1993; Kaji 2000). However, the promotional and inhibitory condition of water surface and the optimal timing for effective ISI promotion have not yet been investigated in PBT.

The purpose of this study is to investigate the effect of the flow control of rearing water on larval survival and larval sinking velocity to prevent sinking death (Chapter 1), the influence of ISI failure on larval vertical distribution and their survival (Chapter 2), the promotional and inhibitory conditions of water surface, the optimal timing of ISI promotion, and the relationship between ISI promotion and occurrence of surface death (Chapter 3) to improve early larval mortality due to sinking death and surface death (Fig. 1). Additionally, in this study, the influence of swimbladder inflation failure on mortality, growth and lordotic deformity in postflexion larvae and juveniles is also investigated as a series of study on the influence of swimbladder inflation failure to obtain the information for improvement of the technique of fingerling mass production (Chapter 4).

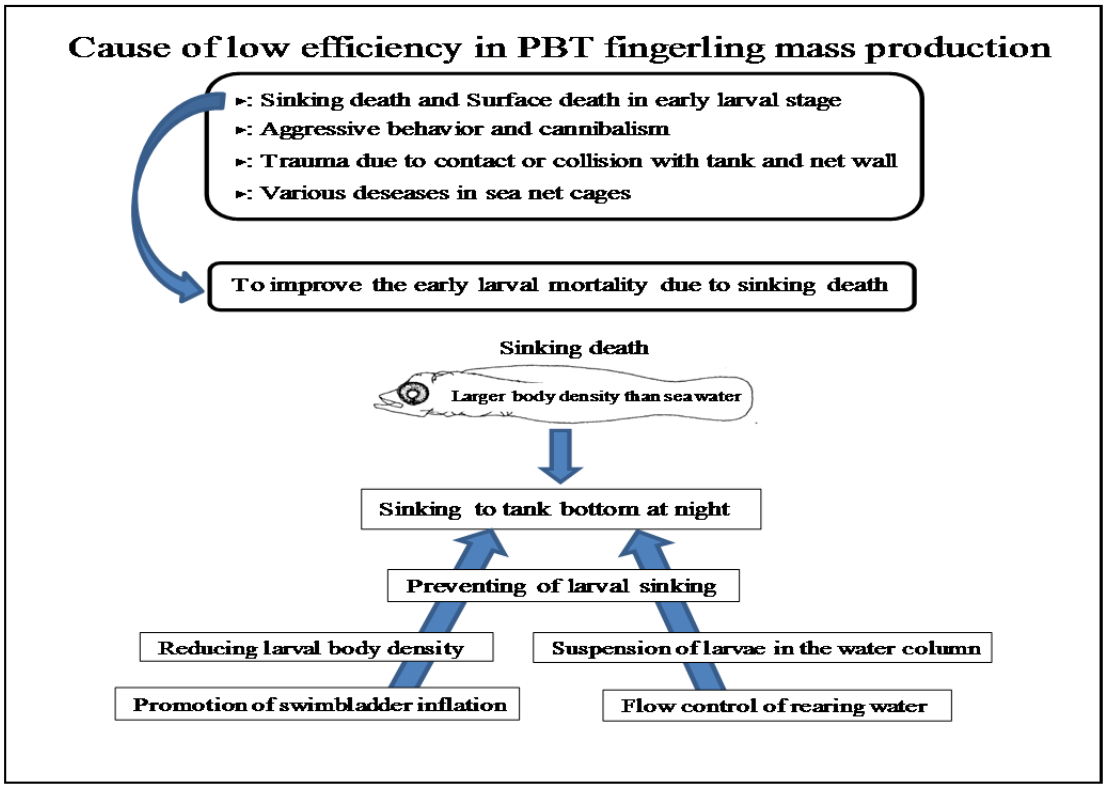

Fig. 1. Problem of low efficiency in PBT fingerling mass production and the purpose of this study to improve the early larval mortality due to sinking death.

Chapter 1

Flow control by aeration to prevent sinking death in Pacific bluefin tuna, *Thunnus orientalis*, larvae

1.1 Introduction

PBT larval body density is greater than that of rearing water, and is believed to be the primary cause of sinking death (Takashi et al. 2006). Additionally, larvae swim in the mid-water to feed during the day after first feeding, but swimming activity decreases at night (Takashi et al. 2006). As a result, techniques to maintain larval suspension within the rearing water column during the nighttime are considered to be necessary to prevent larval sinking to tank bottom.

At FLKU, the air supply rate to PBT larval rearing tanks is increased during the nighttime to increase larval survival via prevention of sinking death on an empirical basis. However, it has not yet been examined in detail its enhancement effect of survival in PBT.

Bubbles from the aerators drag water upwards and increase water circulation. The flow pattern is typically characterized by an inner segment of relatively low flow and an outer segment of higher flow, often shaped like a doughnut. Larvae remain suspended in the water column segments where larval sinking velocity is balanced by upward flow velocity.

Although increased air supply rate likely increases larval suspension, higher air input and water flow during the nighttime may also decrease larval feeding during the day and cause skeletal deformities or injuries as a result of collisions with air bubbles

(Tucker 1998). However, there is no method of determining the ideal air supply rate, and control depends on staff experience. An understanding of the effects of air supply rate on water circulation and larval downward velocity would allow more accurate control of water movement in larval rearing tanks.

The aim of this study is to evaluate the effects of different air supply rate during the nighttime on larval survival and water circulation of rearing water, and to determine the larval sinking velocity, and then, based on the results, the danger zones for larval sinking were estimated and the relationship between larval survival and water circulation was determined in order to improve larval mortality due to larval sinking death.

1.2 Materials and methods

Larval rearing

Fertilized eggs were obtained from brood stock fish in a sea net-cage at the FLKU. Two trials were conducted, in which PBT larvae were reared in essentially the same conditions except for the number of eggs per tank (Table 3.1). Between 5000 and 6000 fertilized eggs were introduced into each of nine 500 *l* cylindrical polycarbonate tanks (diameter 100 cm, height 62 cm). Hatching rates were 92.3% in Trial 1 and 99.0% in Trial 2.

Water was replenished daily at 10% of tank volume × days-post-hatch (dph). Seawater was filtered through a high-speed fiber filter (≤2 μm) system (Unitica Co. Ltd., Osaka, Japan) and sterilized with a UV lamp.

The photoperiod was 12 h light (07:00–19:00) via fluorescent lights with 500

lx luminance during the rearing period. PBT larvae were fed S-type rotifers (*Brachionus plicatilis* sp. complex) at 10:00 and 14:00 and condensed *Nannochloropsis oculata* (Marine Fresh, Marine Bio Co., Ltd., Yatsushiro, Japan) from 2 dph onwards. The *N. oculata* were added to the rearing tank before 07:00 to maintain a density of 0.75 million cells/*ml*.

The rotifers were enriched with commercial supplements to enhance n-3 highly unsaturated fatty acids (n-3HUFA) (Marine Gloss and Marine Gloss EX, Nisshin Marine Tech Co., Ltd., Yokohama, Japan), and taurine and vitamin content (Aquaplus ET, Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan). The rotifers were supplied to maintain a density of 5–7 rotifers/*ml*.

In both trials, 0.1 *ml* of feed oil (Ueda Oils And Fats MFG, Co., Ltd., Kobe, Japan) was added to the tank from just after hatching to 3 dph to prevent surface death. To promote the larval swimbladder inflation, the surface film on the rearing water was removed from 3 to10 dph, using paper in Trial 1 and a handcrafted surface skimmer in Trial 2.

Air was supplied through a circular ceramic air stone (32mm diameter) placed 25 mm above the bottom in the centre of each tank. All tanks were supplied with air at 300 *ml*/min during the day between 07:00 and 19:00, using a rotameter (ModelRK1250, Kojima Instruments Inc., Kyoto, Japan). After first feeding (2 dph), air supply rate was changed to 0, 300, or 900 *ml*/min during the nighttime (n = 3 tanks/flow rate). During the rearing period, temperature (°C), dissolved oxygen (mg/*l*), pH and salinity were measured with a stick thermometer and a multiple water quality sensor (556 MPS, YSI Inc., Yellow Springs, OH, USA) at 16:00 every day. The water conditions during larval rearing are shown in Table 1.1. At the end of each trial (10 dph), and before turning on

the light, the flow rate in all tanks was set to 900 *ml*/min, except for the tanks that were set at 0 *ml*/min. Then, 20–30 live larvae were collected from the surface water of each tank using a 3*l* beaker and observed their swimbladders under a microscope. After turning on the light (10 dph), the total number of live PBT larvae in each tank was counted and survival was calculated. Because there were only low numbers of larvae in the tanks set at 0 *ml*/min, larvae for swimbladder observation was collected at the time of counting the total larval number in the tank. Then, the percentage of larvae with inflated swimbladder (WIS) was calculated.

Table 1.1. Rearing conditions for larviculture of Pacific bluefin tuna, *Thunnus orientalis*

Conditions	Trial 1	Trial 2
Number of eggs introduced per tank	6000	5000
Photoperiod	07:00–19:00	
Luminance (lx)	500	
Water exchange ratio (%/day)	10% of tank volume × days-post-hatch	
Density of *Nannochloropsis oculata* (cells/*ml*)	0.75 million	
Density of *Brachionus plicatilis* sp. complex (ind./*ml*)	5–7	
Water temperature (°C)	24.0–24.9	25.6–26.9
Dissolved oxygen (mg/*l*)	6.1–8.2	5.3–6.6
pH	7.96–8.28	7.97–8.31
Salinity	32.8–33.9	33.0–33.5

Measurements and observations

Larval sinking velocity

In Trial 2, the sinking velocity of larvae was measured at night (20:00–22:00) between 1 and 9 dph. Thirty larvae were selected at random from a rearing tank in which the air was supplied at 900 *ml*/min. The larvae were anesthetized with 200 ppm eugenol (FA-100, Mitsubishi Tanabe Pharma Co., Ltd., Osaka, Japan), their swimbladder inflation, standard length and total length were examined under a microscope, then each fish was dropped gently into a 2 *l* cylinder filled with water from the rearing tank. This sinking velocity measurement was conducted in an air conditioned room (26 °C) which was equal to the larval rearing temperature. It was assumed that terminal velocity was achieved 5 cm below the surface; therefore, the time for each larva to sink was measured from 5 to 15 cm depth.

Flow field measurements

The flow fields in a 500 *l* rearing tank were measured at two air supply rates (300 and 900 *ml*/min) with an acoustic Doppler flow meter (velocimeter, Vectorino, Nortec AS, Rud, Norway). The velocimeter was fixed to a movable trestle that allowed the sensor to take horizontal and vertical measurements. Measurements were taken in three planes: horizontal (*x*-, *y*-) and vertical (*z*-) axes, at 137 points at 25 and 50 mm intervals. Fossil shell powder (Fish Green, Green Culture Co., Ltd., Takaoka, Japan) was added to the test tank to improve sonic reflection. The flow rate at each point in both experiments was measured at 25 Hz for 3 min. Preliminary data suggested that the velocity reached a steady state within 2 to 3 min. For the analysis, therefore, only the

data over the 1 min period between 2 and 3 min was analyzed. Because air bubbles, physical structures, and the surface/air interface may interfere with measurements, all measurement points were >5 cm from the tank wall and >5 cm below the surface.

Estimated danger zone

The risk of larval sinking death is highest when the larval sinking velocity (*V*l) is higher than the upward water velocity (*V*z) during the nighttime. The "Estimated danger zone" (EDZ) was defined as the area within the cross-section of the rearing tank where, based on flow readings, the upward flow velocity was less than the larval sinking velocity (*V*l > *V*z). The EDZ between 3–9 dph was calculated daily from flow field measurements and mean larval sinking speed. The velocity at each sample point was compared with daily mean larval sinking speed between 3 and 9 dph. The EDZ was plotted as a cross-sectional spatial percentage of the vertical section of the tanks with flow rates of 300 and 900 *ml*/min.

Statistical analysis

The significance of differences in survival or swimbladder inflation frequency among different air supply rates were tested using one-way ANOVA followed by Scheffe's test. Data were assumed to be normally distributed. The effect of swimbladder inflation on sinking velocity was evaluated using a *t*-test. The analyses were performed in Stat View (SAS Institute, Cary, NC, USA) and $P < 0.05$ was considered to represent a significant difference.

1.3 Results

Survival

In both trials, survival was higher at higher flow rates (ANOVA, Trial 1, $P < 0.05$; Trial 2, $P < 0.05$; Table 1.2). Larval survival was highest in the tanks with a nighttime air supply rate of 900 *ml*/min (Trial 1, 48.6 ± 4.2%; Trial 2, 43.2 ± 4.5%) (Table 1.2). In both trials, more larvae developed a fully inflated swimbladder by the final day of rearing when held at higher flow rates (ANOVA, Trial 1, $P < 0.05$; Trial 2, $P < 0.05$; Table 1.2). The swimbladder inflation frequencies of Trial 1 were higher than those of Trial 2.

Table 1.2. Survival and swimbladder inflation frequency (SB %, mean with standard deviation) for Pacific bluefin Tuna, *Thunnus orientalis,* at 10 days-post-hatch in both trials

Trial	Air supply at night time		
	0 (*ml*/min)	300 (*ml*/min)	900 (*ml*/min)
Trial 1			
Survival (%)	0.4 ± 0.1^{a}	18.2 ± 13.1^{a}	48.6 ± 4.2^{b}
SB (%)	0^{a}	5.0 ± 0.0^{b}	6.7 ± 2.9^{b}
Trial 2			
Survival (%)	1.8 ± 1.0^{a}	26.6 ± 15.7^{ab}	43.2 ± 4.5^{b}
SB (%)	5.6 ± 6.9	54.4 ± 23.4	21.0 ± 22.2

$n = 3$ tanks at each air supply rate.

Survival and SB% values with different superscripts are significantly different within each row (Scheffé's test, $P < 0.05$).

Flow field

Fig. 1.1 showed the measured flow fields in a 500 *l* at two air supply rates (300 and 900 *ml*/ min).

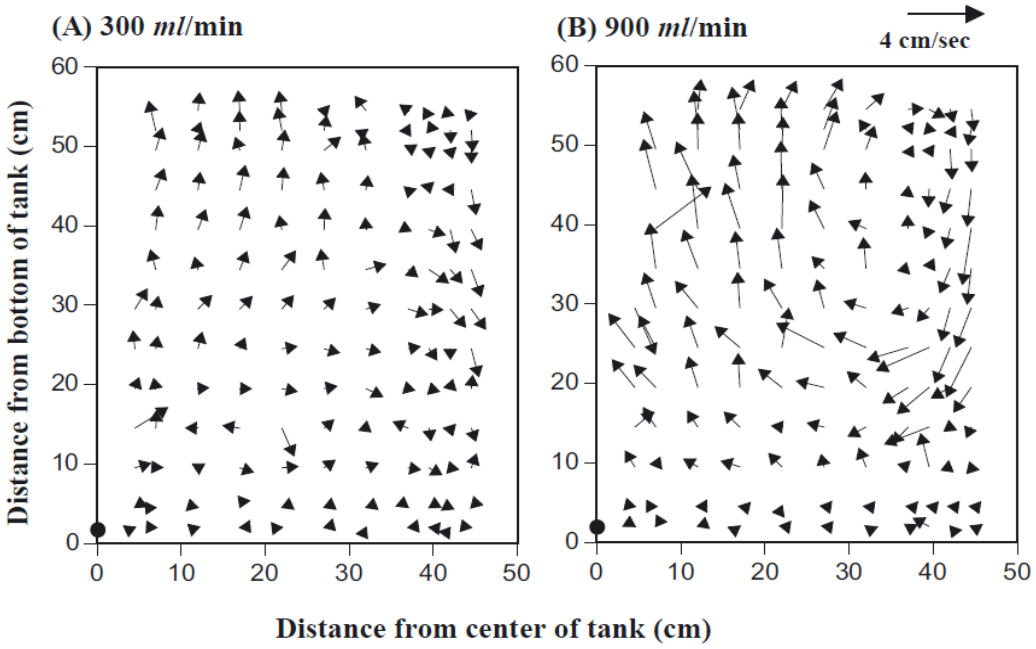

Fig. 1.1. Half of a horizontal, cross sectional view of mean flow velocity in 500 *l* larval rearing tanks at air flow rates of (A) 300 and (B) 900 *ml*/min. The arrows indicate mean flow velocity (length) and direction for a point in the rearing tank. The black circle indicates the location of the aerator at the bottom centre of the tank. The arrow at the top of the figure indicates the speed scale.

Vertical flows were generated as bubbles from the aerator at the centre of the tank bottom rose, dragging water upwards until the bubbles burst at the surface. At the surface, the flow direction changed from vertical to horizontal. Water flowed horizontally across the surface to the side of the tank, and then flowed down the side wall. Upon reaching the bottom, the flow moved towards the centre of the tank, completing the circulatory pattern. Circulation velocity increased and the thickness of the boundary layer between the turbulent zone and the bottom of the tank decreased at

higher air supply rate (Fig. 1.1). At 300 *ml*/min, the boundary layer was 30–40 cm above the bottom, whereas at 900 *ml*/min the boundary layer was 20–30 cm above the bottom.

Larval sinking velocity

The sinking velocity of larvae increased as the larvae grew (from 0.12 ± 0.05 cm/s 1 dph to 0.20 ± 0.04 cm/s 3 dph) (Fig. 1.2).

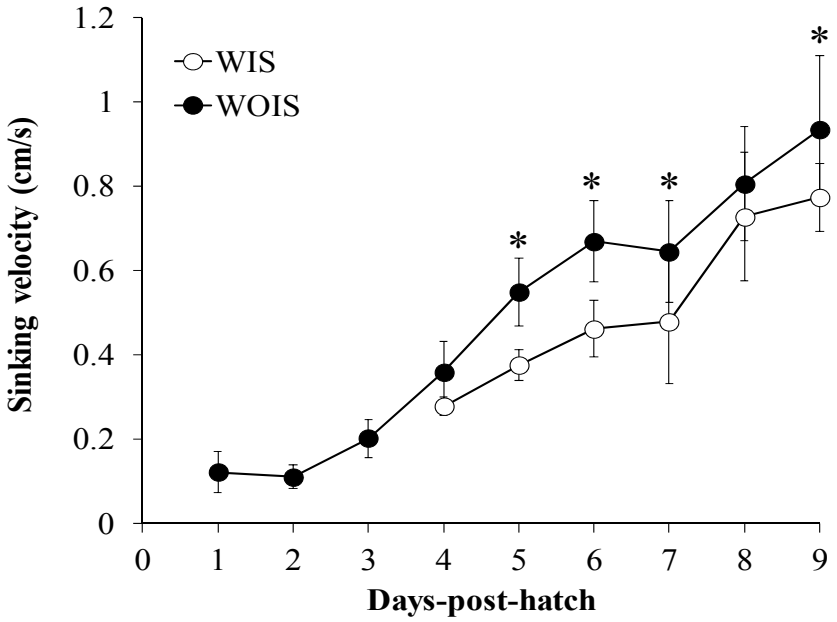

Fig. 1.2. Average sinking velocities (cm/s) of Pacific bluefin tuna, *Thunnus orientalis* larvae with (WIS: white circles) or without (WOIS: black circles) inflated swimbladder during Trial 2 between 1-9 days–post-hatch. Vertical bars indicate standard deviations. Asterisks indicate significant differences between WIS and WOIS according to age.

At 4 dph, some larvae were found to have inflated swimbladders; their mean sinking velocity was 0.28 ± 0.02 cm/s, whereas the WOIS had a mean sinking velocity

of 0.36 ± 0.07 cm/s (Fig. 1.2). Approximately 6.7 % of larvae completed swimbladder inflation at 4 dph (Fig. 1.3). From 5 dph, the sinking velocities of WOIS were significantly higher than those of WIS. At 9 dph, the sinking velocity was 0.77 ± 0.16 cm/s for WIS and 0.93 ± 0.14 cm/s for WOIS (Fig. 1.2), with the former accounting for 20% of the total number of larvae (Fig.1.3).

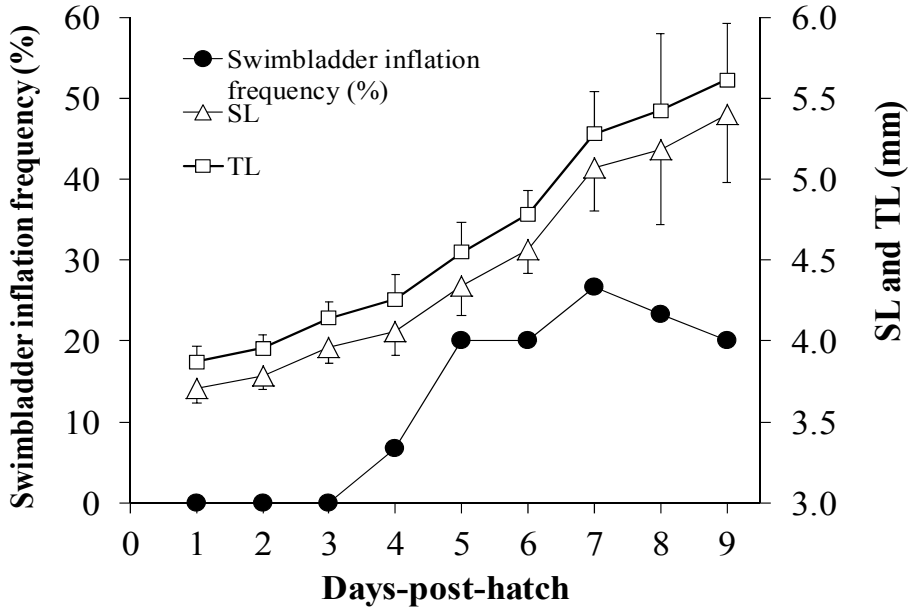

Fig. 1.3. Swimbladder inflation frequency, standard length (SL) and total length (TL) (*n*=30) in Pacific bluefin tuna, *Thunnus orientalis,* larvae during larval sinking velocity measurements between 4–9 days-post-hatch.

Estimated danger zone

At all air supply rates, the area and the percentages of the EDZ increased with larval growth, additionally, the area and the percentages of the EDZ of WIS tended to be smaller than that of WOIS (Fig. 1.4, 1.5, 1.6). For all dph, the area and the percentages of the EDZ at 300 *ml*/min air supply rate were much larger than those at 900 *ml*/min (Fig. 1.4, 1.5, 1.6).

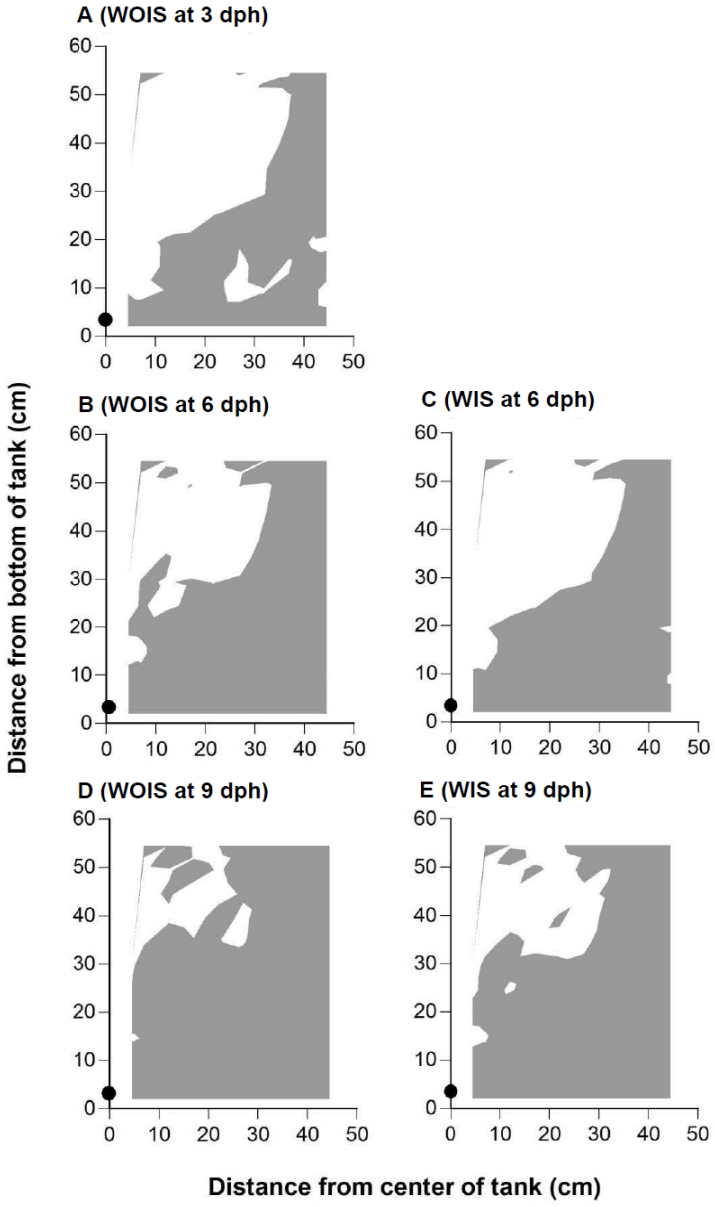

Fig. 1.4. Half of a horizontal, cross sectional view of the estimated danger zone (EDZ) at 3, 6, and 9 days-post-hatch (dph) at an air supply rate of 300 *ml*/min. A: Pacific bluefin tuna, *Thunnus orientalis*, larvae without inflated swimbladders (WOIS) at 3 dph; B: WOIS at 6 dph; C, larvae with inflated swimbladders (WIS) at 6 dph; D, WOIS at 9 dph; E, WIS at 9 dph. Grey areas indicate the EDZ, where larval sinking velocity on each dph was higher than water upward flow velocity. The black circle at the bottom centre indicates the location of the aerator.

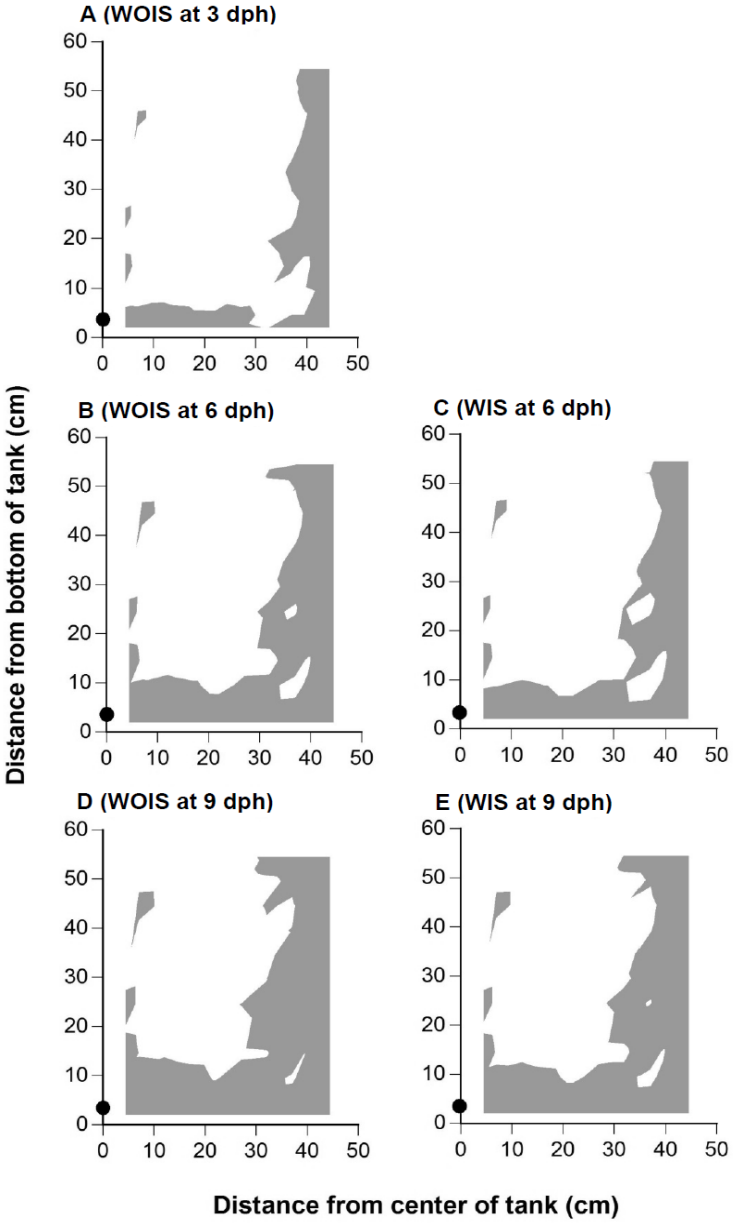

Fig. 1.5. Half of a horizontal, cross sectional view of the estimated danger zone (EDZ) at 3, 6, and 9 days-post-hatch (dph) at an air supply of 900ml/min. A: Pacific bluefin tuna, *Thunnus orientalis* larvae without inflated swimbladders (WOIS) at 3 dph; B, WOIS at 6 dph; C, larvae with inflated swimbladders (WIS) at 6 dph; D,WOIS at 9 dph; E,SB at 9 dph. Grey areas indicate the EDZ, where larval sinking velocity at each dph was higher than water upward flow velocity. The black circle at the bottom centre indicates an aerator.

The depth of the EDZ above the tank bottom on 9 dph was 30–40 cm from the tank bottom at 300 *ml*/min and 10–20 cm from the tank bottom at 900 *ml*/min (Figs. 1.4, 1.5). There was a significant difference in larval survival between 0 and 300 or 900 *ml*/min in Trial 1, but not in Trial 2 (Scheffe's test, $P < 0.05$).

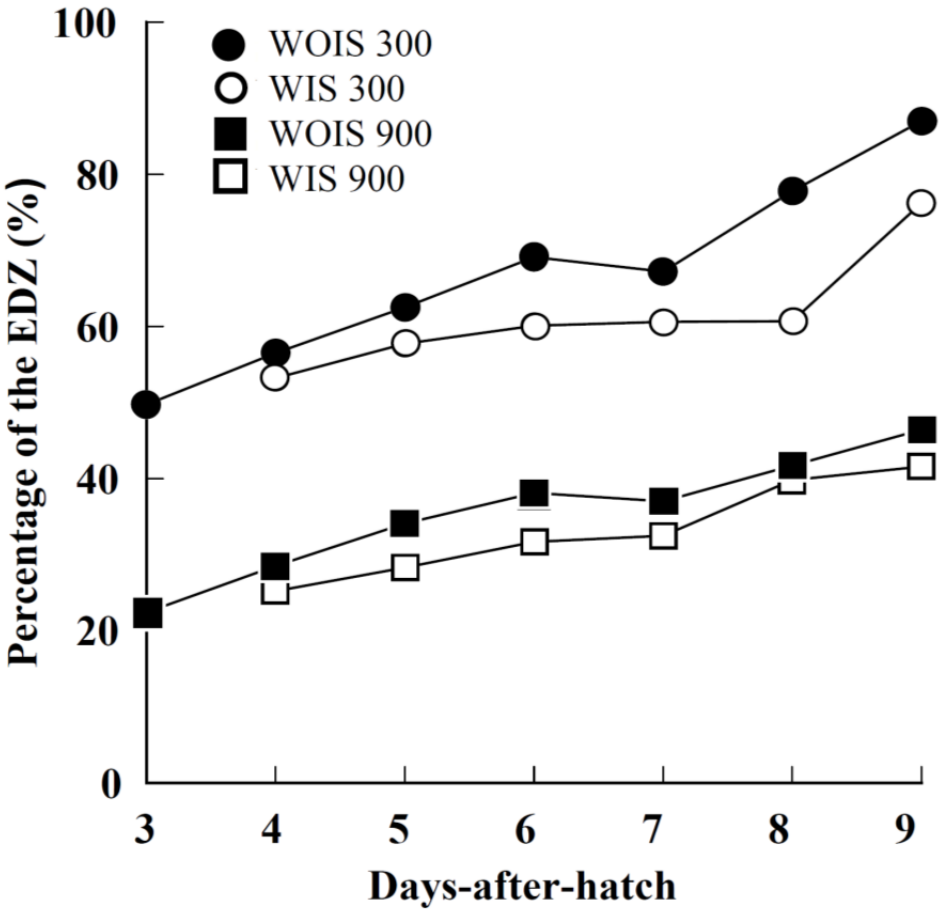

Fig. 1.6. Percentage of the estimated danger zone (EDZ) relative to the area of the vertical tank section under different nighttime air supply rate conditions: Pacific bluefin tuna, *Thunnus orientalis* larvae with inflated (WIS 300, open circle) and without inflated swimbladders (WOIS 300, closed circle) at 300 *ml*/min; larvae with inflated (WIS 900, open square) and without inflated swimbladders (WOIS 900, closed square) at 900 *ml*/min.

1.4 Discussion

This study demonstrated that the air supply rate during the nighttime plays a role in preventing sinking death among PBT larvae. Larval survival in both trials increased with increasing air supply rate during the nighttime (Table 1.2), although there were a few statistical discrepancies between the trials. These discrepancies may have been caused by differences in the quality of eggs or by small variations in environmental conditions, such as water temperature and dissolved oxygen concentration (Table 1.1). Despite these differences, it was concluded that both experiments demonstrated a clear relationship between PBT larval survival and characteristics of the flow field in the rearing tank.

These results are also consistent with larval survival reported for other aquaculture fish species. Kayaba (2003) reported that aeration reduced mortality in barfin flounder *Verasper moseri* larvae at early stage settlement. Similarly, larval sinking and survival in seven-band grouper *Epinephelus septemfasciatus* is related to aerator distribution and air supply rate (Shiotani et al. 2003), and to the pattern of circulation in the rearing tank (Sakakura et al. 2006). Teruya et al. (2009) quantified the flow field that prevented the sinking of amberjack larvae, and Tanaka et al. (2009) demonstrated that the number of sunken PBT larvae was decreased by vertical turbulent mixing of the rearing water through aeration.

It was surmised that higher circulation rates might retain larvae within the water column, thus reducing mortality. This may be a result of the balance between downward larval sinking and upward flow velocities. If this hypothesis is valid, it may be possible to describe typical larval movement based on flow velocity in the tank and

larval sinking speed in the following way. In the early stages of development, fish larvae are generally unable to maintain a single position within a water column under normal rearing conditions. Swimming ability increases as larvae grow and PBT larvae swim actively during the day (Kawamura 2003). However, larvae are less active at night and, consequently, sink (Sakamoto 2005; Takashi et al. 2006).

Larvae appear to be less active at night during early developmental stages. As a result, it may be difficult for larvae that sink into the EDZ during the nighttime to re-enter the upper layers. Because increased air supply rates reduce the depth of the EDZ, the risk of larvae sinking into the EDZ might also decrease. Further, higher air supply rates produce stronger horizontal flow, which may move larvae towards the centre of the tank and, thus, back into circulation.

Takashi et al. (2006) have shown that larvae larger than 4.6mm TL have the ability to change their density by swimbladder inflation; however, larval body density is greater than that of rearing water even in WIS. In this study, the sinking velocities of WOIS were significantly higher than those of WIS from 5 dph (4.6 mm TL), and this result are consistent with those of Takashi et al. (2006). Therefore, it follows that WOIS will sink faster, and will have greater risk of sinking death than WIS.

There was a significant difference in larval survival between 300 and 900 *ml*/min in Trial 1, but not in Trial 2 (Scheffe's test, $P < 0.05$). In Trial 2, the higher swimbladder inflation frequency was observed (Table 1.2). Additionally, in this study, the sinking velocities of WOIS were significantly higher than those of WIS from 5 dph. Therefore, the higher swimbladder inflation frequency perhaps contributed to higher larval survival under lower flow conditions (300 *ml*/min) in Trial 2. Although other factors are likely to affect larval survival, it is clear that increasing the air supply rate

during the nighttime increases water circulation, reduces the size of the EDZ (Figs. 1.4, 1.5, 1.6), and enhances PBT larval survival. These results provide insight into the relationship between PBT larval survival and flow field characteristics, as well as techniques to prevent larval sinking death in mass production tanks. Further studies should be examined on flow field and the means of controlling flow field in larger-scale tanks to improve larval survival in mass production of PBT fingerlings.

Chapter 2

Influence of initial swimbladder inflation (ISI) failure on survival and vertical distribution in Pacific bluefin tuna, *Thunnus orientalis,* larvae

2.1 Introduction

Chapter 1 demonstrated that increased air supply rate to rearing water during the nighttime mitigate larval mortality due to sinking death in PBT larviculture. However, larval survival was still low in the experimental and especially in mass-scale PBT larviculture even when nighttime aeration is increased (Sawada 2012). Therefore, further investigation is required in order to develop a method of preventing these deaths more effectively.

PBT larval body density is greater than that of rearing water, even when larvae possess an inflated swimbladder, and is believed to be the primary cause of sinking death during the night-time on ceasing swimming (Takashi et al. 2006). On the other hand, swimbladder is an important organ which controls buoyancy by reducing fish body density relative to that of surrounding water (Magnuson 1978; Itazawa 1991; Alexander 1993). Body density of the larvae with inflated swimbladder (WIS) is lower than that of the larvae without inflated swimbladder (WOIS) at every ontogenetic stage in PBT (Takashi et al. 2006) as reported in striped trumpeter, *Latris lineata* (Bloch and Schneider), (Trotter et al. 2005b). Additionally, Chapter 1 demonstrated that WOIS sink faster than WIS, and suggested that WOIS have a greater risk of sinking death than WIS in PBT larvae even when the flow control to prevent the sinking death is employed during the nighttime. Therefore, success of ISI during the larval stage and subsequent

normal swimbladder development is considered to be important biological factors capable of contributing to larval survival improvement by prevention of sinking death via control of body density. Indeed, poor survivals in cohorts with low swimbladder inflation frequency have often been observed in PBT mass-scale larviculture in our hatchery. In addition, for all the above mentioned species with the exception of barfin flounder, a species which lacks a swimbladder, the relationship between larval swimbladder inflation and vertical distribution within rearing tanks has not yet been investigated.

In this study, the ability of two methods (Experiment 1, Experiment 2) to prevent mass mortality caused by sinking death were evaluated in order to develop more effective rearing methods for use in mass-scale larviculture. In Experiment 1, the influence of ISI failure on survival in early larval stage PBT in a mass-scale tank was examined, while in Experiment 2, whether WOIS actually had a greater tendency to sink than WIS in experimental and mass-scale tanks was determined through the examination of larval vertical distribution. In this study, the influence of ISI failure on larval growth was also examined.

2.2 Materials and methods

Eggs and larvae

Fertilized PBT eggs were obtained from the spontaneous spawning of cultivated PBT brood stock fish in a sea net-cage at the FLKU. Eggs were introduced into experimental tanks and incubated at 24°C prior to hatching. Hatched larvae were reared at 25.0°C prior to commencement of feeding at 2 days-post-hatch (dph). To

prevent larval surface death, approximately 1.0 to 1.5 *ml* of feed oil (Fish oil for fish feed: Nice Feed Oil DA-22; Ueda Oils and Fats Mfg Co. Ltd., Kobe, Japan) was deposited onto the surface of rearing water daily from the time of hatching (0 dph) up until 2 dph in order to form a surface film according to the ordinary PBT larviculture procedure in our laboratory.

Experiment 1

Experiment 1 was conducted in order to elucidate the influence of ISI failure on survival in early larval stage PBT within a mass-scale tank. During Experiment 1, two trials (Trial 1 and Trial 2), which differed with respect to tank volume and shape, were carried out using different batches of fertilized eggs at the Ohshima Hatchery, Fish Nursery Center, Kinki University.

Eggs were introduced into quadrangular concrete 20 *kl* tanks (4.5 m × 4.5 m × 1.0 m depth) in Trial 1, and circular fiberglass 30 *kl* tanks (6.0 m internal diameter, 1.1 m depth) in Trial 2 at a density of 5000 eggs/*kl*. Resulting larvae were subjected to rearing experiments within those tanks beginning 2 dph until the end of the experiment (9 dph in Trial 1, 8 dph in Trial 2).

PBT larvae were reared using the following two types of treatment during both trials: one treatment in which ISI was promoted by removing the surface film on rearing water using a surface skimmer (Fig. 2.1) from the period 3 dph until the end of the experiment (PS group) and another treatment lacking ISI promotion by allowing the surface film formation on rearing water without employing the surface skimmer from 3 dph to the end of the experiment (NPS group). Swimbladder inflation frequency and larval survival were compared between PS and NPS groups.

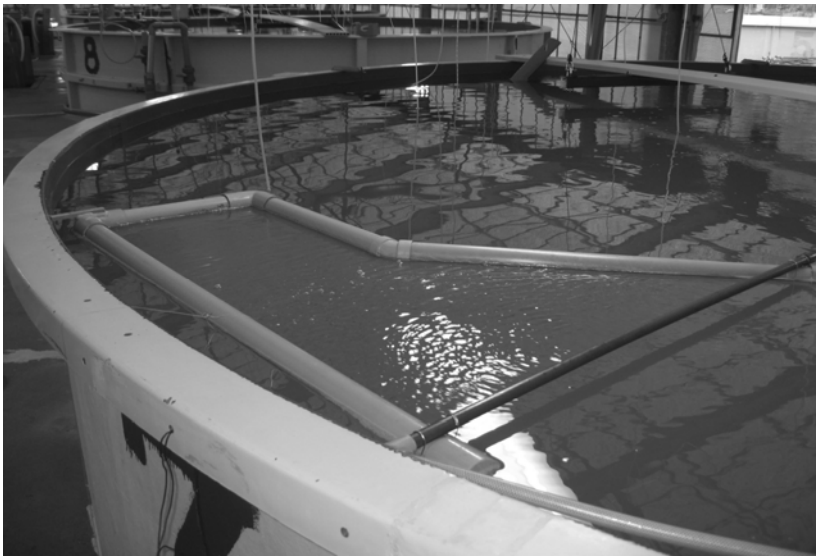

Fig. 2.1. Surface skimmer for the surface film removal on rearing water used in Experiment 1.

In Trial 1, two iterations with two replicates per treatment were carried out (n = 4) using different batches of fertilized eggs. Hatching rates of each fertilized eggs used in each iteration were 93.6% and 93.9% respectively. In Trial 2, a single iteration with two replicates per treatment was carried out (n = 2). Hatching rates of fertilized eggs used in Trial 2 was 93.2%.

Larvae were fed S-type rotifers *Brachionus plicatilis* sp. complex (Hagiwara et al. 2007), enriched for 18 h with *Nannochloropsis oculata* of 1.5 × 10^7 cells/*ml*, a commercial product (Marine Glos EX, Nisshin Marinetech Co. Ltd., Yokohama, Japan), and taurine (Japan Nutrition Co., Ltd., Tokyo, Japan) of 0.4 g/*l*, from 2 dph onwards, and were reared under a natural photoperiod (14 h light period: 5:00 to 19:00). In addition to the *N. oculata* used in enrichment of rotifer, it was added to the rearing water from 2 dph to the end of experiment.

Aeration was achieved using four air stones with air-flow rates of 1.7 *l*/min/air stone$^-$ placed on tank bottom at the center of each tank bottom in both trials. At night

(19:00 to 5:00), 8 and 12 additional air stones with air-flow rates of 3.0 *l*/min/air stone were placed evenly at the bottom of tanks in trial 1 and 2, respectively, from 2 dph until the end of the experiment in order to prevent sinking deaths resulting in mass mortality (Tanaka et al. 2009).

Other rearing conditions employed during Experiment 1 were as follows; Trial 1: temperature, 26.5 ± 0.1°C; dissolved oxygen, 101 ± 3.5%; salinity, 31.9 ± 0.4; pH, 7. 8 ± 0.2, and Trial 2: temperature, 26.5 ± 0.1°C; dissolved oxygen, 105 ± 3.1%; salinity, 32.1 ± 0.5; pH, 7.9 ± 0.3.

Experiment 2

Experiment 2 examined the relationship between vertical larval distribution and swimbladder inflation frequency in order to determine whether WOIS have a stronger tendency to sink to tank bottom than WIS.

Swimbladder inflation frequency and distributional density of larvae within sampling water were compared among larvae sampled within the upper (Upper larvae) and middle layers of experimental tanks (Middle larvae) as well as larvae sampled at tank bottom (Bottom larvae). Examinations were taken within three circular fiberglass 1.6 *kl* tanks (1.4 m internal diameter, 1.0 m water depth) and a single circular fiberglass 30 *kl* tank (6.0 m internal diameter, 1.1 m water depth) at night (21:00) 5 dph, when density of sunken larvae at tank bottom was highest (Tanaka et al.2009).

Within three circular fiberglass 1.6 *kl* tanks, larvae were sampled using a siphon tube made of soft PVC hose and PVC pipe (13 mm inner diameter). Upper and Middle larvae were sampled along a tank diameter within the upper (approximate depth of 15 cm) and middle (approximate depth of 55 cm) layers of the rearing water column,

respectively. Bottom larvae were sampled along a line representing the diameter of the bottom face of the tank. In order to eliminate rearing water flow, aeration and sea water in letting were stopped 30 min prior to sampling within each of the three replicate tanks.

Within a 30 *kl* tank, larval sampling was conducted without elimination of the flow of rearing water under continuous aeration and sea water inletting. Sampling methodology was otherwise consistent with that used in the sampling of larvae within 1.6 *kl* tanks.

Eggs were introduced into three 1.6 *kl* tanks (n = 3) and a single 30 *kl* tank at a density of 5000 eggs/*kl*. Resulting larvae were reared prior to sampling at 5 dph. Rearing methodology used was the same as that used for the PS group in Experiment 1 with the exceptions being the aeration methods employed within 1.6 *kl* tanks and the running of a surface skimmer in both 1.6 *kl* and 30 *kl* tanks, both of which are outlined below.

In 1.6 *kl* tanks, aeration was provided by air stones with air-flow rates of 150 *ml*/min located at the center of each tank's bottom. Additionally, an air stone with an air-flow rate of 1300 *ml*/min was added near the center of each tank every night from 2 dph through to 5 dph in order to mitigate sinking death fatalities. A surface skimmer was employed from 3 dph through to 5 dph in order to promote ISI; however, it was used with restraint in order to generate cohorts consisting of WIS and WOIS for the experiment.

Other rearing conditions employed during Experiment 2 were as follows; 1.6 *kl* tanks: temperature, 26.6 ± 0.1°C; dissolved oxygen, 96.3 ± 4.2%; salinity, 32.3 ± 0.4; pH, 7.7 ± 0.1; 30 *kl* tank: temperature, 26.8 ± 0.2°C; dissolved oxygen, 104.3 ± 3.7%; salinity, 31.3 ± 0.1; pH, 7.9 ± 0.2.

Measurements and observations

Experiment 1

Thirty larvae were sampled in order to determine swimbladder inflation frequency. Larval sampling was conducted at night (21:00–22:00) because PBT larvae deflate the swimbladder in daytime (Takashi et al. 2006) and this makes it difficult to determine the correct swimbladder inflation frequency. Fifteen and twenty larvae were also sampled during Trials 1 and 2, respectively, in order to measure standard length (SL: length from the rostral tip to the end of the notochord) within each tank at 6 and 9 dph (Trial 1), and at 5 and 8 dph (Trial 2). Larval swimbladder inflation was determined through observation under a stereomicroscope. SL was measured using digital images of samples taken with a digital camera (Moticam 2000, Shimadzu Rika Corp., Tokyo, Japan) and processed using a software package developed for image analysis (Motic Images Plus 2.2s, Shimadzu Rika Corp., Tokyo, Japan).

Survival at 9 dph (Trial 1) and 8 dph (Trial 2) was estimated using the volumetric method (Abdo-de la Parra et al. 2010; Yoseda et al. 2008) with a handcrafted PVC columnar sampler (105 mm in diameter, 1200 mm in length). Larvae were sampled with the surrounding water at three points of each rearing tanks with enhanced aeration at night (21:00–22:00) and counted within sampled water. Survival rate was calculated using the following equation:

$$\text{Survival rate (\%)} = 100 \times Ls / Ws \times Wt / Li$$

where Ls represented the number of larvae sampled by the columnar sampler, Ws represented the total volume of sampled water, Li represented the number of larvae at

the commencement of the rearing experiment, and *Wt* represented the volume of rearing water within the tank. In the present study, *Li* was obtained using the number of introduced eggs and the hatching rate mentioned previously.

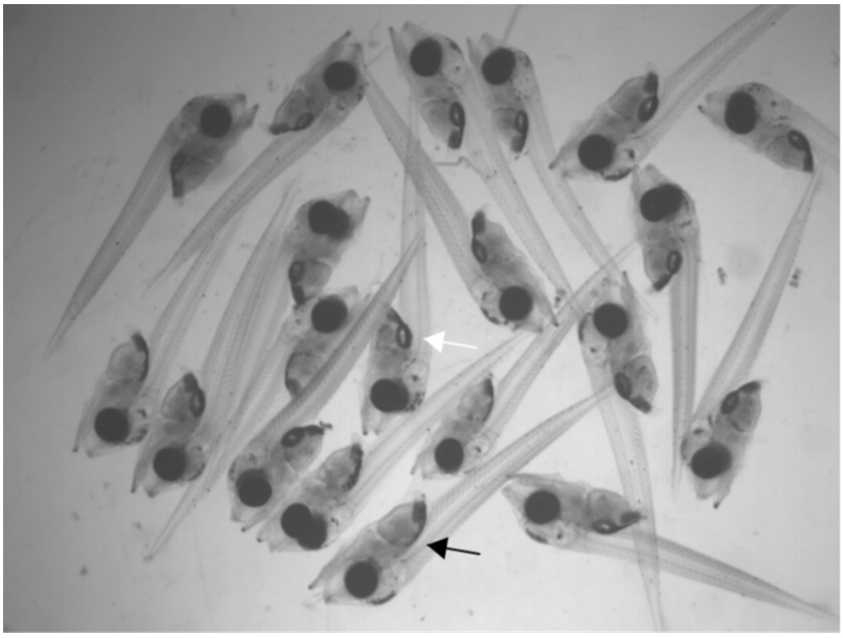

Fig. 2.2. Larvae sampled at night on 6 days-post-hatch. White and black arrows show the larvae with and without inflated swimbladder respectively.

Experiment 2

Twenty larvae were determined for swimbladder inflation frequency, and 10 larvae were measured SL for Upper, Middle and Bottom larvae in each1.6 and 30 *kl* tank. Observation of swimbladder inflation and SL measurement were carried out using the methodology outlined for Experiment 1.

Statistical analysis

A Welch's *t*-test and a Wilcoxon–Mann–Whitney test were employed in order to test for significance of differences in survival and swimbladder inflation frequency between PS and NPS groups in Experiment 1.

A Tukey–Kramer test was used to test for significance in the differences of swimbladder inflation frequency among Upper, Middle and Bottom larvae, and a Steel–Dwass test was used to test for significance in the differences of larval distributional density of Upper, Middle, and Bottom larvae within sampling water in Experiment 2. The Pearson product moment correlation coefficient was used in order to measure the strength of the relationship between swimbladder inflation frequency and survival in Experiment 1. Statistical analyses were performed using statistical software (Kyplot 5.0 for Windows, KyensLab, Tokyo, Japan). In the present study, $P < 0.05$ was used as the criteria of significant difference in these tests.

2.3 Results

Experiment 1

In Trial 1, larval SL was significantly greater in the PS group than it was in the NPS group at 6 ($P = 0.017$, $n = 60$) and 9 dph ($P = 0.009$, $n = 60$). Swimbladder inflation frequency was 97% and 99% in the PS group at 6 and 9 dph, respectively. Results from the NPS group were significantly lower at both 6 (22%, $P = 0.002$, $n = 4$) and 9 dph (19%, $P = 0.001$, $n = 4$; Table 2.1). Survival rate at the end of experiment was significantly higher in the PS group than in the NPS group ($P = 0.013$, $n = 4$; Table 2.1).

Table 2.1. Swimbladder inflation frequency, growth rate and survival of Pacific bluefin tuna, *Thunnus orientalis*, larvae in each experimental group within 20 *kl* tanks (Experiment 1, Trial 1)

Group	Swimbladder inflation frequency (%)		Standard length (mm)		Survival (%)
	6 dph	9 dph	6 dph	9 dph	9 dph
Promoted ISI (PS)	96.8 ± 3.7^{a}	99.2 ± 1.7^{a}	5.3 ± 0.3^{a}	6.2 ± 0.4^{a}	26.0 ± 9.1^{a}
No promoted ISI (NPS)	22.0 ± 16.6^{b}	19.1 ± 11.5^{b}	5.1 ± 0.3^{b}	6.1 ± 0.3^{b}	8.3 ± 4.7^{b}

Values of swimbladder inflation frequency and survival rate are presented as means with standard deviation ($n = 4$).

Values of standard length are presented as means with standard deviation ($n = 60$). Different lower case letters indicate significant differences within each days-post-hatch (dph, $P < 0.05$).

Table 2.2. Swimbladder inflation frequency, growth rate and survival of Pacific bluefin tuna, *Thunnus orientalis*, larvae in each experimental groups within 30 *kl* tanks (Experiment 1, Trial 2)

Group	Swimbladder inflation frequency (%)		Standard length (mm)		Survival (%)
	5 dph	8 dph	5 dph	8 dph	8 dph
Promoted ISI (PS)	100.0	96.7	4.7 ± 0.2	6.0 ± 0.4^{a}	19.2
	100.0	100.0			21.5
No promoted ISI (NPS)	26.7	33.3	4.7 ± 0.2	5.7 ± 0.4^{b}	1.6
	16.7	26.7			0.0

Values of swimbladder inflation frequency and survival rate presented here were obtained from each tank ($n = 2$) and represent PS and NPS groups.

Values of standard length are presented as means with standard deviation ($n = 40$).

Different lower case letters indicate significant differences within each days-post-hatch (dph) in standard lengths ($P < 0.05$).

In Trial 2, SL was significantly greater in the PS group than it was in the NPS group at 8 dph ($P = 0.001$, $n = 40$), while no significant difference in SL was found between the two groups at 5 dph (Table 2.2). Swimbladder inflation frequency at 6 and

9 dph, and the survival rate at 9 dph were higher in the PS group than they were in the NPS group (Table 2.2).

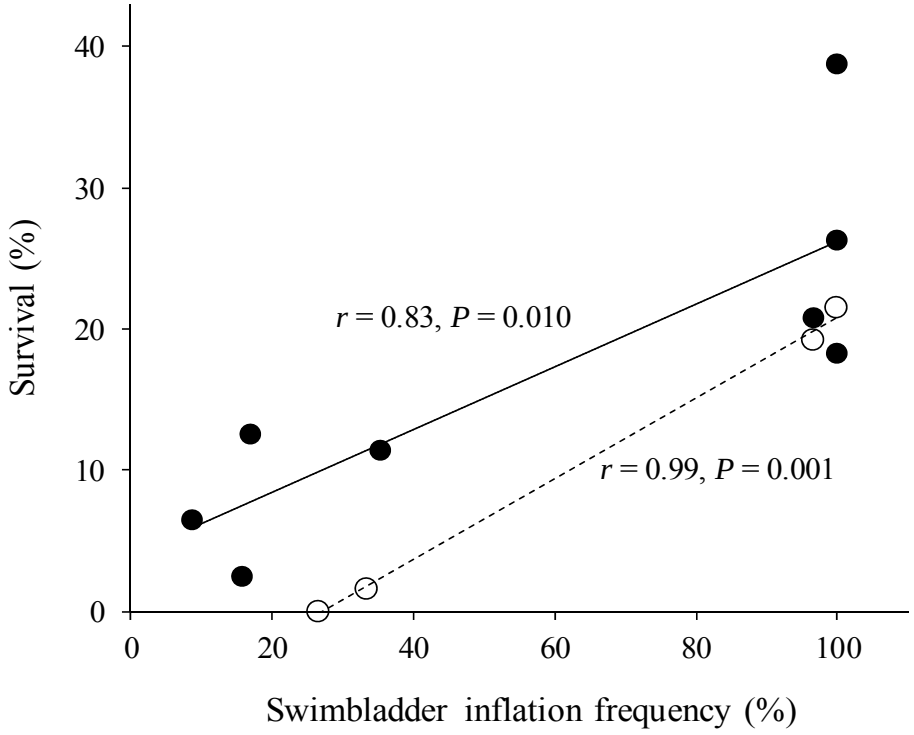

Fig. 2.3. Relationship between survival and swim bladder inflation frequency in Pacific bluefin tuna, *Thunnus orientalis*, larvae within 20 *kl* tanks (Trial 1) and 30 *kl* tanks (Trial 2). Black circles along with a solid regression line, and white circles along with a dashed regression line show the relationship between survival and swimbladder inflation frequency at 9 dph in 20 *kl* tanks (Trial 1, n = 8) and at 8 dph in 30 *kl* tanks (Trial 2, n = 4), respectively. r = Pearson's product moment correlation coefficient.

A strong significant correlation was found between swimbladder inflation frequency and survival at the end of each trial. Pearson's product moment correlation coefficient between swimbladder inflation frequency and survival was 0.83 (P = 0.01, n = 8) in Trial 1, and 0.99 in Trial 2 (P = 0.001, n = 4) (Fig. 2.3).

Experiment 2

Within the 1.6 *kl* tanks, larval density in the sampling water (ind./*l*) of Bottom larvae was greater than that of Upper and Middle larvae, although differences among larval groups were not statistically significant at night 5 dph (n = 3; Table 2.3).

Table 2.3. Swimbladder inflation frequency, larval density in sampling water, and standard length in Upper, Middle, and Bottom Pacific bluefin tuna, *Thunnus orientalis*, larvae at night in 5 days-post-hatch within 1.6 *kl* tanks and a 30 *kl* tank

	Swimbladder inflation frequency (%)		Larval density in sampling water (ind./*l*)		Standard length (mm)	
	1.6 *kl*	30 *kl*	1.6 *kl*	30 *kl*	1.6 *kl*	30 *kl*
Upper larvae	42.1 ± 9.7[a]	45.0	1.0 ± 0.4	0.8	4.4 ± 0.2	4.5 ± 0.3
Middle larvae	81.4 ± 10.8[b]	50.0	0.5 ± 0.2	0.6	4.6 ± 0.3	4.5 ± 0.3
Bottom larvae	7.0 ± 7.6[c]	26.3	7.8 ± 2.0	4.7	4.3 ± 0.4	4.3 ± 0.4

Upper and Middle larvae were sampled from the upper and middle layers of the rearing water column, respectively, while Bottom larvae were sampled at tank bottom.

Values of swimbladder inflation frequency and larval density in sampling water are presented as means with standard deviations for both 1.6 *kl* tanks (n = 3) and a values of those for a 30 *kl* tank (n = 1).

Different lower case letters indicate significant differences (P < 0.05).

Values of standard length are presented as means with standard deviations (n = 30 for 1.6 *kl* tanks and n = 10 for a 30 *kl* tank).

Swimbladder inflation frequency of Bottom larvae was significantly lower than that of Upper (P = 0.010, n = 3) and Middle larvae (P = 0.0002, n = 3), and swimbladder inflation frequency of Middle larvae was significant higher than that of Upper larvae (P = 0.005, n = 3; Table 2.3). Patterns of larval density and swimbladder inflation frequency at 5 dph within the 30 *kl* tank were similar to those observed in the 1.6 *kl* tanks (Table 2.3).

No significant differences were found in SL among larval groups within the 1.6 *kl* tanks and the 30 *kl* tank at 5 dph (Table 2.3).

2.4 Discussion

This study demonstrated that larval ISI failure reduces survival in PBT larviculture in mass production tanks, even when flow field was controlled by aeration that enhanced vertical circular current in order to prevent larval sinking. This study also indicated that swimbladder inflation frequency was significantly lower at night within larvae found at tank bottom than it was within larvae in both the upper and middle layers of the rearing water column. These results suggest that reduction of larval survival due to ISI failure caused by associated increases in the sinking death ratio of larvae exhibiting incomplete swimbladder inflation.

During weaning of sea bass, *Dicenrrarchus labrax* (Linnaeus), juveniles, selective mortality of individuals without inflated swimbladders has been observed, and a direct relationship between swimbladder inflation frequency and survival has been shown (Chatain 1989; Chatain and Dewavrin 1989). Failure of ISI during the larval stage has also been shown to reduce survival in both Black Sea turbot, *Scophthalmus maeoticus maeoticus* (Pallas), and zebrafish, *Danio rerio* (Hamilton) (Spectorova and Doroshev 1976; Goolish and Okutake 1999).

Although PBT larval body density is greater than that of rearing water even with inflated swimbladder, it has been demonstrated that body density of PBT larvae decreases along with swimbladder inflation at every ontogenetic stage (Takashi et al.

2006). Moreover, Chapter 1 demonstrated that WOIS sink faster than WIS, and suggested that WOIS have a greater risk of sinking death than WIS in PBT larvae even when the flow control to prevent the sinking death is employed during the nighttime. Therefore, ISI success during the larval stage and subsequent normal swimbladder development is seem to be crucial for improvement of PBT larval survival by prevention of sinking death via low body density.

On the other hand, Tanaka et al. (2009), who have demonstrated that distributional density of sunken larvae on tank bottom peaks at 5 dph, had a converse view with ours about that the larval sinking is the ordinary event in the rearing tanks, not due to the developmental defect of swimbladder, because swimbladder volume was not correlated with larval body density due to its relatively small size at 5 dph, and the fact that this peak in distributional density coincided with the timing of the consumption of a yolk and oil globule that is known to have an effect on buoyancy. In Experiment 1 of this study, however, it was determined that survival and swimbladder inflation frequency at the end of the experiment in the NPS group were significantly lower than they were in the PS group within 20 *kl* tanks (Trial 1), and that a similar pattern was observed in swimbladder inflation and survival within 30 *kl* tanks (Trial 2). Moreover, a strong significant correlation was found between survival and swimbladder inflation frequency at the end of the experiment in both trials of this study.

Regarding on the relationship between larval simbladder inflation and their vertical distribution, in Experiment 2 of this study, larvae with low swimbladder inflation frequencies were found distributed along the tank bottom with high distributional density. This result suggests the presence of sunken larvae at tank bottom as shown by Tanaka et al. (2009). Moreover, this result demonstrates that WOIS indeed

have a stronger tendency to sink to tank bottom than WIS. Although body densities of the larvae sampled in Experiment 2 were not examine in this study, the greater body density and faster sinking velocity in WOIS than those in WIS described above would presumably lead to larval sinking to tank bottom in Experiment 2. The presence of sunken larvae at tank bottom with survival reduction was also observed in Black Sea turbot, and zebrafish, (Spectorova and Doroshev 1976; Goolish and Okutake 1999). These results imply that the three species mentioned above share sinking death due to ISI failure as common process of larval mortality.

Chapter 1 demonstrated that increased air supply rate to rearing water during the nighttime mitigate larval mortality due to sinking death in PBT larviculture. Tanaka et al. (2009) have also shown that distributional density of PBT sunken larvae at tank bottom was reduced significantly more during the night in a 50 *kl* mass-scale tank when flow was enhanced by both aeration and a water pump than it was when only a water pump was employed. These results indicate the effectiveness of flow control of rearing water in the prevention of PBT larval sinking. However, the results of Experiment 1 were obtained despite aeration being enhanced overnight through the use of 8 air stones in Trial 1, and 12 air stones in Trial 2 in mass-scale production tanks.

Based on the discussion above, it was concluded that ISI failure resulted in reduced larval survival via increases in sinking death ratio in mass-scale production tank, even if preventive measure of sinking death using flow control was employed.

Mass mortalities in PBT larviculture during the early larval stage have been considered to cause by both surface death and sinking death. Surface death can be effectively prevented by surface film such as oil film, while it is encouraged by surface film removal to promote ISI (Yamaoka et al. 2000; Kaji et al. 2003). In Experiment 1,

indeed, more larval surface death was observed in PS group with surface film removal than NPS group without surface film removal. Therefore, the mortality due to surface death was expected to be higher in PS group than in NPS group in Experiment 1.

However, the results of Experiment 1 showed higher survival in PS group than in NPS group. Although allocation mortality by sinking death and surface death remained unclear in Experiment1, the mortality due to sinking death via ISI failure would presumably surpassed the difference of mortality due to surface death between PS and NPS groups in Experiment 1. Other authors have same opinion that sinking death is more serious problem than surface death and largely affect survival in early stage larviculture (Sakamoto et al. 2005; Miyashita 2006; Tanaka et al. 2009).

In Experiment 1 of this study, swimbladder inflation frequency in the PS group reached a plateau of almost 100% at 6 dph in Trial 1 and 5 dph in Trial 2; however, swimbladder inflation frequency in the NPS group was approximately 22% at 6 and 5 dph in Trial 1and Trial 2 respectively. Tanaka et al. (2009) have demonstrated that the distributional density of PBT larvae settled at tank bottom is highest at around 5 dph. These results suggest that high swimbladder inflation frequency in the PS group at 6 (Trial 1) and 5 dph (Trial 2) contributed to increases in larval survival by preventing larval sinking. For PBT larvae, rapid increase in swimbladder inflation frequency towards 5 dph is likely to be important to effectively prevent larval mortality caused by sinking death.

Although further investigation is necessary to elucidate the effectiveness of flow control within mass-scale production tank on larval survival and selecting suitable tank shape in relation to ISI failure, this study suggested that larval survival should also be

improved by mitigation of sinking death through larval ISI success, without depending only on flow control, in mass-scale PBT larviculture.

In Experiment 1, larval SL was significantly larger in the PS group than it was in the NPS group at 6 and 9 dph in Trial 1, and at 8 dph in Trial 2; however, no difference was observed in SL between both experimental groups at 5 dph in Trial 2. In Experiment 2, no significant difference was found among SL in Upper, Middle and Bottom larvae at 5 dph within both the 1.6 *kl* and the30 *kl* tank. These results suggested that the larval growth retardation due to ISI failure appeared at 8–9 dph, although it was varied and not clear at 5 dph. Growth retardation in PBT larvae due to ISI failure may be apparent after 5 dph; however, these results should be confirmed through further investigation.

In conclusion, larval ISI failure reduces survival in PBT larviculture; therefore, more suitable method to promote larval ISI should be developed to improve larval PBT survival in the hatchery.

Chapter 3

Promotion of initial swimbladder inflation (ISI) in Pacific bluefin tuna, *Thunnus orientalis*, larvae

3.1: Conditions of water surface and optimal period to promote ISI in Pacific bluefin tuna, *Thunnus orientalis*, larvae

3.1.1 Introduction

Chapter 1 demonstrated that the larvae without inflated swimbladder (WOIS) sink faster than the larvae with inflated swimbladder (WIS), and suggested that WOIS have a greater risk of sinking death than WIS in PBT larvae even when the flow control to prevent the sinking death is applied. Additionally, Chapter 2 demonstrated that larval ISI failure indeed increase larval mortality due to sinking death via contributing larval sinking to tank bottom in PBT larviculture in mass production tanks. Moreover, the results of Chapter 2 suggested that ISI failure cause larval growth retardation. Therefore, the development of suitable method to promote larval ISI is considered to be crucial to improve larval PBT survival and growth in the hatchery.

Physoclistous fishes have physostomous swimbladder in the early larval stage. Such fish larvae (transient-physostomes) are considered unable to initially inflate their swimbladder if they cannot gulp atmospheric air by swimming up to the water surface covered by surface film originating from enriched oily live feeds, larval feces, and dead larvae in the practical production and by a liquid-paraffin-layer under experimental

condition (Kitajima et al. 1981, 1994; Chatain and Ounais-Guschemann1990; Trotter et al. 2005a).

In the practical larviculture of various aquaculture species, the surface skimmer has also been used to remove surface film for the promotion of ISI thereby making it possible for larvae to make contact with the air (Chatain and Ounais-Guschemann 1990; Battaglene et al. 1994; Moretti et al. 1999). Additionally, the period for air gulping to achieve ISI, i.e., the period when effective promotion of ISI is possible (so-called "window"), is finite and species specific (Bailey and Doroshov 1995; Friedmann and Shutty 1999; Trotter et al. 2005a; Kitajima et al. 1981). However, suitable promotional methods and the "window" for ISI have not yet been investigated in PBT larvae.

On the other hand, making oil film on the water surface is carried out in practical PBT larviculture to prevent surface death on an empirical basis (Munday et al. 2003). Therefore, If PBT larvae require the air gulping at water surface to achieve their ISI as other fish species, the promotion measure of ISI and the prevention measure of surface death by making the surface oil film will conflict each other in larviculture. To assess this conflict, detailed information is necessary on both the promotional and inhibitory conditions of water surface for ISI and identification of the "window" for ISI i.e., optimal period for the effective promotion of ISI.

Therefore, this study aimed to verify the promoting effect of surface film removal using surface skimmers and the inhibitory effect of the coverage of water surface with liquid-paraffin-layer or oil film on ISI (Experiment 1), and to elucidate the proper day of larval age to start skimming for promoting ISI with four different periods of oil film removal (Experiment 2), and to elucidate the essential period for ISI promotion with four different periods of oil film removal (Experiment 3). Moreover, the

effect of oil film removal on the incidence of surface death in the PBT larviculture was investigated in relation with the window for ISI in Experiment 2. In this study, the influence of ISI failure on larval growth was also examined continuing from Chapter 2.

3.1.2 Materials and methods

Larvae and larval rearing

The PBT larvae used in the present study hatched from the eggs spontaneously spawned by cultivated PBT bloodstock fish in a sea net-cage at the FLKU. The eggs were introduced into cylindrical fiberglass tanks (1.0 *kl*; 135 cm in internal diameter: Fig. 3.1.1) at density of 6000 eggs per tank, and incubated in 23°C until hatching. They had a high normal hatch rate of 94.8% and 93.0% both in Experiment 1 and Experiment 2. Hatched larvae were reared at 25.0°C until the commencement of feeding on 2 days-post-hatch (dph) and were subsequently subjected to the rearing experiment in the same tanks. The dph was defined to hatching day as 0 dph.

Fig. 3.1.1. Experimental tanks (1.0 *kl*) used in Experiment 1, 2 and 3.

The larvae were fed rotifers (*Brachionus plicatilis* sp. complex) enriched with a commercial product (Marine Glos, Nisshin Marinetech Co. Ltd., Yokohama, Japan) from 2 dph onwards, and were reared under natural and artificial fluorescent lighting (05:00–18:30; a 40 W lamp per tank). Air was supplied using an air stone with air-flow rate of 130 *ml*/min at the bottom centre of the rearing tanks. In this study, the aeration was increased to prevent sinking deaths which caused mass mortality at night (Sakamoto et al. 2005; Takashi et al. 2006; Ishibashi et al. 2009; Tanaka et al. 2009), i.e. an air stone with air-flow rate of 1200 *ml*/min was added (18:00–05:30) at the bottom centre of the rearing tanks from 2 dph to the end of the experiment. Other rearing conditions in the experimental period were as follows: salinity, 31.5–33.1 (Experiment 1), 30.2–31.7 (Experiment 2), 30.5–33.0 (Experiment 3); dissolved oxygen, > 89.6% (Experiment 1), >95.2% (Experiment 2), >98.1% (Experiment 3); pH, 7.9–8.3 (Experiment 1), 7.8–8.1 (Experiment 2), 7.8–8.3 (Experiment 3); temperature, 26.5 ± 0.1°C (Experiment 1), 26.5 ± 0.1°C (Experiment 2), 26.5 ± 0.1°C (Experiment 3).

Experimental design

Experiment 1

The PBT larvae were reared with the following four treatment: removing autogenous surface film formed during larval rearing with surface skimmer (SS group), covering the water surface with liquid-paraffin layer (LP group; HI-CALL K-300, Kaneda Co., Ltd., Tokyo, Japan) and oil film (OF group; Nice Feed Oil DA – 22; Ueda Oils and Fats Mfg Co. Ltd., Kobe, Japan), and non-treatment to the water surface (NT group) as a control. The rearing experiment was carried out in triplicates from 2 to 10 dph. The SS group was designed to verify the promoting effect of removing autogenous

surface film from the rearing water on ISI. The LP group was designed to verify an inhibitory effect on ISI by the prevention of larval air gulping at the water surface. The OF group was designed to compare the inhibitory effect of oil film, which is applied to prevent surface death, with LP group on ISI. In the LP group, the water surface was covered with a 4 mm thickness liquid-paraffin-layer which maintained its thickness during the experiment. The oil film was formed with feed oil that was dropped several times onto the water surface using a pipette, between 08:00 and 18:00 every day and the total volume of the feed oil dropped per day was 0.3 *ml*. The surface skimmer worked from 08:00 to 18:00. The autogenous surface film accumulated in the surface skimmer was removed several times per day.

Experiment 2

Experiment 2 was conducted to elucidate proper day of larval age to start skimming for promoting ISI with four different periods with surface oil film removal. The examined period was from 3 dph and later days because PBT larvae with the inflated swimbladder were not found on 2 dph in the result of Experiment1. The PBT larvae were reared with the following four treatments differing by the commencement dphs of surface skimmer usage to remove artificially formed oil film: from 3 to 8 dph (SF3D group); from 4 to 8 dph (SF4D group); from5 to 8 dph (SF5D group); from 6 to 8 dph (SF6D group). The rearing experiment was carried out in triplicates from 2 to 8 dph. The surface of the water in each treatment tank was sealed with oil film from 0 dph until the use of surface skimmer to prevent surface death according to the ordinary PBT larviculture procedure in our laboratory. The oil film was formed with feed oil that was dropped several times onto the water surface using a pipette, from 08:00 to 18:00 every

day; the total volume of the feed oil dropped per day was 0.3 *ml*. The surface skimmer worked from 08:00 to 18:00 in each treatment period. The oil film accumulated in surface skimmer was removed several times per day.

Experiment 3

Experiment 3 was conducted to elucidate the essential period for ISI promotion continuing from Experiment 2. The PBT larvae were reared with the following four different periods of removing the artificially formed surface oil film using surface skimmer: one day of 3 dph (SF3 group), from 3 to 4 dph (SF3–4 group), from 3 to 5 dph (SF3–5 group) and from 3 to 8 dph (SF3–8 group). The rearing experiment was carried out in triplicates from 2 to 8 dph. The water surface in each treatment tank was sealed with oil film from 0 to 8 dph except for the period with removing the surface oil film. The method of oil film formation on the water surface was the same as the previous experiments.

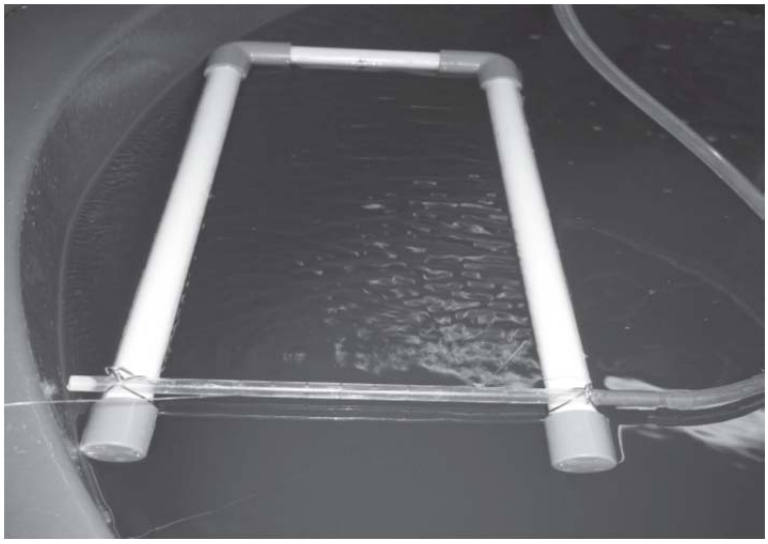

Fig. 3.1.2. The handcrafted surface skimmer which consisted of a rectangular floating trap and an air blower used in Experiment 1 and 2.

The surface skimmer used in this study was handcrafted and consisted of a rectangular floating trap (20 cm × 45 cm) and an air blower (Fig. 3.1.2).

Measurements and observations

The larvae (30–40 individuals) were examined to determine the swimbladder inflation frequency and 15 larvae were measured standard length (SL: length from the rostral tip to the end of the notochord) with the observation of their swimbladder inflation on 2, 4, 6, 8, 10 dph. Larval sampling was conducted at night (21:00–22:00) because the swimbladder of the PBT larvae inflates at a greater volume at night compared with during the day (Takashi et al. 2006), which makes it easier for obtaining the rate of WIS. The observation of swimbladder inflation was conducted under a stereomicroscope. SL was measured in digital images of samples taken by a digital camera (Moticam 2000, Shimadzu Rika Corp., Tokyo, Japan) using a software package for image analysis (Motic Images Plus 2.2 s, Shimadzu Rika Corp., Tokyo, Japan). In Experiment 1, the survival rate was estimated on 10 dph using a handcrafted PVC columnar sampler (105 mm in diameter, 750 mm in length). The larvae were sampled with the surrounding water at 3 points of each rearing tank at night (21:00–22:00), and the total number was counted. The strong aeration in night and caesura of swimming of PBT larvae during nighttime (Takashi et al. 2006; Tanaka et al. 2009) could provide random distribution of larvae and then enable a unbiased estimate of larval survival using such type of columnar sampler (Yoseda et al. 2008; Abdo-de la Parra et al. 2010).

The survival rate was calculated using the following equation.

Survival rate (%) = 100 × Ls/Ws/Li × Wt.

where, Ls: the number of larvae sampled using the columnar sampler, Ws: the volume of the surrounding water sampled together using the columnar sampler, Li: is the number of the larvae at the commencement of the rearing experiment, and Wt: the volume of rearing water in the tank (1000 *l*).

In the present study, estimated Li was 5700, which was obtained from the number of the introduced eggs (6000 eggs) and the hatch rate (94.8%) mentioned above. In Experiment 2, at the end of the experiment (8 dph), all surviving larvae were fixed in 5% formalin solution and the counted numbers recorded. In the evaluation of survival, the initial number of larvae was estimated 5600 in each tank according to the number of eggs (6000) and the hatch rate of 93.0%.

The larvae trapped in the surface of the rearing water and the surface skimmer were taken up and counted as the surface death larvae (Fig. 3.1.3) at 10:30, 13:00, 15:30 and 18:00 every day from 2 to 8 dph.

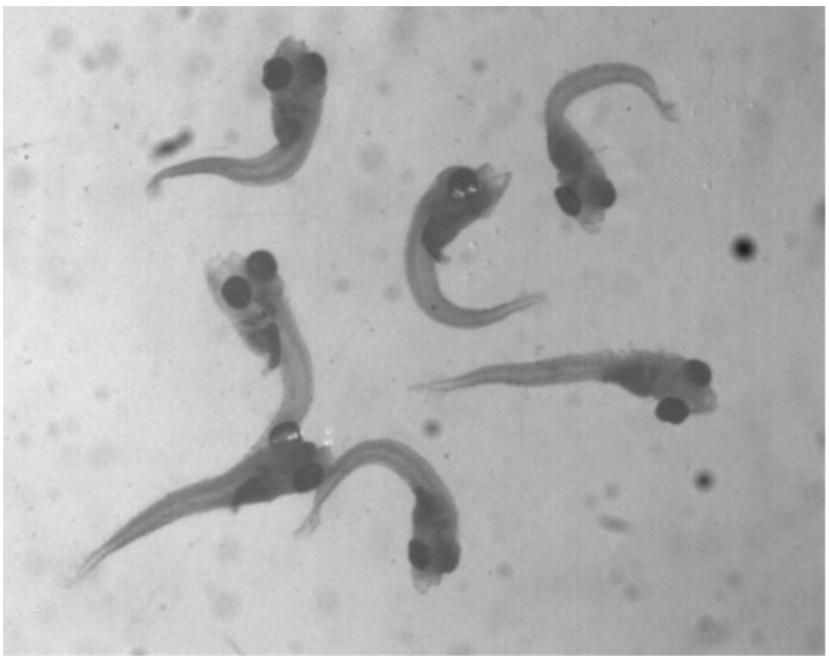

Fig. 3.1.3. Surface death larvae at 4 days-post-hatch.

Statistical analysis

In Experiment 1, the significance of difference between the NT (control) group and other groups and the homogeneity of the variance were tested using Dunnett's test and Bartlett's test respectively. The homoscedasticity was not observed in the swimbladder inflation frequency; therefore, the data were subjected to arcsine transformation prior to Dunnett's test. In Experiment 2, the significance of differences among the groups and the homogeneity of the variance were tested using Tukey–Kramer test and Bartlett's test respectively. Significance of differences between WIS and WOIS on SL was tested using Student's t-test or Welch's modified t-test, and the homogeneity of the variance was tested using F-test. To compare the SL between WIS and WOIS, the SL data were randomly selected in each of WIS and WOIS in Experiment 1 and 2. The sample size was standardized to 45 as possible same as each treatment in Experiment 1 and 2. Statistical analyses were performed using statistical software (Kyplot 5.0 for Windows, KyensLab, Tokyo, Japan). In the present study, differences at $P < 0.05$ were considered to be significant.

3.1.3 Results

Experiment 1

Swimbladder inflation

No WIS were found on 2 dph in all the groups (Fig. 3.1.4). The swimbladder inflation frequency in SS group increased after 2 dph and reached a plateau of approximately 60% on 4 dph, on the other hand, such an increase in swimbladder inflation frequency was not observed in other groups. The inflation frequency at the end

of the rearing experiment was 62.2 ± 27.9% (mean ± SD, *n* = 3) in SS group, which was significantly higher than that in NT (11.9 ± 6.0%), LP (2.7 ± 4.7%), and OF (3.9 ± 2.7%) groups where no significant differences were detected among these groups (Fig. 3.1.4).

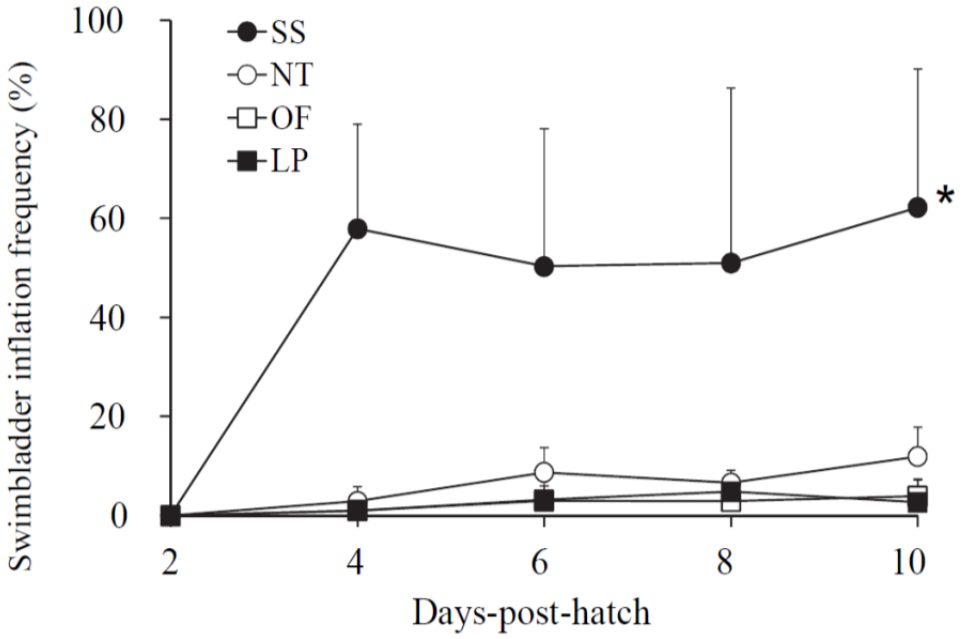

Fig.3.1.4. Changes in the swimbladder inflation frequency of Pacific bluefin tuna, *Thunnus orientalis*, larvae reared with the following four types of treatment: removing autogenous surface film with surface skimmer (SS group), covering the water surface with liquid-paraffin-layer (LP group), oil film (OF group), and non-treatment (NT group, control). Means of three replicate tanks are shown for each group (*n* = 3). Error bars indicate standard deviations. Asterisk indicates statistically significant difference ($P < 0.05$).

Growth and survival

The initial average SL was approximately 4.0 mm in all groups. Each group showed similar growth, and no significant difference in SL were found between NT and other groups (SS, OF, LP) until the end of the rearing experiment (10 dph) (Table 3.1.1).

Fig. 3.1.5 shows the comparative results of SL between WIS and WOIS. SL was significantly greater in WIS than in WOIS on 10 dph ($P = 0.014$).

No significant difference was detected in the survival between NT and other groups (SS, OF, LP) at the end of the rearing experiment, although OF group showed a higher survival rate than other groups (Table 3.1.1).

Table 3.1.1. Growth and survival of Pacific bluefin tuna, *Thunnus orientalis*, larvae in different water surface conditions; use of surface skimmer to remove autogenous surface film (SS), covering the water surface with oil film (OF), liquid-paraffin-layer (LP) and control (non- treatment, NT) in Experiment 1

Treatment	Standard length (mm)					Survival (%)
	2 dph	4 dph	6 dph	8 dph	10 dph	10 dph
NT	3.9 ± 0.1	4.5 ± 0.2	5.3 ± 0.3	6.0 ± 0.5	6.8 ± 0.5	22.2 ± 12.1
SS	3.9 ± 0.2	4.7 ± 0.2	5.3 ± 0.3	6.1 ± 0.4	7.0 ± 0.5	25.6 ± 12.3
OF	4.0 ± 0.1	4.6 ± 0.2	5.4 ± 0.2	6.1 ± 0.4	7.0 ± 0.4	42.3 ± 8.6
LP	3.9 ± 0.1	4.5 ± 0.2	5.2 ± 0.2	5.8 ± 0.5	6.8 ± 0.6	27.5 ± 19.0

Values of standard length are mean and standard deviation ($n = 45$ per treatment).
Values of survival are mean with standard deviation of three replicate tanks ($n = 3$).
dph: days-post-hatch.

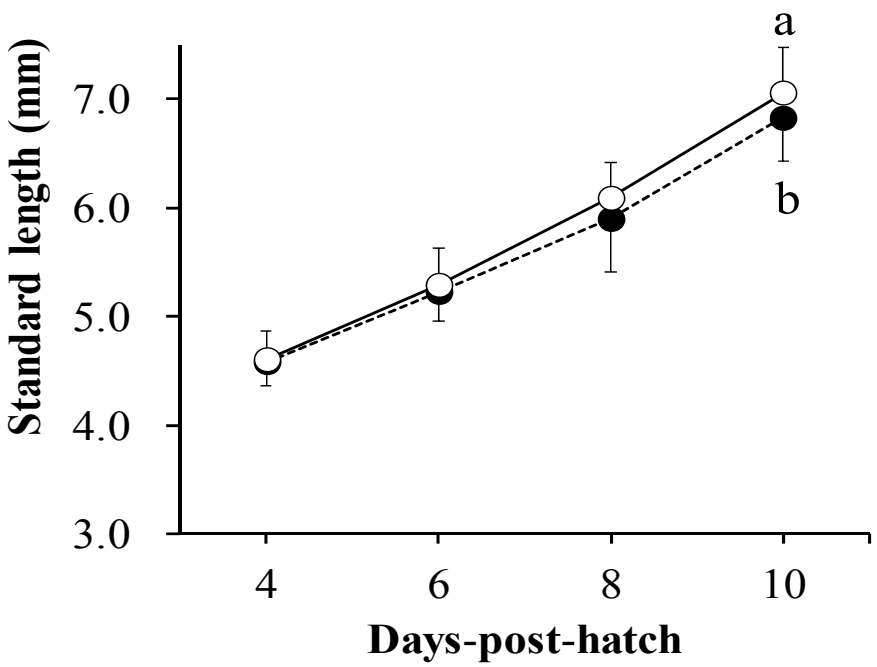

Fig. 3.1.5. Comparison of growth between the larvae with (WIS) and without (WOIS) inflated swimbladder in Pacific bluefin tuna, *Thunnus orientalis,* by the reorganized standard length data in Experiment 1. Open circles and closed circles show WIS and WOIS respectively, and error bars indicate standard deviations (n = 45 in WOIS, n = 25 to 39 in WIS). Different lower case letters indicate significant differences ($P < 0.05$).

Experiment 2

Swimbladder inflation

The WIS appeared from 3 dph (the yolk-sac disappeared: “D” stage shown in Kawakami et al. 2008), but the inflation frequency varied among the groups. Inflation frequency in SF3D group was 25.1 ± 20.7% on 3 dph, and thereafter continued to increase until the end of the experiment. Inflation frequency in other groups remained low during the experiment, although SF4D group tended to maintain relatively higher frequency than SF5D and SF6D groups. Inflation frequency at the end of the experiment

was significantly higher in SF3D group (80.2 ± 7.3%) than in other groups (7.5 ± 10.4%–17.8 ± 10.5%) (Fig. 3.1.6).

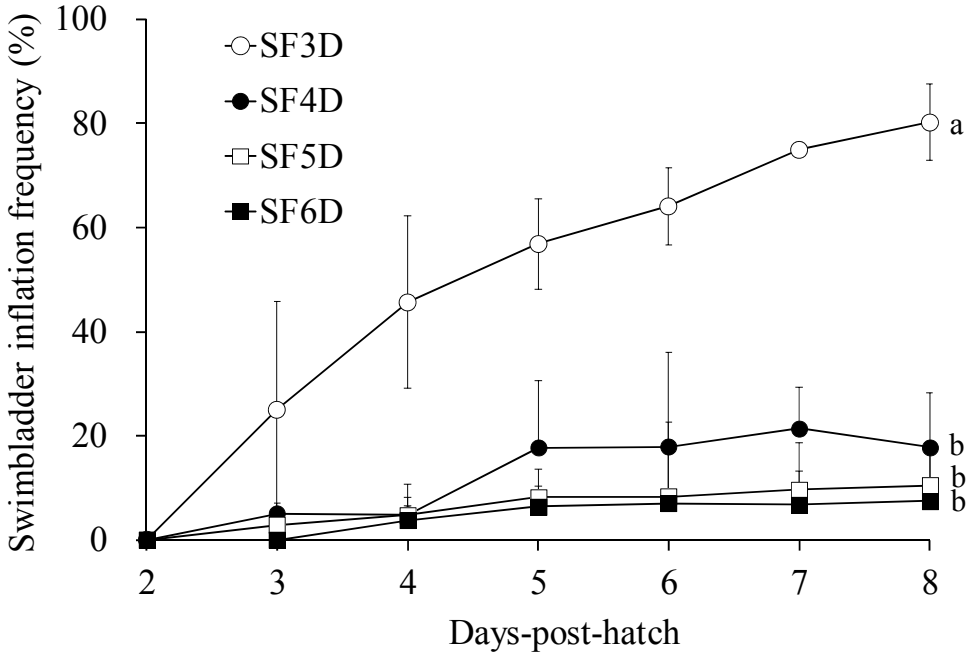

Fig. 3.1.6. Change in the swimbladder inflation frequency of the Pacific bluefin tuna, *Thunnus orientalis*, larvae reared with four different periods of oil film removal using surface skimmer: from 3 to 8 days-post-hatch (dph) (SF3D group); from 4 to 8 dph (SF4D group); from 5 to 8 dph (SF5D group); from 6 to 8 dph (SF6D group) in Experiment 2. Frequencies are shown by the means with standard deviations of three replicate tanks (n = 3). Different lower case letters indicate statistically significant difference ($P < 0.05$).

Number of surface death individuals

The number of surface death individuals was below 100 in each treatment on 2 dph, and no significant difference was found among the groups (Fig. 3.1.7). On 3 dph, when oil film removal started, 379 individuals ± 33.5 were counted in the SF3D which was significantly higher than those in other treatments. The number of surface death

individuals in SF3D decreased on 4 dph, but it was still significantly more (124 individuals ± 51.5) than that in other treatments (SF5D, 11 individuals ± 5.1 and SF6D, 13 individuals ± 7.1) (Fig. 3.1.7). In the SF4D group, it increased on 4 dph when oil film removal started and had more individuals (146 individual ± 26.7) than those in the groups of SF5D and SF6D. The increase in the number of the surface death individuals occurred in SF3D and SF4D was not observed in SF5D and SF6D after the commencement of oil film removal on 5dph and 6 dph respectively. The numbers of surface death individuals were much less from5 to 8 dph than on the former dph, and no significant difference was found among the groups (Fig.3.1.7).

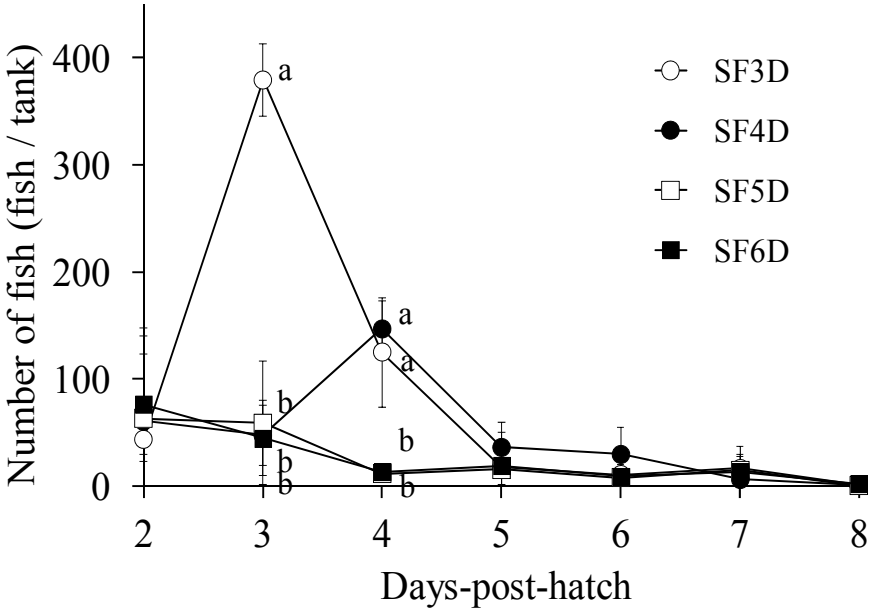

Fig. 3.1.7. Change in the number of surface death individuals in SF3D, SF4D, SF5D, and SF6D group in Experiment 2. Numbers of fish are shown by the means of three replicate tanks (n = 3). Error bars indicate standard deviations. Different lower case letters indicate statistically significant difference ($P < 0.05$).

Growth and mortality

The initial SL was approximately 4.0 mm in all groups on 2 dph. Each group showed similar growth, and no significant difference was found among all groups until 7dph. At the end of the rearing experiment (8 dph), SL was significantly smaller in SF5D group; however, no significant difference was found among other three groups (SF3D, SF4D, SF6D; Table 3.1.2). Fig. 3.1.8 shows the comparative results of SL between WIS and WOIS. SL was significantly greater in WIS than in WOIS on 8 dph ($P = 0.0001$).

The total mortality for each treatment was similar, approximately 50%, and there was no significant difference among other treatments (Table 3.1.2). Mortality due to surface death was highest inSF3D and the next in SF4D. It was significantly lower in SF5D, SF6D (Table 3.1.2).

Table 3.1.2. Growth, total mortality and mortality due to surface death of Pacific bluefin tuna, *Thunnus orientalis*, larvae in different periods of oil film removal; from 3 to 8 (SF3D), 4 to 8 (SF4D), 5 to 8 (SF5D), 6 to 8 (SF6D) days-post-hatch (dph)

Treatment	Standard length (mm)							Total mortality (%)	Mortality due to the surface death (%)
	2 dph	3 dph	4 dph	5 dph	6 dph	7 dph	8 dph		
SF3D	4.0 ± 0.1	4.1 ± 0.2	4.4 ± 0.2	4.8 ± 0.2	5.2 ± 0.3	5.6 ± 0.3	6.1 ± 0.4a	44.7 ± 14.3	10.6 ± 2.6a
SF4D	4.0 ± 0.1	4.1 ± 0.2	4.5 ± 0.2	4.8 ± 0.3	5.2 ± 0.3	5.5 ± 0.3	6.0 ± 0.4a	52.3 ± 24.3	5.9 ± 0.8ab
SF5D	4.0 ± 0.1	4.1 ± 0.2	4.5 ± 0.2	4.8 ± 0.2	5.2 ± 0.2	5.7 ± 0.3	5.8 ± 0.3b	45.8 ± 31.2	3.1 ± 2.7b
SF6D	3.9 ± 0.1	4.0 ± 0.2	4.5 ± 0.2	4.9 ± 0.3	5.2 ± 0.3	5.7 ± 0.3	6.0 ± 0.3a	42.3 ± 9.7	3.2 ± 1.7b

Values of standard length are mean and standard deviation ($n = 45$ per treatment).

Values of total mortality and mortality due to surface death are mean with standard deviation of three replicate tanks ($n = 3$).

Different lower case letters indicate significant differences within dph ($P < 0.05$).

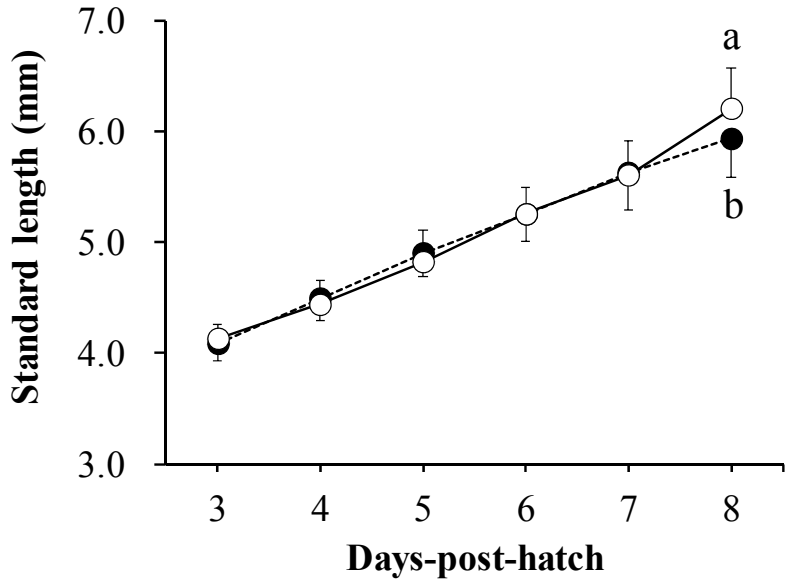

Fig. 3.1.8. Comparison of growth between the larvae with (WIS) and without (WOIS) inflated swimbladder in Pacific bluefin tuna, *Thunnus orientalis,* by the reorganized standard length data in Experiment 2. Open circles and closed circles show WIS and WOIS respectively, and error bars indicate standard deviations (n = 45 in each WIS and WOIS except for n = 28 in WIS on 3 dph). Different lower case letters indicate significant differences ($P < 0.05$).

Experiment 3

Swimbladder inflation

The WIS appeared from 3 dph (the yolk-sac disappeared: “D” stage shown in Kawakami et al. 2008). Swimbladder inflation frequencies in all the groups were approximately 50% on 3 dph, and it increased until 4 dph, and reached a plateau with no clear change thereafter except for S3–8 group. At the end of experiment (8 dph), no significant differences were found in swimbladder inflation frequency among experimental groups (SF3: 57.0 ± 14.6%, S3–4: 62.0 ± 9.1%, S3–5: 61.8 ± 4.9%, S3–8: 71.0 ± 13.5%); however, SF3 group tended to maintain relatively lower frequency and

the frequency in SF3–8 group tended to be higher than those of SF3–4 and SF3–5 groups at 7 and 8 dph (Fig. 3.1.9).

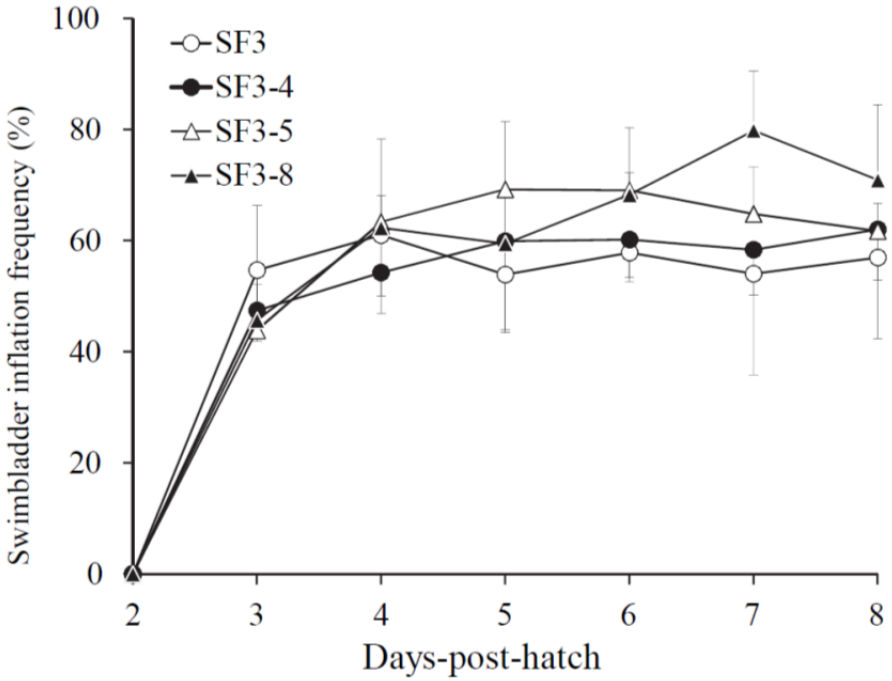

Fig. 3.1.9. Changes in the swimbladder inflation frequency of the Pacific bluefin tuna, *Thunnus orientalis*, larvae reared with four different periods of oil film removal using surface skimmer: one day of 3 days-post-hatch (dph) (SF3 group), from 3 to 4 dph (SF3–4 group), from 3 to 5 dph (SF3–5 group) and from 3 to 8 dph (SF3–8 group) in Experiment 3. The swimbladder inflation frequency shown by the means of three replicate tanks (n = 3). Error bars indicate standard deviations.

Growth and survival

Each group showed similar growth as in Experiment 2, and no significant difference was found among the groups until 7 dph, however at the end of the rearing experiment (8 dph), SL was significantly smaller in SF3–5 and SF3–8 groups than those in other groups (Table 3.1.3).

The survival for each treatment varied largely, and no significant difference was observed among the groups (Table 3.1.3).

Table 3.1.3. Growth and survival of Pacific bluefin tuna, *Thunnus orientalis*, larvae reared with four different periods of oil film removal using surface skimmer: one day of 3 days-post-hatch (dph) (SF3 group), from 3 to 4 dph (SF3–4 group), from 3 to 5 dph (SF3–5 group) and from 3 to 8 dph (SF3–8 group) in Experiment 3

Treatment	Standard length (mm)							Survival (%)
	2 dph	3 dph	4 dph	5 dph	6 dph	7 dph	8 dph	
SF3	3.8 ± 0.2	3.9 ± 0.1	4.3 ± 0.2	4.8 ± 0.2	5.2 ± 0.1	5.5 ± 0.3	6.0 ± 0.2a	55.7 ± 8.8
SF3–4	3.8 ± 0.1	3.9 ± 0.2	4.3 ± 0.2	4.8 ± 0.2	5.2 ± 0.2	5.5 ± 0.2	6.0 ± 0.2a	42.6 ± 14.7
SF3–5	3.8 ± 0.2	3.9 ± 0.2	4.4 ± 0.2	4.8 ± 0.2	5.2 ± 0.2	5.5 ± 0.2	5.8 ± 0.3b	50.6 ± 6.6
SF3–8	3.8 ± 0.1	3.9 ± 0.2	4.2 ± 0.2	4.8 ± 0.3	5.2 ± 0.2	5.4 ± 0.3	5.7 ± 0.3b	39.1 ± 7.1

Values of standard length are mean and standard deviation (n = 45 per treatment).
Values of survival are mean with standard deviation of three replicate tanks (n = 3).
Different lower case letters indicate significant differences within dph ($P < 0.05$).

3.1.4 Discussion

This study was conducted to focus on improvement of mortality caused by both surface death and sinking death during the PBT early larval stage. In this study, the promoting and inhibitory conditions of water surface and the period for effective promotion of the ISI in the PBT larviculture was investigated.

In Experiment 1, ISI in PBT larvae was inhibited not only by the liquid-paraffin-layer and the feed oil film but also by autogenous surface film that were formed in the process of larval rearing on the water surface. In addition, it was possible to promote ISI by surface film removal using the surface skimmer. These results suggest that the PBT larvae require air gulping for ISI as reported in other marine fish.

In Experiment 2, skimming from 3 dph effectively promoted the ISI whereas such a promotion effect was not found when skimming begins on 4 dph. Furthermore, skimming on only 1 day of 3 dph had the similar promotional effect for ISI to skimming on 3 dph and later in Experiment 3. These mean that there is a specific day when ISI can be effectively promoted in larval PBT production. On the other hand, the skimming caused highest incidences of surface death on 3 dph in Experiment 2, which indicates that the promotional method of ISI and the preventive method of surface death conflict with each other on 3 dph. Such a conflict would induce considerable mortality due to surface death in practical PBT larviculture.

The significant improvement in swimbladder inflation frequency in SS group in Experiment 1 indicates that the surface skimmer effectively promoted ISI in PBT larvae (Fig. 3.1.2). The low frequencies in OF and LP groups as well as NT group indicate that these substances on the water surface inhibited ISI. Similar results have been reported in red sea bream, *Pagrus major* (Temminck and Schlegel) (Kitajima et al. 1981), gilthead sea bream, *Sparus aurata* (Linnaeus) (Chatain and Ounais-Guschemann 1990), striped bass, *Morone saxatilis* (Walbaum) (Friedmann and Shutty 1999), and striped trumpeter, *Latris lineata* (Bloch and Schneider) (Trotter et al. 2005a). These studies have suggested that the larvae require air gulping for ISI and the presence of some substances on the water surface as a surface film inhibits larval access to the atmospheric air and subsequently prevents the air gulping. The results of this study strongly suggest that PBT larvae have the same mechanism of ISI by air gulping. The ISI by gulping air can be considered to be common in physoclists with physostomous larvae including scombrid fish to which the PBT belong. In the process of practical larval rearing, autogenous surface film may originate in enriched oily live feed and/or dead larvae and

have a similar adverse effect on the ISI, as observed in NT group. Therefore, the surface film removal on water surface with devices such as the surface skimmer is considered to aid the larval air gulp and to eventually promote ISI in PBT larviculture.

The WIS were not found on 2 dph in Experiment 1 but appeared on 3 dph (the yolk-sac disappeared: “D” stage shown in Kawakami et al. 2008) in Experiment 2 and 3. These results indicate that the ISI began on 3 dph under the present rearing condition, and this present result is not contradictory to observations in the practical PBT larviculture at FLKU. In contrast, Kaji (2000) reported with the histological observation that the swimbladder of PBT larvae began to differentiate on 2 dph but the inflated swimbladder was firstly detected in the larvae on 7 dph. Although Kaji (2000) did not describe in detail the rearing condition, rearing water temperature, which could be considered as a major determinant of the larval development, was higher in his study (26.7–28.6°C) than that in the present study (25.0–26.5°C). Therefore, the reason behind the difference with his study is unclear; however, it may be partly attributed to other factors such as the nutritional condition and genetic back ground as other researchers have reported in other fish species (Kanazawsa et al. 1982; Kitajima et al. 1994; Peruzzia et al. 2007).

In Experiment 2, the swimbladder inflation frequency at the end of the experiment was significantly improved in SF3D group than in other groups (Fig. 3.1.3). This improvement indicates that ISI promotion cannot be effectively achieved if the skimming does not begin on 3dph, under a rearing condition as in the present study. Thus, there is little doubt that a specific period exists for the promotion of ISI in PBT larvae. Moreover, in Experiment 3, there was no significant difference in the swimbladder inflation frequency between SF3 group and other groups at the end of the

experiment (Fig. 3.1.5). These results suggest that ISI promotion can be effectively achieved by surface film removal in 1 day of 3 dph. Such a limited period, so-called 'window' when effective promotion of ISI is possible, has also been reported in many studies; e.g. striped bass, *M. saxatilis*: <48 h at 20°C and c. 3 days at 17°C (Bailey and Doroshov 1995; Friedmann and Shutty1999), striped trumpeter, *L. lineata*: 4 days at 16.2°C (Trotter et al. 2005a), red sea bream, *P. major*: 3 days (water temperature was not given) (Kitajima et al. 1981). The window in larval PBT seems to be extremely narrow, which can imply the need for running surface skimmer without missing this narrow window, 1 day of 3 dph, to promote ISI effectively in practical PBT larviculture. In these experiments, the larval developmental stage on 3 dph is "D" stage shown in Kawakami et al. 2008: the yolk-sac disappeared). However, it requires attention to consider the speed of larval development and growth under different temperatures.

On the other hand, the swimbladder inflation frequency continued to increase after 3 dph in SF3D group in Experiment 2 (Fig. 3.1.3) whereas that in SS group in Experiment 1 reached a plateau on 4 dph (Fig. 3.1.2), regardless of the similar rearing condition between these groups. In addition, inter treatment variation in the frequency decreased in the former group, while that in the latter group maintained relatively high, during each experiment. Moreover, the swimbladder inflation frequency in SF3 group tended to be lower than those of other groups, while that of SF3–8 group tended to be higher than those of other groups without significant differences. Additionally, swimbladder inflation frequency did not achieve 100% in Experiment 2 and 3. Therefore, further study should be performed to elucidate whether the period of 1 day of 3 dph is completely sufficient for achieving of 100% ISI.

Subsequent process of the ISI after the air gulping may be susceptible to simple differences among rearing cohorts, the aforementioned factors such as nutritional condition and genetic background, and/or unknown factors. In the present study, the surface skimmer, which was run during daytime, significantly improved the swimbladder inflation; however, this may also influence the changing pattern of the frequency and the variation. Additional researches on mechanism of the ISI as well as on operating time of the surface skimmer are required to obtain high and stable swimbladder inflation frequency. The possibility that strong aeration in nighttime could influence the ISI is also a topic of further research since the resultant water current might disturb the air gulping which may occur in nighttime as well.

It has been reported that surface death occurs between 1 and 4 dph in PBT larvae (Takashi et al. 2006). From a practical point of view, this period partly overlaps with the window identified herein for the promotion of ISI. Making oil film on the water surface is carried out in practical PBT larviculture to prevent surface death on an empirical basis (Munday et al. 2003). However, oil film removal for promoting the ISI could induce a high incidence of surface death, as shown in Experiment 2. The oil film removal on 3 dph in SF3D group resulted in the highest mortality due to surface death among the experimental groups, and it accounted for 24% of the total mortality. These results indicate the effectiveness of making oil film to prevent surface death and the improvement of larval survival by prevention of surface death, and that the promotional method for ISI and the preventive method of surface death conflict with each other on 3 dph. Finding solutions to this conflict will help to improve the production efficiency of practical PBT larviculture.

Failure of ISI has an adverse effect on the larval growth in gilthead sea bream, *S. aurata,* sea bass, *Dicentrarchus labrax* (Linnaeus), striped trumpeter, *L. lineata* (Chatain 1989; Chatain and Ounais-Guschemann 1990; Trotter et al. 2005a), and this adverse effect has been considered to be partially caused by the lack of a stable buoyancy and/or equilibrium of the larvae (Chatain 1989; Trotter et al. 2005a). The poor swimbladder inflation frequency did not stunt the growth of the PBT larvae until 10 dph in Experiment 1 and until 7 dph in Experiment 2 in the treatment groups (Table 3.1.1, 3.1.2). Although only the difference was detected on 8 dph in Experiment 2 (Table 3.1.1, 3.1.2), no clear relationship between swimbladder inflation frequency and growth was found. However, when larval SL was compared between WIS and WOIS, the SL was significantly greater in WIS than WOIS on 10 dph in Experiment 1 and on 8 dph in Experiment 2 (Fig. 3.1.5, 3.1.8). These results indicate that the ISI failure significantly affects growth from 8 dph. Further study with an extended rearing period is necessary to verify the influence of failure of ISI on PBT growth.

Chapter 1 demonstrated that WIS has a smaller sinking velocity than WOIS in PBT larvae. Moreover, Chapter 2 demonstrated that WOIS indeed have a stronger tendency to sink to tank bottom than WIS, and demonstrated that ISI failure resulted in reduced larval survival via increases in sinking death ratio in mass-scale production tank, even if preventive measure of sinking death using flow control was employed during the nighttime. However, there was no significant difference in the survival (Experiment 1 and 3) or total mortality (Experiment 2) among all treatment groups in both the experiments, and no correlations between these parameters and the swimbladder inflation frequency were found, although the flow control to prevent the sinking death is

employed during the nighttime to obtain enough numbers of larval specimens for the comparison of treatments by avoiding mass mortality via sinking death.

Regarding this contrast, there are certain considerations in the previous knowledge and results of this study. Sumida et al. (2011) reported that the aspect ratio (water depth/the half width or radius of tank: AR) of a tank affects flow patterns generated by aeration within that tank, and suggested that high AR prevents larval sinking death (Sumida et al. 2011). Furthermore, upwelling flow generated by aeration was reportedly faster within higher AR tanks than within lower AR tanks (Shiotani et al. 2005). Results of these study suggest that tanks with high AR possess a greater capacity to prevent sinking death of PBT larvae than do other tanks. In this study, survival in small experimental tanks tended to be higher (from 43.2% to 48.6% in 500 *l* tanks in Chapter 1; from 22.2% to 57.7% and 28.1% to 63.7% in 1.0 *kl* tanks in Section 3.1 and Section 3.2 in this chapter) than that in mass-scale tanks (from 0.8% to 26.0% in 30 and 20 *kl* tanks in Chapter 2; 19.3% in 50 *kl* tank by Tanaka et al. 2009). Additionally, the AR of the tank was higher in small experimental tanks (1.0 *k l* tank: 1.04 and 500 *l* tank: 1.20) than in mass-scale tanks (20 *kl* tank: 0.44 and 30 *kl* tank: 0.35). Therefore, the differences in survival observed between mass-scale and experimental tanks could be the result of differences in tank AR, i.e. WOIS could be lifted up in the tanks with high AR. The results of Chapter 2, that ISI failure reduced the larval survival, imply that the generation of enough vertical circular current to prevent the larval sinking in mass-scale tank with low AR is more difficult than in small experiment tank with high AR. Further investigation is necessary to elucidate the effectiveness of flow control in mass-scale tank on larval survival and selecting suitable tank shape in relation to ISI failure.

3.2: Optimal timing in the day to promote ISI in Pacific bluefin tuna, *Thunnus orientalis*, larvae

3.2.1 Introduction

For the ISI promotion method in PBT larvae, Section 3.1 in this chapter demonstrated that surface film removal (SFR), using a cleaning device such as surface skimmer, effectively promotes PBT larval ISI, and elucidated that the period when effective ISI promotion is possible (window) is extremely narrow of 1 day of 3 dph under 26.5°C. This is much shorter than other reported fish species. Moreover, Section 3.1 in this chapter elucidated that high incidence of surface death was induced by oil film removal for ISI promotion, and it corresponded with the window for the promotion of ISI identified in this study, i.e., the promotional method for ISI and the preventive method of surface deaths conflict with each other on 3 dph.

Additionally, it is empirically known that missing an opportunity of ISI promotion by removal surface film in the evening often results poor swimbladder inflation frequency in mass-scale PBT larviculture in hatchery. This suggests the possibility of presence of optimal timing to promote ISI even within the day in PBT larviculture.

Therefore, in this section, a series of three experiments (Experiment 1, 2 and 3) was conducted with different time schemes of SFR to verify the optimal timing of the day to promote ISI. In addition, diel change in the occurrence frequency of surface death was examined to elucidate the relationship between optimal timing of the day to promote ISI and occurrence of surface death continuing from the study of Section 3.1 in

this chapter. In this study, the influence of ISI failure on larval growth was also examined continuing from Section 3.1 in this chapter.

3.2.2 Materials and methods

Eggs and larval rearing

PBT fertilized eggs were obtained by spontaneous spawning of cultivated brood stock fish in a sea net-cage at the FLKU. Eggs of the late embryonic stage were placed in cylindrical fibreglass tanks (1.0 *kl;* 135 cm in internal diameter and 70 cm in depth) at a density of 6000 eggs per tank. They had a normal hatching rate of 98.7, 88.3 and 93.8% in Experiment 1, 2 and 3 respectively. Larvae were subsequently subjected to each rearing experiment, detailed below, in the same tanks. The dph was defined to hatching day as 0 dph.

As in study of Section 3.1 in this chapter, incubation temperature of eggs and hatched larvae before feeding was set at 23 and 25°C, respectively, and larval rearing temperature until the end of the each experiment after feeding was set at 26.5°C.

To prevent larval surface death in prelarvae rearing, 0.3 *ml* per tank of feed oil added as drops every day onto the rearing water surface to form a surface film from hatching to 2 dph as in the previous report and section (Munday et al. 2003; Section 3.1 in this chapter).

The larvae were fed with S-type rotifers *Brachionus plicatilis* sp. complex (Hagiwara et al. 2007), enriched with *Nannochloropsis oculata* of 1.5×10^7 cells/*ml*, a commercial product (Marine Glos EX; Nisshin Marinetech, Yokohama, Japan) and taurine of 0.4 g/*l* (Japan Nutrition, Tokyo, Japan), from 2 dph onwards at a food density

of 5–15 ind./*ml*. Water exchange in each tank was provided by flow through sand filtered sea water at 1000 *l*/day from feeding to the end of the each experiment. Natural sunlight was attenuated using a plastic sheet, which covered the experimental facilities. In addition, subsidiary fluorescent lighting was used within the natural light period, which includes fade-in and fade-out of light intensity after the start and before the end of light period respectively. In this study, dark period and light period were defined as the period when the light intensity was 0 μmols/m^2, and the period other than the dark period respectively. Light intensity was measured using alight photon meter (MDS-MkV/L; Alec Electronics, Kobe, Japan) every 10 min during light period for experimental period in Experiment 3 (refer to Fig. 3.2.3). Light period was 14 h (natural light period; 05:00–19:00). *N. oculata* was added every day to the rearing water from 2 dph to the end of experiment.

Other rearing conditions were as follows: rearing water temperature, 26.5 ± 0.1 ºC; salinity of 31.2 ± 0.3; dissolved oxygen, 107.4 ± 6.3 %; and pH, 7.88 ± 0.36 in Experiment 1;-temperature, 26.5 ± 0.1ºC; salinity, 31.6 ± 0.3; dissolved oxygen, 101.0 ± 2.2 %; and pH, 8.13 ± 0.03 in Experiment 2; temperature, 26.5 ± 0.1 ºC; salinity, 31.6 ± 0.3; dissolved oxygen, 100.3 ± 1.9 %; and pH, 8.17 ± 0.10 in Experiment 3.

Experimental design

A series of three rearing experiments (Experiment1, 2, 3) was conducted to verify the optimal timing of the day to promote ISI using different batches of fertilized eggs, which were spawned on different days. The examined timings in the experiments were narrowed down based on the results of preceding experiment (Fig. 3.2.1). The rearing experiments with SFR were carried out from 3 to 9 dph as in our previous study.

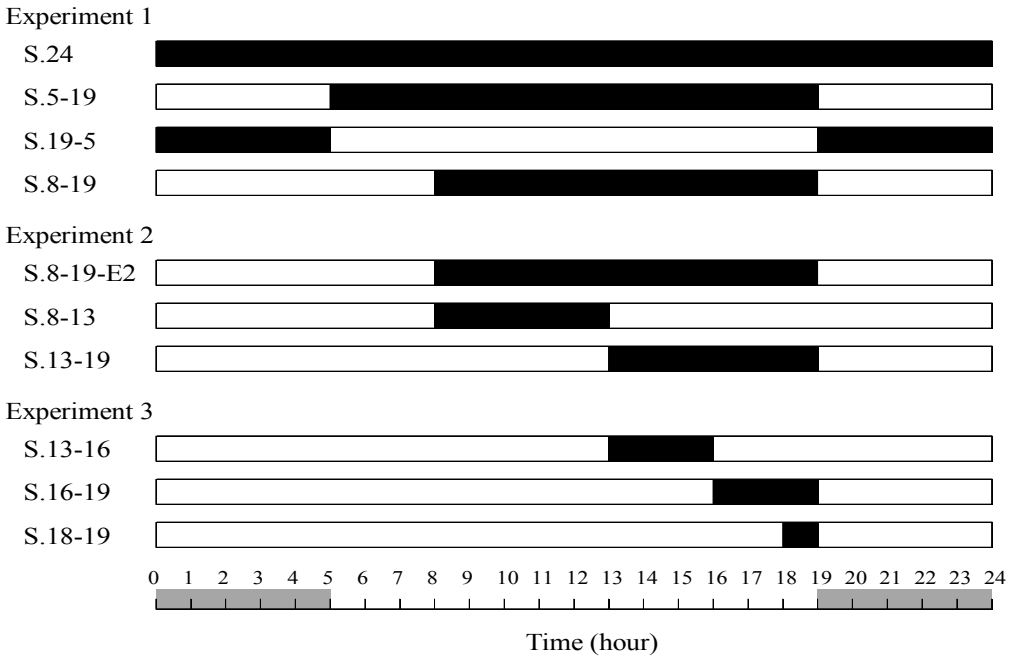

Fig. 3.2.1. Timing of surface film removal to promote ISI in each experiment. Black areas and grey areas represent the timing of surface film removal and the dark period respectively.

Initially, in Experiment 1, larval swimbladder inflation frequency was compared among the following different SFR timings of the day: from 05:00 to 19:00 (i.e. during the light period: S.5–19), from 19:00 to 05:00 (i.e. during the dark period: S.19–5), from 08:00 to19:00 (the general running period of surface skimmer in hatchery: S.8–19) and the entire day (i.e. during 24 h: S.24).

After getting the results of Experiment 1, Experiment 2 was conducted to refine the necessary period of SFR during the light period, where the effect of SFR on larval swimbladder inflation frequency was compared among the following different SFR timings of the day in: from 08:00 to 19:00 (same as the treatment of S.8–19 in Experiment 1:S.8–19-E2), from 08:00 to 13:00 (S.8–13) and from 13:00 to 19:00 (S.13–19).

Based on the results of Experiment 2, Experiment 3 was conducted to refine the necessary period of SFR in the afternoon, where larval swimbladder inflation frequency

was compared among the following different SFR timings of the day: from 13:00 to 16:00 (S.13–16), from 16:00 to 19:00 (S.16–19) and from 18:00 to 19:00 (S.18–19).

In these experiments, the feed oil was added during the time period other than SFR timings from 3 to 9 dph to form the surface film to inhibit larval air gulping for ISI. The surface skimmers for SFR used in this study were same as that used in Section 3.1 in this chapter.

There were three replicates per treatment for Experiment 1 and 3, two replicates per treatment in Experiment 2.

Aeration

Aeration was provided at the centre of tank bottom using an air stone (100 mm long, 23 mm diameter: Ibuki air stone #100; KING Stone, Tokoname, Japan) with air-flow rate of 130 *ml*/min in the light period. In the dark period, another air stone with air-flow rate of 1,300 *ml*/min was additionally placed at the centre of tank bottom from 2 to 9 dph to mitigate sinking death (Tanaka et al. 2009; Nakagawa et al.2011).

In S.5–19, S.19–5 and S.24 of Experiment 1, aeration was provided at the centre of tank bottom using an air stone with air-flow rate of 650 *ml*/min during 24 hours through the experimental period without additional aeration during the dark period to examine the effect of timing of SFR on swimbladder inflation frequency under a constant aeration condition.

Measurements and observations

Thirty larvae were examined to determine the swimbladder inflation frequency and 15 larvae were measured standard length (SL: length from the rostral tip to the end

of the notochord) for each tank at the end of the experiment (9 dph), and 6 dph when success of swimbladder inflation is likely to be important to prevent larval mortality caused by sinking death in PBT larviculture in mass production tanks (Chapter 2). Larval sampling was conducted in nighttime (21:00–22:00) because PBT larvae deflate the swimbladder in daytime (Takashi et al. 2006) and this makes it difficult to determine the correct swimbladder inflation frequency. Larvae were anaesthetized in 0.06% 2-phenoxyethanol (Trotter et al. 2005) to observe swimbladder inflation under a stereomicroscope. SL was measured in digital images of samples taken by a digital camera (Moticam 2000, Shimadzu Rika, Tokyo, Japan) using software package for image analysis (MoticImages Plus 2.2s, Shimadzu Rika).

At the end of the experiment, all surviving larvae were collected and fixed in 5% formalin solution for counting the number to evaluate the survival. In the evaluation of survival, the initial number of larvae in each tank was estimated by multiplying the number of introduced eggs by the hatching rate as stated above.

To examine the diel change in occurrence frequency of surface death, larvae trapped at the rearing water surface and in the surface skimmer were counted as the surface death larvae every 2 h for 24 h on 3 dph when is the effective period to promote ISI and the number of surface death larvae reached the maximum level (Section 3.1 in this chapter), in the three tanks of S.24 treatment in Experiment 1 where the running of surface skimmer and air-flow rate of aeration were constant for 24 h (n = 3).

Statistical analysis

Significance was tested using Tukey-Kramer test in differences among the treatments on SL in each experiment, on survival and swimbladder inflation frequency

in Experiment 1 and 3, and among each time on the number of surface death larvae in Experiment 1. The homogeneity of the variance was tested using Bartlett's test. Significance was tested using Student's t-test or Welch's modified t-test in differences between the larvae with inflated swimbladder (WIS) and without inflated swimbladder (WOIS) on SL, and the homogeneity of the variance was tested using F-test. To compare the SL between WIS and WOIS, the SL data were randomly selected in each of WIS and WOIS on every dph in Experiment 1, 2 and 3. The sample size was standardized to 45 same as each treatment in Experiment 1 and 3. (In Experiment 2, the total numbers of data in WIS were 39 and 40 in 6 and 9 dph, respectively, due to lack of the number of sample). Statistical analyses were performed using statistical software (Kyplot 5.0 for Windows, KyensLab, Tokyo, Japan). In this study, differences at $P < 0.05$were considered to be significant.

3.2.3 Results

Experiment 1

No significant difference was found in larval SL among the treatments on 6 dph and between S.8–19 and S.19–5 on 9 dph, while it was significantly greater in S.24, S.5–19 than in S.19–5 on 9 dph ($n = 45$; Table 3.2.1). Survival rates were similar among the treatments without significant differences (30.7 ± 7.6 to 36.1 ± 14.6, $n = 3$; Table 3.2.1).

Swimbladder inflation frequency was significantly higher in S.24 (81.1 ± 7.0%), S.5–19 (78.9 ± 13.5%) and S.8–19 (86.7 ± 8.8%) than in S.19–5 (10.0 ± 3.3%) on 6 dph, and also on 9 dph, it was significantly higher in S.24 (91.1 ± 5.7%), S.5–19 (92.2 ± 5.1%) and S.8–19 (93.3 ± 3.4%) than in S.19–5 (11.1 ± 5.1%) ($n = 3$, $P < 0.001$

on both 6, 9 dph); however, no significant differences were found among S.24, S.5–19 and S.8–19 on both 6 and 9 dph (Table 3.2.1).

Table 3.2.1. Effect of timing in surface film removal on growth, survival and initial swimbladder inflation frequency of Pacific bluefin tuna, *Thunnus orientalis*, larvae in each treatment in Experiment 1

	Treatment			
	S.24	S.5-19	S.19-5	S.8-19
Standard length (mm)				
6 dph	5.3 ± 0.2	5.3 ± 0.2	5.3 ± 0.2	5.4 ± 0.2
9 dph	6.9 ± 0.3^{a}	6.9 ± 0.3^{a}	6.6 ± 0.4^{b}	6.8 ± 0.4^{ab}
Survival (%)	30.8 ± 7.3	32.0 ± 5.6	30.0 ± 7.6	36.1 ± 14.6
Swimbladder inflation frequency (%)				
6 dph	81.1 ± 7.0^{a}	78.9 ± 13.5^{a}	10.0 ± 3.3^{b}	86.7 ± 8.8^{a}
9 dph	91.1 ± 5.7^{a}	92.2 ± 5.1^{a}	11.1 ± 5.1^{b}	93.3 ± 3.4^{a}

Timing in surface film removal is from 5:00 to 19:00 in S.5–19, from 19:00 to 5:00 in S.19–5, from 8:00 to19:00 in S.8–19 and during 24 h in S.24.

Surface film removal was carried out from 3 to 9 days-post-hatch (dph).

Standard length are shown as means ± standard deviation (n = 45 per treatment).

Survival rate and swimbladder inflation frequency are mean ± standard deviation of three replicate tanks (n = 3).

Within each dph, different lower case letters indicate significant differences ($P < 0.05$).

Time change in the number of surface death larvae

The number of surface death larvae was significantly more at 18:00 (135.3 ± 45.2) than that at other time (69.0 ± 15.6 at 14:00 to 11.7 ± 4.7 at 04:00) except for 16:00 (98.0 ± 25.6), and no significant difference was found in the number of surface death larvae between 16:00 and 18:00 (Fig. 3.2.2).

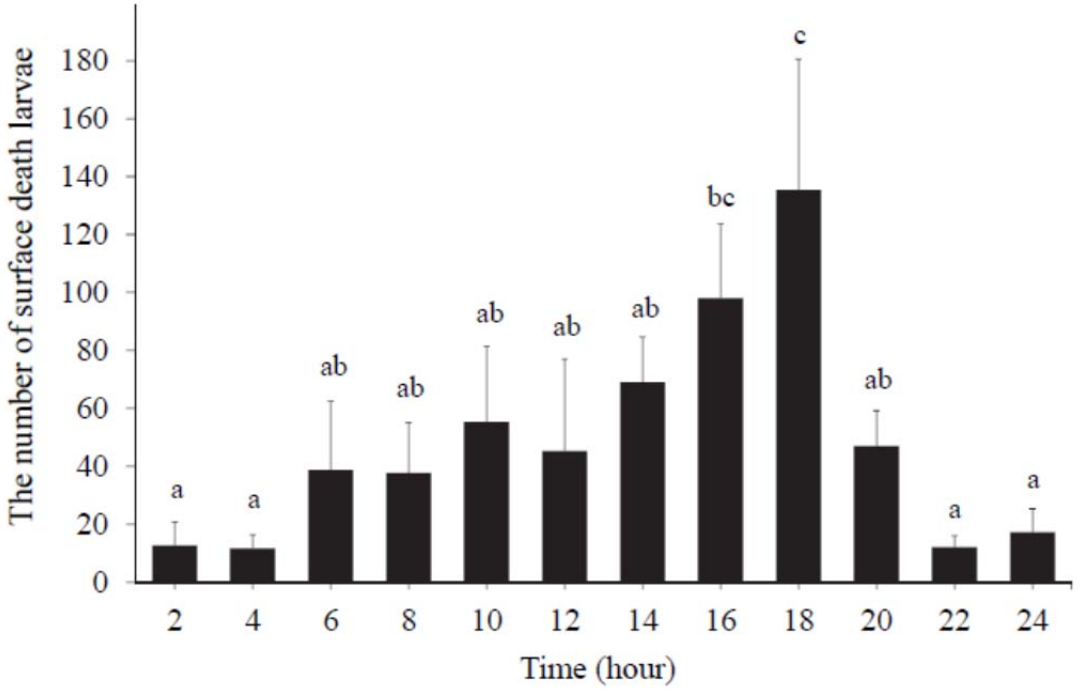

Fig. 3.2.2. Time change in the number of surface death larvae of Pacific bluefin tuna, *Thunnus orientalis*, on 3 days-post-hatch under continuous running of surface skimmer in S.24 of Experiment 1. The numbers of surface death larvae are shown as means with standard deviations of three replicate tanks (n = 3). Different lowercase letters indicate significant differences (P < 0.05).

Experiment 2

No significant difference was found in larval SL among the treatments on 6 and 9 dph (n = 30; Table 3.2.2). Survival rate in S.8–13 (63.7%: mean of two replicate tanks) was higher than in other treatments (40.4% in S.8–19-E2, 28.3% in S.13–19); however, it was varied among the treatments (Table 3.2.2). Swimbladder inflation frequency was remarkably higher in S.8–19-E2 (83.3%: mean of two replicate tanks) and S.13–19 (83.3%) than in S.8–13 (1.7%) on 6 dph, and also on 9 dph, it was remarkably higher in S.8–19-E2 (81.7%) and S.13–19 (88.3%) than in S.8–13 (0.0%); however, it showed similar value betweenS.8–19-E2 and S.13–19 on both 6 and 9 dph (Table 3.2.2).

Table 3.2.2. Effect of timing in surface film removal on growth, survival and initial swimbladder inflation frequency of Pacific bluefin tuna, *Thunnus orientalis*, larvae in each treatment in Experiment 2

	Treatment		
	S.8-19-E2	S.8-13	S.13-19
Standard length (mm)			
6 dph	5.2 ± 0.3	5.1 ± 0.3	5.3 ± 0.3
9 dph	6.5 ± 0.4	6.2 ± 0.4	6.4 ± 0.4
Survival (%)	40.4 (45.3, 35.5)	63.7 (66.4, 60.9)	28.3 (24.5, 32.1)
Swimbladder inflation frequency (%)			
6 dph	83.3 (80.0, 86.7)	1.7 (0.0, 3.3)	83.3 (86.7, 80.0)
9 dph	81.7 (73.3, 90.0)	0.0 (0.0, 0.0)	88.3 (80.0, 96.7)

Timing in surface film removal is from 8:00 to 19:00 in S.8–19-E2, from 8:00 to 13:00 in S.8–13, from 13:00 to 19:00 in S.13–19.

Surface film removal was carried out from 3 to 9 days-post-hatch.

Standard lengths are shown as means with standard deviations (n = 30 per treatment).

Survival rate and swimbladder inflation frequency are mean of two replicate tanks and value of each tank (in parentheses).

Experiment 3

No significant difference was found in larval SL among the treatments on 6 and 9 dph (n = 45; Table 3.2.3). Survival rates were varied among the treatments without significant differences (41.6 ± 11.4% in S.13–16, 28.1 ± 12.8% in S.16–19 and 31.9 ± 9.5% in S.18–19, n = 3: Table 3.2.3).

Table 3.2.3. Effect of timing in surface film removal on growth, survival and initial swimbladder inflation frequency of Pacific bluefin tuna, *Thunnus orientalis*, larvae in each treatment in Experiment 3

	Treatment		
	S.13-16	S.16-19	S.18-19
Standard length (mm)			
6 dph	5.0 ± 0.2	5.0 ± 0.2	4.9 ± 0.3
9 dph	6.1 ± 0.4	6.3 ± 0.3	6.2 ± 0.4
Survival (%)	41.6 ± 11.4	28.1 ± 12.8	31.9 ± 9.5
Swimbladder inflation frequency (%)			
6 dph	3.3 ± 3.4[a]	72.2 ± 1.9[c]	50.0 ± 6.7[b]
9 dph	7.8 ± 3.9[a]	84.4 ± 5.1[b]	70.0 ± 12.0[b]

Timing in surface film removal is from 13:00 to 16:00 in S.13–16, from 16:00 to 19:00 in S.16–19, from18:00 to 19:00 in S.18–19, and surface film removal was carried out from 3 to 9 days-post-hatch (dph).

Standard lengths are shown as means with standard deviations (n = 45 per treatment). Survival rate and swimbladder inflation frequency are mean with standard deviation of three replicate tanks (n = 3).

Within each dph, different lower case letters indicate significant differences ($P < 0.05$).

Swimbladder inflation frequency was significantly higher in S.16–19 (72.2 ± 1.9%, n = 3) than in S.13–16 (3.3 ± 3.4%), S.18–19 (50.0 ± 6.7%), and it was significantly lower in S.13–16 (3.3 ± 3.4%) than in other treatments on 6 dph (n = 3, $P < 0.001$). On 9 dph, swimbladder inflation frequency was significantly lower in S.13–16 (7.8 ± 3.9%) than in S.16–19 (84.4 ± 5.1%), S.18–19 (70.0 ± 12.0%) same as 6 dph (n = 3, $P < 0.001$). No significant difference in swimbladder inflation frequency was found between S.16–19 and S.18–19; however, the swimbladder inflation frequency in S.16–19 showed higher tendency than in S.18–19 on 9 dph (Table 3.2.3).

Change in light intensity at rearing water surface of experimental tank

The change in the light intensity at rearing water surface of experimental tank in the light period (05:00–19:00) in Experiment 3 was shown as the mean value of each day in addition to the value on 3 dph when effective promotion of ISI is possible (Fig. 3.2.3). After the start of the light period, these light intensities gradually increased. Around noon, although these light intensities were varied with weather condition, they reached maximum of c.a. 200 μmol/s/m^2, and then decreased to less than 50 μmol/s/m^2 after 16:00, and to 0 μmol/s/m^2 at the end of the light period.

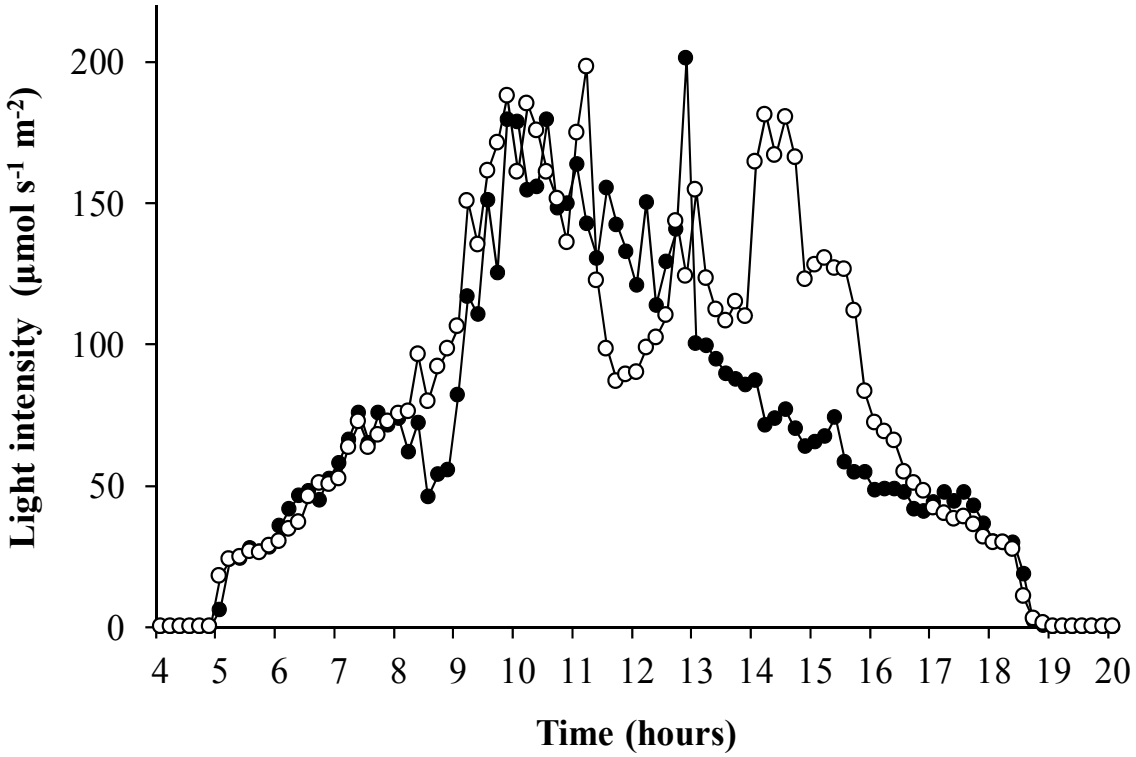

Fig. 3.2.3. Change in light intensity at rearing water surface of experimental tank in Experiment 3. Closed and open circle represent the mean value of each day in the experimental period and the value on 3 days-post-hatch, when effective promotion of ISI is possible, respectively.

Comparison of SL between WIS and WOIS

Table 3.2.4 shows the comparative results of SL between WIS and WOIS. SL was significantly greater in WIS than in WOIS on both 6 and 9 dph in each experiment.

Table 3.2.4. Comparison of standard lengths between the larvae with (WIS) and without (WOIS) inflated swimbladder in Pacific bluefin tuna, *Thunnus orientalis*

	Standard length (mm)	
	WOIS	WIS
Experiment 1		
6 dph	5.2 ± 0.2^{b}	5.3 ± 0.2^{a}
9 dph	6.6 ± 0.3^{b}	6.9 ± 0.3^{a}
Experiment 2		
6 dph	5.1 ± 0.3^{b}	5.2 ± 0.3^{a}
9 dph	6.2 ± 0.4^{b}	6.5 ± 0.3^{a}
Experiment 3		
6 dph	4.9 ± 0.3^{b}	5.0 ± 0.2^{a}
9 dph	6.1 ± 0.4^{b}	6.3 ± 0.3^{a}

Forty-five data were randomly selected in WIS and WOIS, respectively, within each days-post-hatch (dph) in each experiment using random numbers (In Experiment 2, the total numbers of data in WIS were 39 and 40 in 6 and 9 dph, respectively, due to lack of the number of sample.)

Standard lengths are shown as means with standard deviations (n = 45 in Experiment 1 and 3, n = 39 to 45 in Experiment2).

Within each dph and the experiment, different lower case letters indicate significant differences.

3.2.4 Discussion

This study have demonstrated that SFR during 16:00–19:00, when is twilight just before the end of light period, is effective to promote ISI in PBT larviculture, whereas SFR during both the dark period and the time from morning to early afternoon had no effect.

In Experiment 1, swimbladder inflation frequency for the SFR for 24 h (S.24; 81.1 ± 7.0% on 6 dph and 91.1 ± 5.7% on 9 dph) and during the light period (S.5–19; 78.9 ± 13.5% on 6 dph and 92.2 ± 5.1% on 9 dph) was significantly higher than that for dark period (S.19–5; 10.0 ± 3.3% on 6 dph and 11.1 ± 5.1% on 9 dph) without significant difference among S.24, S.5–19 and S.8–19 on both 6 and 9 dph (Table 3.2.1). This result indicates that the SFR during light period is effective to promote larval ISI, while the SFR during the dark period had no effect to promote larval ISI. This result also suggests that PBT larvae can achieve ISI by gulping air at the water surface during the light period.

In addition, swimbladder inflation frequency for the SFR during the ordinary SFR time period in hatchery without early morning from 05:00 to 08:00 (S.8–19; 86.7 ± 8.8% on 6 dph and 93.3 ± 3.4% on 9 dph) was the same as that for the SFR during the light period. This result indicates that the SFR during light period without early morning produce same effect as the SFR during light period to promote larval ISI, and suggest that the SFR during early morning is not necessary for promotion of larval ISI.

Strong aeration has been reported to reduce larval ISI frequency in Australian bass, *Macquaria novemaculeata* (Steindachner) (Battaglene and Talbot 1990, 1993), red sea bream and Japanese sea bass, *Lateolabrax japonicus* (Cuvier) (Kitajima et al. 1994). Vigorous water currents generated by strong aeration was considered to inhibit larval

access to the water surface to gulp air (Battaglene and Talbot 1990, 1993; Kitajima et al. 1994). In contrast, strong aeration enhanced swimbladder inflation in striped bass larvae (Doroshev and Cornacchia 1979). These findings indicate that larval ISI is affected by aeration, and optimal air-flow rate appears to be species specific.

On the other hand, in Experiment 1, the air-flow rate of tank aeration of three treatments of S.24, S.5–19 and S.19–5 was constant at 650 *ml*/min during 24 hours to compare the promotional effect of ISI among different timings in the day and to prevent larval sinking death in the night time. As a result, the aeration air-flow rate during the light period was larger in S.5–19 (650 *ml*/min) than in S.8–19 (130 *ml*/min), however, the swimbladder inflation frequency in these treatments showed similar value in Experiment 1 (Table 3.2.1). Therefore, it is concluded that such a difference of the air-flow rate in the light period did not affect the larval ISI. Furthermore, even if the ISI success is susceptible to strong aeration in PBT larvae, the strong aeration in the dark period to prevent sinking death would not affect the success of ISI in PBT larvae, because PBT larvae successfully achieve ISI during the light period as demonstrated in Experiment 1. However, further study is necessary to verify the influence of aeration intensity for ISI success.

Regarding the optimal timing to promote ISI, greater amberjack, *Seriola dumerili* (Risso), larvae can achieve the ISI only in photocycle with light and dark period and cannot achieve their ISI in continuous darkness (Hirata et al. 2009). In contrast, in striped trumpeter, larval ISI was promoted by providing a dark period, and larval swim-up behavior to gulp air at the water surface for their ISI was predominantly observed during the dark period (Trotter et al. 2003). These indicate that the optimal timing to promote ISI is species specific.

From the results of Experiment 2 and 3, the optimum timing of SFR can be more confined. In Experiment 2, the swimbladder inflation frequency for afternoon SFR (S.13–19; 83.3% on 6 dph and 88.3% on 9 dph) was same as that during the ordinary SFR timing in hatchery including morning time (S.8–19-E2; 83.3% on 6 dph and 81.7% on 9 dph) on both 6 and 9 dph. However, the swimbladder inflation frequency for morning to noon SFR (S.8–13; 1.7 % on 6 dph and 0.0% on 9 dph) showed extremely lower swimbladder inflation frequency than S.13–19 and S.8–19-E2 on both 6 and 9 dph, although no statistical analysis was done due to small sample size (n = 2; Table 3.2.2). These results indicate that the afternoon SFR from 13:00 to19:00 was concluded to be substantial to promote ISI. In Experiment 3, where the necessary period of SFR could be more confined, SFR during 16:00–19:00 gave significantly higher swimbladder inflation frequency than that during the early afternoon on both 6 and 9 dph (S.13–16; Table 3.2.3). In addition, although the time length of SFR in S.18–19 is one third of that in S.16–19, no significant difference of swimbladder inflation frequency was found between S.18–19 (84.4 ± 5.1%) and S.16–19 (70.0 ± 12.0%) on 9 dph in Experiment 3. This indicates the significance of a very short time of only 1 hour between 18:00 and 19:00 to promote ISI of larval PBT by SFR. However, swimbladder inflation frequency in S.18–19 (50.0 ± 6.7%) was significantly lower than that in S.16–19 (72.2 ± 1.9%) on 6 dph. This indicates that the SFR only for 1 hour between 18:00 and 19:00 is insufficient to promote ISI of larval PBT on 6 dph.

Results of this study indicate that the optimal timing of SFR to promote ISI is 16:00–19:00 in PBT larviculture. This short time period is twilight just before the end of light period, and the light intensity at rearing water surface of the experimental tank gradually decreased from ca. 50 to 0 μmol/s/m^2 (Fig. 3.2.3). Therefore, the light

intensity decrease before the end of light period may be a promotional factor for ISI. Moreover, the optimal SFR timing to promote ISI is considered to change with seasonal change in light period. This is supported by the result in Experiment 1 that is SFR during the dark period had no effect on ISI promotion. Further investigation should be performed on the effect of light condition and other factors on ISI promotion.

The ISI of PBT larvae began on 3 dph, and 'window' for ISI is extremely narrow 1 day of 3 dph under 26.5°C (Section 3.1 in this chapter) as mentioned in the Introduction section. Therefore, SFR should be done without missing this extremely finite term of a few hours before the end of light period on 3 dph to promote ISI effectively in PBT larviculture under the rearing temperature of 26.5°C.

In Experiment 1, the number of surface death larvae was largest at 18:00 (Fig. 3.2.2), and it corresponded to the optimal timing of the day to promote ISI as demonstrated in this study. In addition, surface death occurrence peaks on 3 dph and also corresponds to the 'window' for ISI (Section 3.1 in this chapter). Moreover, PBT larval swim up and their activity near the water surface was observed more frequently in a few hours before the end of light period on 3 dph than other times and dphs in this experiment, and similar behavior is usually observed also in the mass production tanks in hatchery. Therefore, these behaviors are considered to be for air gulping at water surface for ISI, and to trigger their surface death. In contrast, in PBT larvae, surface death can effectively be prevented by an oil film, while it is enhanced by SFR using a skimmer to promote ISI (Section 3.1 in this chapter); therefore, there is a contradiction between surface death prevention and ISI promotion on the optimal timing for ISI promotion of a few hours before the end of light period on 3 dph. Consequently, larval surface death will not avoid in ISI promotion by existing SFR using surface skimmer.

Therefore, further efforts to find effective solutions in order to avoid the a contradiction between surface death prevention and ISI promotion should be taken, although, the minimized operation of SFR within the optimal timing for ISI promotion during the few hours before the end of light period on 3 dph is only solution to mitigate surface death at present.

The ISI failure has an adverse effect on the larval growth in gilthead sea bream, *Sparus aurata* (Linnaeus), sea bass, *Dicentrarchus labrax* (Linnaeus), striped trumpeter (Chatain 1989; Chatain and Ounais-Guschemann 1990; Trotter et al. 2005). In this study, larval SL showed a greater tendency in treatments with higher swimbladder inflation frequency at 9 dph, while there was no significant difference among treatments in larval SL at 6 and 9 dph in each experiment except for Experiment 1. However, when larval SL was compared between WIS and WOIS, the SL was significantly greater in WIS than WOIS at both 6 and 9 dph in each experiment (Table 3.2.4). This result indicates that the ISI failure significantly affects growth during the early larval stage.

In this study, larval survival at 9 dph was not significantly different among treatments in Experiment 1 and 3, while it was varied among the treatments in Experiment 2. In addition, a clear relationship was not found between survival and swimbladder inflation frequency in each experiment. No clear relationship between survival and swimbladder inflation frequency in experimental tanks has been also found in the Section 3.1 of this chapter. In contrast, Chapter 2 demonstrated that larval ISI failure reduces survival via enhancing sinking death in mass culture tanks even when preventive measure of sinking death by enhanced aeration was employed. Sumida et al. (2011) reported that the aspect ratio (water depth/the half width or radius of tank: AR) of a tank affects flow patterns generated by aeration within that tank, and suggested that

high AR prevents larval sinking death. Moreover, tanks AR used in study were higher in small experimental tank in Chapter 1 (1.20) and Section 3.1, 3.2 in this chapter (1.04) than the mass-scale tanks used in Chapter 2 (0.35–0.44). Therefore, the inconsistency on survival in this study may be attributable to the difference of tank AR used in each experiment. The possibility to improve larval survival by suitable tank AR is a topic of research in PBT mass-scale larviculture.

In conclusion, this study demonstrated that effective ISI promotion by SFR can be achieved only in extremely limited term of a few hours before the end of light period in PBT larvae. SFR should be done without missing this timing on 3 dph to promote ISI effectively in PBT larviculture.

Chapter 4

Influence of swimbladder inflation failure on mortality, growth and development of lordotic deformity in Pacific bluefin tuna, *Thunnus orientalis*, postflexion larvae and juveniles

4.1 Introduction

Chapter 2 and 3 demonstrated that swimbladder inflation (SBI) failure increases mortality due to sinking death and reduces growth in early larval stage of PBT larviculture. However, SBI failure has been reported to result not only in poor survival and growth in early larval stage but also in growth and/or development of lordotic deformity, which is characterized by the abnormal V-shaped sagittal curvature of vertebra, in later larval and juvenile stage (Spectorova and Doroshev 1976; Kitajima et al. 1981; Chatain 1989, 1994; Kitajima et al. 1994; Goolish and Okutake 1999; Jacquemond 2004; Trotter et al. 2005). These negative influences of SBI failure largely reduce fingerling production efficiency in many other aquaculture fish species. Therefore, it is essential to elucidate the influence of SBI failure on growth and development of lordotic deformity to establish the technology of fingerling production. However, the relationship between SBI failure and mortality, growth, and lordotic deformity in PBT postflexion larvae and juveniles has not yet been investigated.

In this chapter, it was investigated that the influence of SBI failure on mortality and growth in PBT postflexion larvae and juveniles (Experiment 1), lordotic deformity

and growth in PBT juveniles (Experiment 2) to obtain information for the improvement of fingerling production technology in PBT.

4.2 Materials and methods

Experiment 1

Experiment 1 was conducted to examine the relationships between SBI failure and mortality and growth in PBT postflexion larvae and juveniles. Rearing experiment was conducted from postflexion to juvenile stage (from 18 to 30 dph: Fig. 4.1) with two replications. In Experiment 1, a cohort was used including both fish with (WIS) and without (WOIS) inflated swimbladder, which were reared without any artificial SBI promotion, because the sorting live anesthetized larval individuals into WIS and WOIS based on the body density differences (Chatain and Corrao 1992; Jacquemond 2004) is difficult to apply to PBT.

Eggs and preparatory larval rearing

Fertilized eggs were obtained from spontaneous spawning of cultivated PBT brood stock fish in a sea net cage. Eggs of the late embryonic stage were introduced into a circular fiberglass 30 *kl* tank (6 m internal diameter, 1.1 m depth) at a density of 5000 eggs/*kl*, and incubated at 23°C prior to hatching. Hatched larvae were reared at 25.0°C until commencement of feeding on 2 days-post-hatch (dph). Eggs had a high normal hatching rate of 93.2%. In this study, the dph was defined to hatching day as 0 dph. To prevent larval surface death, 1.0 to 1.5 *ml* of feed oil was added as drops every day onto

the rearing water surface to form a surface film from hatching to 2 dph as in Chapter 2 and 3.

The larvae were fed with S-type rotifers *Brachionus plicatilis* sp. complex (Hagiwara et al. 2007) enriched with *Nannochloropsis oculata*, a commercial product (Marine Glos EX, Nisshin Marinetech Co. Ltd., Yokohama, Japan) and taurine (Japan Nutrition Co., Ltd., Tokyo, Japan) from 2 to 10 dph. From 10 dph, the larvae were fed with enriched *Artemia franciscana* (Kellogg), and yolk-sac larvae of Japanese parrot fish *Oplegnathus fasciatus* (Temminck and Schlegel). *N. oculata* or commercial concentrated microalgae (Fresh Chlorella V-12, Chlorella Industry Co., Ltd., Tokyo, Japan) were added every day to the rearing water from 2 to 15 dph.

Air was supplied to the rearing water using four air stones with air-flow rates of 1.7 *l*/min and placed at the center of each tank bottom. During the night (19:00 to 05:00), 12 additional air stones with air-flow rates of 3.0 *l*/min were added on the tank bottom from 2 to 9 dph in order to prevent mass mortality due to sinking death as in Chapter 2. Surface film formation was allowed without employing a surface skimmer to promote swimbladder inflation in order to generate the cohorts failed swimbladder inflation which includes WOIS based on the results in Chapter 3. Other rearing conditions in 30 *kl* larval rearing tank were as follows: temperature, 26.5 ± 0.8°C; dissolved oxygen, 106.6 ± 5.1%; salinity, 31.7 ± 0.3; pH, 7.8 ±0.3.

Rearing experiment of postflexion larvae and juveniles

On 18 dph (the transitional period from postflexion to juvenile stage: pectoral fin rays appear, or they are complete: “M” to “O” stage shown in Kawakami et al. 2008; Fig. 4.1), when SBI observation in live and dead fish became possible by soft X-rays,

postflexion larvae and juveniles were transferred from a 30 *kl* larval rearing tank to two 500 *l* volume cylindrical polycarbonate tanks (diameter 100 cm, height 62 cm) at a density of 200 individuals per tank. They were fed with enriched *A. franciscana* and yolk-sac larvae of Japanese parrot fish, and formulated feed according to their growth, and reared until juvenile stage on 30 dph (the preopercle spines disappear, or the first and second dorsal fins are completely separated: "P" to "Q" stage shown in Kawakami et al. 2008). Rearing water was gently agitated by aeration using an air stone with air-flow rate of 130 *ml*/min placed at the center of tank bottom to maintain homogeneity of environment and food distribution. Dead fishes during the experimental period were taken up and cryopreserved until soft X-ray examination. Other rearing conditions in the 500 *l* rearing tanks were as follows: temperature, 26.9 ± 0.2°C; dissolved oxygen, 106.6 ± 4.9%; salinity, 31.8 ± 0.2; pH, 7.8 ± 0.1.

Experiment 2

Experiment 2 was conducted to examine the relationships between SBI failure and lordotic deformity, and growth in juveniles. Two trials (Trial 1 and 2) were carried out using a different cohort reared from a different batch of eggs, and these cohorts included both WIS and WOIS as in Experiment 1.

Examination of SBI, vertebral deformity, and growth were carried out on the early juvenile stage of 22 dph (pectoral fin rays develop completely: "O" stage shown in Kawakami et al. 2008) and on the juvenile stage of 37, 54, 78 dph in Trial 1, and on the juvenile stage of 62 dph in Trial 2 (Fig. 4.1).

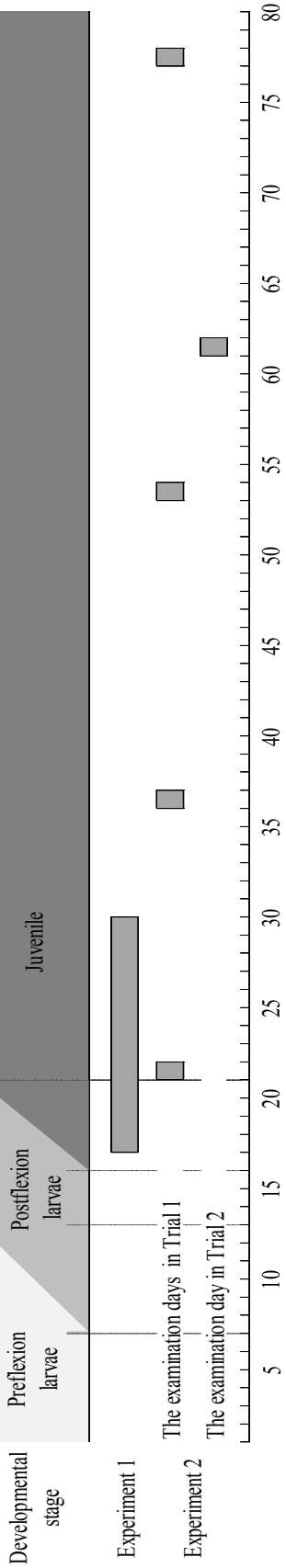

Fig. 4.1. Developmental stage of Pacific bluefin tuna, *Thunnus orientalis*, and the experimental periods of Experiment 1 and the examination days in Experiment 2.

Eggs and preparatory larval rearing and juvenile rearing

In Trial 1 of Experiment 2, after providing fish partly for Experiment 1 at 18 dph, the remaining cohort in the 30 *kl* larval rearing tank was continuously reared with feeding of enriched *A. franciscana*, yolk-sac larvae of Japanese parrot fish, and formulated feed according to the growth. On 38 dph, 1400 juveniles were transferred to a 30 *kl* circular fiberglass weaning tank (6 m internal diameter, 1.1 m depth), and were reared with formulated feed until the final examination on 78 dph.

In Trial 2, eggs were introduced into a quadrangular concrete 20 *kl* tank (4.5 m × 4.5 m × 1.0 m) at a density of 5000 eggs/*kl*. Eggs had a high normal hatching rate of 94.1%. Methods of egg incubation and larval and juvenile rearing were the same as in Experiment 1 and Trial 1. On 36 dph, 300 juveniles were transferred to a 30 *kl* circular fiberglass weaning tank (6 m internal diameter, 1.1 m depth), and were reared with formulated feed until the final examination on 62 dph.

Other conditions in the larval rearing tank and weaning tank (in parentheses) were as follows: temperature, 26.6 ± 0.5°C (24.1 ± 4.1°C); dissolved oxygen, 104.9 ± 4.4% (100.1 ± 3.1%); salinity, 31.7 ± 0.4 (31.9 ± 0.4); pH, 7.9 ± 0.2 (8.1 ± 0.1) in Trial 1, and temperature, 26.1 ± 0.7°C (25.5 ± 1.0°C); dissolved oxygen, 105.7 ± 6.5% (103.2 ± 6.1%); salinity, 31.2 ± 0.3 (31.5 ± 0.5); pH, 7.7 ± 0.3 (8.0 ± 0.1) in Trial 2.

In this study, *A. franciscana* was enriched with a commercial product (Marine Glos EX, Nisshin Marinetech, Co. Ltd., Yokohama, Japan), and a commercial formulated feed was fed to PBT larvae and juveniles (Magokoro, Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan: Takii et al. 2007; Ji et al. 2008; Biswas et al. 2009).

Measurements and observations

In the early larval stage, 30 larvae were used to determine the proportion of WIS*, and 20 larvae each of WIS and WOIS were used to measure the standard length (SL: length from the rostral tip to the end of the notochord) on 6 and 9 dph in Experiment 1 and 2 (in Trial 2 of Experiment 2, only 10 larvae were obtained in WIS at 8 dph due to the low proportion of WIS). Larval SBI was observed under a stereomicroscope, and SL was measured using digital images of samples taken with a digital camera (Moticam 2000, Shimadzu Rika Corp., Tokyo, Japan) with a software package for image analysis (Motic Images Plus 2.2s, Shimadzu Rika Corp., Tokyo, Japan).

In Experiment 1, 210 fishes in a 30 *kl* source tank at the start of experiment (18 dph), and all the survived fishes at the end of experiment (30 dph), and all the dead fish during the experimental period were used to determine the proportion of WIS and WOIS in juveniles using soft X-ray image, then SL and body weight (BW: wet weight) of these fishes other than the dead fish were measured. In Experiment 2, 110 to 250 juveniles were sampled on 22, 37, 54, 78 dph in Trial 1, and 62 dph in Trial 2 and the proportion of WIS and vertebral deformity were determined using soft X-ray image with the measurement of SL and BW. In Trial 1, dead fish from 51 to 57 dph and from 62 to 68 dph were also examined for the proportion of WIS and vertebral deformity of juveniles.

Statistical analysis

Student's *t*-test or Welch's *t*-test were employed to test the significance of

* In this study, “swimbladder inflation frequency” used in previous chapters was represent “proportion of WIS”, because we discussed the results of this study including the proportion of WOIS.

differences in SL and BW between WIS and WOIS. Fisher's exact test was employed to test the significance of difference in the proportions of WIS and WOIS in Experiment 1 and 2. Statistical analyses were performed using statistical software (Kypolt 5.0 for Windows, KyensLab, Tokyo, Japan). In this study, $P < 0.05$ was used as the criteria of significant difference in these tests.

4.3 Results

Experiment 1

Preparatory larval rearing

For the larvae reared for Experiment 1 and Trial 1 of Experiment 2, larval SL showed the same value in WIS and WOIS on 5 dph, however, it was significantly smaller in those of WOIS than WIS on 8 dph (Table 4.1). The proportion of WIS in larvae was low on both 5 (26.7%) and 8 dph (33.3%) as a result of no promotion of SBI. Survival rate on 8 dph was also low (1.6%; Table 4.1).

Rearing experiment from postflexion larvae to juveniles

The fish developed to postflexion stage from preflexion stage between 8 and 13 dph, and postflexion to juvenile between 17 and 21 dph (Fig. 4.1).

Survival rate at the end of the experiment (30 dph) in each tank was 72.4 and 68.2 (mean: 70.3%, Table 4.2). SL and BW were significantly greater for WIS than for WOIS at both the start of the experiment (18 dph; transition period from postflexion to juvenile stage) and the end of the experiment (30 dph; juveniles stage). However the growth rate during the experimental period (18 to 30 dph) was higher in those of WOIS than WIS (Table 4.2).

Table 4.1. Growth of the fish with (WIS) and without (WOIS) inflated swimbladder, and survival rate, proportion of WIS of Pacific bluefin tuna, *Thunnus orientalis,* larvae during the preparatory larval rearing for Experiment 1 and Trial 1 of Experiment 2

		WOIS	WIS
SL (mm)			
	5 dph*	4.6 ± 0.2	4.6 ± 0.2
	8 dph	5.4 ± 0.4 [a]	5.8 ± 0.4 [b]
Survival (%)	8 dph	1.6	
Proportion of WIS (%)			
	5 dph	26.7	
	8 dph	33.3	

Standard lengths (SL) are shown as mean with standard deviation (n = 20).
Different lower case letters indicate significant differences ($P < 0.01$).
*Days-post-hatch.

During the preparatory rearing, the proportion of WIS in larvae increased from 33.3% on 8 dph (Table 4.1) to 51.9% at start of experiment of 18 dph, and it increased furthermore to 75.2% at end of experiment of 30 dph (Fig. 4.2). The proportions of WIS and WOIS in the dead fish, which were collected during the experimental period from 18 to 30 dph, were 60.7% and 39.3% respectively, and these values did not differ significantly from those at the start of the experiment (18 dph; Fig. 4.2).

The proportion of WIS in survived fish was 75.2%, moreover, the proportion of WIS in all fish (survived and dead fish) was 70.8%. They were both significantly higher than that at the start of the experiment (51.9%, $P < 0.001$; Fig.4.2).

Table 4.2. Growth in the fish with (WIS) and without (WOIS) inflated swimbladder and survival rate in postflexion larvae and juveniles of Pacific bluefin tuna, *Thunnus orientalis,* reared for 12 days from 18 to 30 days-post-hatch (dph) in Experiment 1

	WOIS	WIS
SL (mm)		
18 dph	12.5 ± 1.5 [a]	14.5 ± 1.4 [b]
30 dph	43.9 ± 3.4 [a]	46.5 ± 3.1 [b]
BW (mg wet/fish)		
18 dph	34.2 ± 15.8 [a]	52.3 ± 18.4 [b]
30 dph	1066.6 ± 249.2 [a]	1290.8 ± 347.0 [b]
Growth rate (%)	2946, 3310 (3128)	2255, 2652 (2454)
Survival rate (%)	72.4, 68.2 (70.3)	

Standard length (SL) and body weight (BW) are shown as mean with standard deviation (n = 69 to 208).
SL and BW in the same dph, different lower case letters indicate significant differences ($P < 0.01$).
The data for 18 and 30 dph are values for live fish at the start of experiment and survived fish at the end of experiment respectively.
Growth rate was calculated as follows: BW at 30 dph / BW at 18 dph × 100.
Growth and survival rates are shown for two replicate tanks and the mean value is given in parentheses.

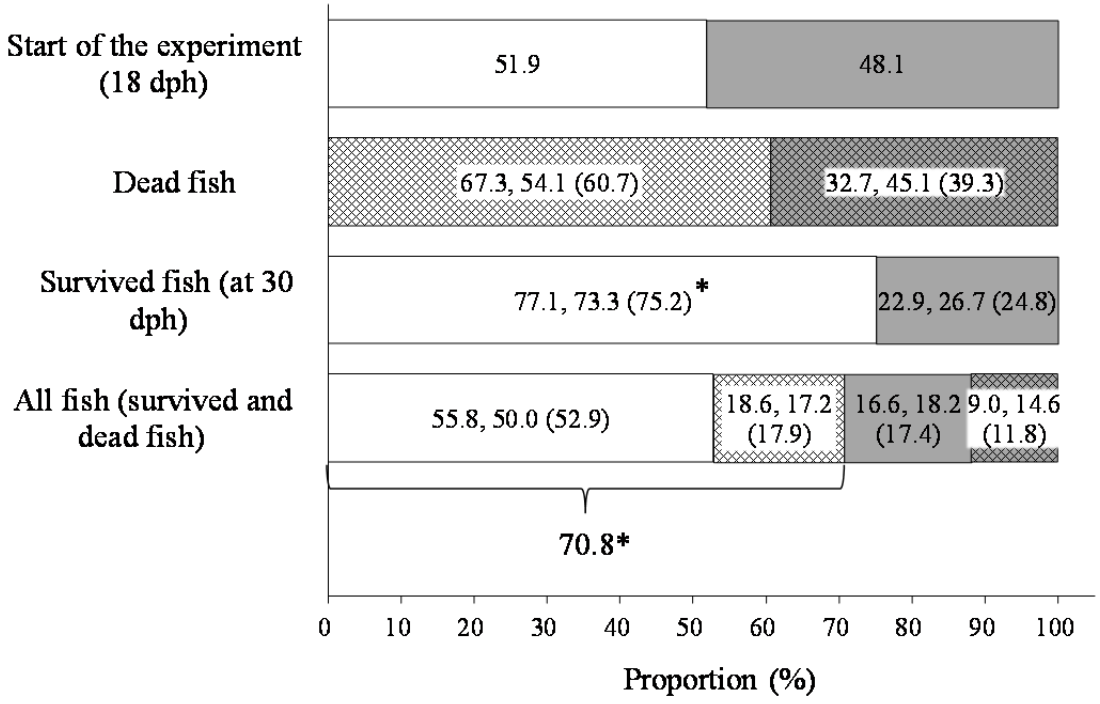

Fig. 4.2. Proportion of the fish with (WIS) and without (WOIS) inflated swimbladder at the start of experiment and in dead fish during the experimental period and survived fish at the end of experiment in Pacific bluefin tuna, *Thunnus orientalis,* in Experiment 1. White and grey indicate WIS and WOIS, respectively. Lattice pattern indicates the dead fish. Each bar represents the mean value of two replicate tanks. Values in the bar show each value of two replicate tanks and the mean in parentheses. Asterisk represents a significant difference to the value of proportion of WIS at the start of the experiment (18 days-post-hatch: dph) ($P < 0.001$; Fisher's exact test).

Experiment 2

Preparatory larval rearing

Data of the preparatory larval rearing for Trial 1 are shown in Table 4.1 (same cohort as used in Experiment 1). In the preparatory larval rearing for Trial 2 (different cohort), SL was significantly smaller in the WOIS larvae than that in those of WIS at the end of the preparatory rearing (9 dph, flexion stage; Table 4.3). The proportions of WIS

were low value of 0.0% at 6 dph and 10.0% on 9 dph as a result of no SBI promotion. Survival rate on 9 dph had a low value of 6.5% (Table 4.3).

Table 4.3. Growth of the fish with (WIS) and without (WOIS) inflated swimbladder, and survival rate, proportion of WIS of Pacific bluefin tuna, *Thunnus orientalis,* larvae in the preparatory larval rearing for Trial 2 of Experiment 2

		WOIS	WIS
SL (mm)			
	6 dph	5.1 ± 0.2	—
	9 dph	6.0 ± 0.3[a]	6.2 ± 0.2[b]
Survival (%)	9 dph	6.5	
Proportion of WIS (%)			
	6 dph	0.0	
	9 dph	10.0	

Standard lengths (SL) are shown as mean with standard deviation. (n = 20 in WOIS, n = 10 in WIS on 9 days-post-hatch (dph) due to low the proportion of WIS).
Different lower case letters indicate significant differences ($P < 0.05$).

Rearing experiment of juveniles

SL and BW of WIS in juveniles were significantly greater than WOIS on 22 dph; however, no significant differences in SL and BW were found between WIS and WOIS on 37, 54, 78 dph in Trial 1, and on 62 dph in Trial 2 (Table 4.4). The proportion of WIS in juveniles was from 66.1 to 70.4% in sampled live fish from 22 to 78 dph, and 66.5% in the dead fish from 51 to 57 dph and from 62 to 68 dph. There were no

significant differences in the proportion of WIS between live and dead fish on each dph (Fisher's exact test).

Lordotic deformities of juveniles were found neither in WOIS nor in the WIS on every examined dph, and in the dead fish obtained in Trial 1, although other vertebral deformities were found such as centrum defects, undersized centrums (Hattori 2004), and dislocation of vertebral column due to collision with the tank wall (Fig. 4.3, Table 4.4).

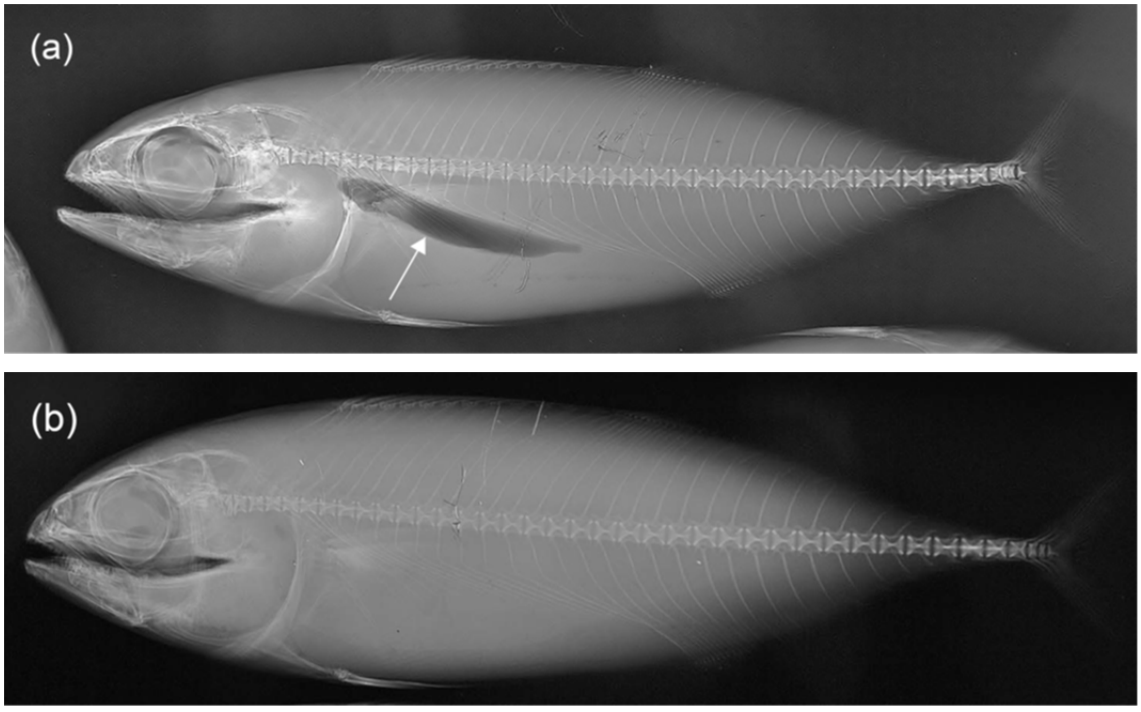

Fig. 4.3. Soft X-rays images of Pacific bluefin tuna, *Thunnus orientalis*, juveniles on 78 days-post-hatch. (a) a juvenile with inflated swimbladder and normal vertebrae (standard length [SL]: 179 mm); arrow indicates the inflated swimbladder. (b) a juvenile without inflated swimbladder and with normal vertebrae (SL: 194 mm).

Table 4.4. Comparison of growth and incidence of vertebral deformity in Pacific bluefin tuna, *Thunnus orientalis,* juveniles with (WIS) and without (WOIS) inflated swimbladders in Experiment 2

	Dph	WOIS					WIS					Proportion of WIS (%)
		SL (mm)	BW (g wet/fish)	Vertebral deformities (%)		*n*	SL (mm)	BW (g wet/fish)	Vertebral deformities (%)		*n*	
				Lordotic deformity	Other vertebral deformities				Lordotic deformity	Other vertebral deformities		
	22	24.1 ± 2.5 [a]	0.2 ± 0.1[a]	0.0	6.5	33	28.8 ± 3.2 [b]	0.4 ± 0.1[b]	0.0	9.1	77	70.0
	37	67.7 ± 8.7	4.3 ±1.8	0.0	9.2	73	69.5 ± 7.0	4.5 ± 1.4	0.0	6.8	174	70.4
Trial 1	54	118.9 ± 13.0	31.4 ± 13.2	0.0	10.0	40	120.4 ± 13.4	34.4 ± 13.3	0.0	7.7	78	66.1
	78	179.0 ± 16.9	120.5 ± 41.9	0.0	10.8	37	176.4 ± 17.6	116.9 ± 42.3	0.0	12.6	87	70.2
	Dead fish	—	—	0.0	12.8	59	—	—	0.0	18.7	117	66.5
Trial 2	62	141.9 ± 12.2	60.2 ± 18.5	0.0	8.5	49	140.5 ± 12.2	58.3 ± 17.5	0.0	10.2	59	54.6

Standard length (SL) and body weight (BW) are shown as mean with standard deviation. Different lower case letters indicate significant differences ($P < 0.001$).

Other vertebral deformities include centrum defects, undersized centrums, and dislocation of vertebral column caused by collision with tank wall.

Dead fish were examined during 51 to 57 and 62 to 68 days-post-hatch (dph).

4.4 Discussion

This study examined the influence of SBI failure on mortality and growth from PBT postflexion larvae to juveniles, and growth and lordotic deformity in juveniles.

SBI failure causes growth retardation until juveniles of 30 dph as in other reported species. However, SBI failure did not cause growth retardation in juveniles after 37 dph and did not affect mortality after the transitional period from postflexion to the juvenile stage. Moreover, lordotic deformities were not observed in WOIS in juveniles differently from various other aquaculture fish species reported. Furthermore, this study showed that SBI occurs in WOIS after the transitional period from the postflexion to the juvenile stage.

Mortality of WOIS in postflexion larvae and juveniles

In Experiment 1, both the proportions of WIS and WOIS were not significantly different between the fishes sampled at the start of the experiment (18 dph) and the dead fishes during the experiment (from 18 to 30 dph; Fig. 4.2). This indicates that no significant difference in mortality between the WIS and WOIS occurs after the transitional period from the postflexion to the juvenile stage. Additionally, there was no significant difference in the proportions of WIS between live and dead juveniles in Experiment 2 (Table 4.4). Therefore, we can conclude that SBI failure does not lead to serious mortality after the transitional period from postflexion to juvenile stage in PBT. In contrast, SBI failure increases mortality caused by sinking death in PBT during the early larval stage (Chapter 2). These indicate that the influence of SBI failure on mortality is stage specific in PBT. On the other hand, in juvenile stage, it has been

reported that 86 to 100% of dead fishes had no inflated swimbladder in sea bass, *Dicentrarchus labrax* (Linnaeus), (Chatain 1989; Chatain and Dewavrin 1989). These indicate that the influence of SBI failure in juvenile stage is species specific.

SBI failure and larval and juvenile growth

Regarding the influence of SBI failure on growth in the early larval stage, growth retardation in WOIS larvae was not observed on 5 dph, while growth retardation significantly appeared in WOIS larvae on 8 and 9 dph in preparatory larval rearing for Experiment 1 and 2 (Table 4.1 and 4.3). Moreover, in Chapter 3, growth retardation was also observed in WOIS on 8 or 10 dph (Fig. 3.1.5, 3.1.8) and on 6 and 9 dph (Table 3.2.4). In addition, in the later stage, SL and BW of WOIS were also significantly smaller than that of WIS in the transitional period from the postflexion to the juvenile stage of 18 dph and juveniles of 30 dph in Experiment 1 (Table 4.2). It was the same in early juveniles on 22 dph in Experiment 2 (Table 4.4). These results indicate that SBI failure causes larval growth retardation from 6 or 10 dph to juvenile stage up to 30 dph.

On the other hand, the growth rate from 18 (transitional period from postflexion to juvenile stage; Fig. 4.1) to 30 dph (juvenile stage) was higher in WOIS than WIS (Table 4.2). Therefore, growth retardation observed on 30 dph was considered to be attributed to the differences in SL and BW which already appeared on 18 dph.

Indeed, on 37 dph and later, no significant differences in SL and BW of juveniles were found between WIS and WOIS (Table 4.4). These results suggest that SBI failure has no effect on the growth of juveniles after 37 dph, and its influence on growth is stage specific in PBT as well as mortality. The WOIS individuals are

presumably able to attain high growth where the influence of lacking functional swimbladders is reduced around the metamorphic stage to juveniles.

Larval growth retardation due to SBI failure has been reported in sea bass and sea bream (Chatain 1989; Chatain et al. 1990), snapper, *Pagrus auratus* (Bloch and Schneider; Battaglene and Talbot 1992), striped trumpeter, *Latris lineata* (Bloch and Schneider; Trotter et al. 2005). Therefore, growth retardation due to SBI failure in the larval stage seems to be common among fish species. On the other hand, in juveniles, the growth of yellowtail amberjack *Seriola lalandi* (Valenciennes) juveniles was affected by SBI failure (Kitajima et al. 1994). Therefore, the influence of SBI failure on growth in the juvenile stage also seems to be species specific.

SBI after the transitional period from postflexion to juvenile stage in PBT

Based on the results of this study, it is suggested that WOIS are able to inflate their swimbladder after the transitional period from postflexion to juvenile. In Experiment 1, the proportion of WIS (70.8%) in all fish (survived and dead fish: not only in the dead fish) was significantly higher than that at the start of the experiment on 18 dph corresponding to the transitional period from postflexion to juvenile stage (50.9%, Fig. 4.2). This result indicates that the SBI in WOIS can occur after the transitional period from postflexion to juvenile stage.

The SBI in the juvenile stage has been reported as "late or secondary inflation of swimbladder" (Chatain 1994) in red sea bream *Pagrus major* (Temminck and Schlegel), sea bream *Sparus auratus* (Linnaeus), perch *Perca fluviatilis* (Linnaeus) (Kitajima et al. 1981; Chatain 1994; Jacquemond 2004). The mechanism of late inflation of the swimbladder is presumed to be different from the initial SBI, which is

induced by air gulping and its supply to the swimbladder via the pneumatic duct, and to involve gas secretion by the gas gland.

The PBT belonging to the Scombridae is a physoclistous fish, but they have a physostomous swimbladder during the larval stage (Itazawa 1991; Alexander 1993; Kaji 2000). In PBT larvae, initial SBI is triggered by air gulping, and starts at 3 dph; moreover the window is extremely narrow of 1 day on 3 dph (Section 3.1 in Chapter 3). Kaji (2000) reported the absence of pneumatic duct in juveniles with the suggestion of the atrophy of pneumatic duct in the flexion larvae, and he observed the forming the gas gland in larvae of 7 dph in PBT. Therefore, the increase in the proportion of WIS in postflexion larvae and juveniles, observed in this study, is considered to be due to the late inflation of the swimbladder independent of air gulping carried out for initial SBI.

In Experiment 1, the proportion of WIS also significantly increased during the preparatory larval rearing from 8 dph (at the start of flextion stage; 33.3%, Table 4.1) to the start of the experiment on 18 dph (at the transition period from postflexion to juvenile stage; 50.9%, Fig. 4.2), although it maintained a low value between 5 and 8 dph in the early larval stage (Table 4.1). SBI start at 3 dph for PBT (Section 3.1 in Chapter 3), and the window is extremely narrow of 1 day on 3 dph as mentioned above. Therefore, the increased proportion of WIS between 8 and 18 dph could also be caused by the occurrence of SBI independent of air gulping. However, another possible cause, e.g. the selective mortality of WOIS individuals, cannot be denied. Further study should be performed to clarify the influence of SBI failure and the cause of the increasing proportion of WIS in this period.

Lordotic deformity

Development of lordotic deformity due to SBI failure has been reported in many cultured species: red sea bream, Japanese sea bass, *Lateolabrax japonicus* (Cuvier and Valenciennes), amberjack (Kitajima et al. 1981, 1994), sea bass, sea bream (Chatain 1989, 1994), wild and cultured perch (Egloff 1996; Jacquemond 2004), and it reduces the fingerling production efficiency in these aquaculture species, because the deformed fish have no value as fingerlings. In contrast, PBT juveniles with lordotic deformity were found neither in the WOIS nor in the WIS in both trials of Experiment 2 (Table 4.4, Fig. 3). Moreover, in this study, lordotic deformity was also not observed in the dead fish (Table 4.4). These indicate that the SBI failure does not cause lordotic deformity in PBT juveniles. In other words, these results mean that there is no reduction of production efficiency of PBT fingerlings via lordotic deformity due to SBI failure unlike in other species.

The authors assume the difference of the effect of SBI failure between PBT and other species is as follows. Continuous swimming in an obliquely upward posture has been observed in WOIS individuals of red sea bream, sea bream, sea bass and wild perch (Chatain 1989; Kitajima et al. 1994; Egloff 1996; Jacquemond 2004). Such swimming is considered to be a compensatory behavior for increased body density due to the lack of functional swimbladder, but it exerts a high pressure on the vertebrae mechanically, and consequently leads to lordotic deformity (Chatain 1989, 1994; Kitajima et al. 1994; Egloff 1996; Jacquemond 2004). Therefore, there should be a specific reason for PBT not to have an adverse effect causing lordotic deformities even if they fail to inflate their swimbladders. The PBT change their swimming mode from intermittent swimming in the larval stage to continuous cruising in the juvenile stage,

and their swimming speed rapidly increases after the transition from larvae to juveniles (Fukuda et al. 2010). Although the body density of scombrid fish including tunas is greater than that of seawater, the submerged weight can be opposed by the lift primarily produced by the pectoral fins with continuous swimming (Magnuson 1970, 1973, 1978; Tamura and Takagi 2009). Therefore, for PBT it may be unnecessary to swim in an obliquely upward posture for compensation of their increased body density due to the lack of a functional swimbladder. In fact, PBT juveniles were observed to swim against the horizontal circular current continuously in the rearing tanks, and swimming in an obliquely upward posture as in red sea bream and perch was not observed at all in Experiment 2. Such characteristic features of PBT seem to be the specific reason why they did not develop lordotic deformity in WOIS. Moreover, the results, that SBI failure did not cause mortality and growth retardation in juveniles, may also involve such characteristic swimming features of PBT.

However, the swimbladder controls the buoyancy of fishes (Itazawa 1991; Alexander 1993); therefore, the influence of SBI failure on energy consumption for swimming cannot be denied. Further investigation is required to clarify the influence of SBI failure and the function of swimbladder including the adult stage in PBT.

This study demonstrated that SBI failure does not cause serious mortality after the transitional period from postflexion larvae to the juvenile stage or cause growth retardation and lordotic deformity in PBT juveniles. However, it reduces survival in the early larval stage and growth in larval to early juvenile stage, therefore initial SBI should be promoted in the early larval stage in PBT fingerling production.

General discussion

1. Achievements in this study

To improve the efficiency of the PBT mass fingerling production, prevention of early larval mortality due to sinking death and surface death is essential.

Chapter 1 demonstrated that increased air supply rate during the nighttime mitigate larval mortality due to sinking death in PBT larviculture. Chapter 2 demonstrated that larval ISI failure increases larval mortality due to sinking death in PBT larviculture in mass-scale tanks and also indicated that significantly lower swimbladder inflation frequency in larvae found at tank bottom at night than that within larvae in both the upper and middle layers of the rearing water column. Therefore, larval mortality due to sinking death can be improved by mitigation of larval sinking via both flow control of rearing water during the nighttime and promotion of larval ISI. These preventive measures of sinking death should be adopted in combination in the practical PBT fingerling production. Moreover, Chapter 3 elucidated that the PBT larvae require air gulping for ISI as reported in other marine fishes, and that the optimal period and timing of the day to effectively promote ISI is extremely finite term of twilight just before the end of light period on 3 dph. Therefore, to promote ISI effectively, SFR should be done without missing extremely finite term of a few hours before the end of light period on 3 dph in PBT larviculture.

If these measures to prevent sinking death do not applied in PBT larviculture, early larval survivals were poor value of fewer than 10% or nearly zero, while in the rearing case with applying these measures, larval survival were improved to around 20% in mass-scale tank as shown in Chapter 2.

Additionally, Chapter 4 elucidated that while, swimbladder inflation (SBI) failure causes growth retardation from larval to early juvenile stage as various other aquaculture fish species reported, SBI failure caused neither significant levels of mortality after the transitional period from postflexion to the juvenile stage nor growth retardation in juveniles after 37 dph and lordotic deformities in juvenile in PBT differently from various other aquaculture fish species reported. However, SBI should be promoted in PBT fingerling production, because SBI failure cause a significant level mortality due to sinking death in PBT early larval stage and growth retardation from larval to early juvenile stage.

In recent years, larval mortality due to sinking death is getting attention and many studies have been performed to prevent sinking death in other aquaculture fish species with larval sinking death. However, the relationship between swimbladder inflation failure and larval survival, vertical distribution within rearing tanks were firstly revealed in aquaculture fish species with larval sinking death in this study. Furthermore, the relationship between ISI promotion and surface death was also firstly investigated in this study. Therefore, such relationships, specific promotional method for larval ISI and study on the flow control method to prevent sinking death should be investigated to improve the mass-production technique also in aquaculture fish species other than PBT. The outcomes of such study would contribute to improve the mass-production technique on these species with larval sinking death.

2. Proposal to improve PBT fingerling production efficiency and research for the future

Here, as the conclusion of this study, the author proposes the effective method to mitigate larval sinking death and recommends the future research to improve PBT mass fingerling production technology.

2-1. Prevention of larval sinking death in PBT larviculture

Chapter 1 demonstrated that increasing the air supply rate during the nighttime enhances PBT larval survival; moreover, it increases rearing water circulation and reduces the size of the EDZ (estimated danger zone for larval sinking). These results indicates that flow control in rearing water by aeration during the nighttime is effective to improve larval survival, and provide insight into the relationship between PBT larval survival and flow field characteristics.

PBT larval body density is greater than that of rearing water, even when larvae possess an inflated swimbladder, and this is believed to be the primary cause of sinking death (Takashi et al. 2006). Tanaka et al. (2009) suggested that the larval sinking to tank bottom is the ordinary event in the rearing tanks and not due to swimbladder inflation failure. However, Chapter 1 elucidated that larval sinking velocity was significantly higher in larvae without inflated swimbladders than in those with inflated swimbladders from 5 dph. Moreover, Chapter 2 demonstrated that larval ISI failure reduces survival in PBT larviculture in mass production tanks despite the larger density of larval body than the rearing water even when they successfully inflate their swimbladders. In addition, the study showed that larvae at tank bottom had higher distribution density and significantly lower swimbladder inflation frequency than those distributed in the upper and middle layers. These results suggest that reduction of larval survival is caused by the increase of sinking death ratio of larvae with ISI failure.

Based on these results, therefore, it can be concluded that larval survival due to sinking death should be improved by mitigation of larval sinking via both flow control of rearing water during the nighttime and promotion of larval ISI. These preventive measures of sinking death should be adopted in combination in the practical PBT fingerling production.

2-2. Promotion of ISI in PBT larviculture

Promotion of larval ISI improves larval mortality due to sinking death by mitigation of larval sinking to tank bottom. Section 3.1 in Chapter 3 showed that ISI in PBT larvae can be promoted by surface film removal on rearing water, and surface film on rearing water inhibits larval ISI. This suggests that the PBT larvae require air gulping for ISI as reported in other marine fish, and it was firstly confirmed in scombrid species in this study.

Moreover, Section 3.1 in Chapter 3 showed that the 'window', when effective promotion of ISI is possible, is extremely narrow compared with other reported fish species 1 day of 3 dph under 26.5°C in PBT larvae. Furthermore, Section 3.2 in Chapter 3 also showed that effective ISI promotion by surface film removal (SFR) can be achieved only in extremely limited term of a few hours of the day before the end of light period in PBT larvae, whereas SFR during the dark period and from morning to early afternoon produces no effect of ISI promotion.

Therefore, SFR should be done without missing this extremely finite term of a few hours before the end of light period on 3 dph to promote ISI effectively in PBT larviculture under the rearing temperature of 26.5°C. In this study, the larval

developmental stage on 3 dph is “D” stage shown in Kawakami et al. 2008: the yolk-sac disappeared). However, it is necessary to pay attention that under different temperature the optimal period of SFR may change due to the different developmental speed of larvae. It is necessary to investigate such relationship between larval development and the optimal period of SFR. Moreover, swimbladder inflation frequency did not stably achieve 100% in this study. Therefore, further study should be performed to stably achieve 100% swimbladder inflation.

Strong aeration has been reported to reduce larval ISI frequency in Australian bass red sea bream and Japanese sea bass (Battaglene and Talbot 1990, 1993; Kitajima et al. 1994). Vigorous water currents generated by strong aeration was considered to inhibit larval access to the water surface to gulp air (Battaglene and Talbot 1990, 1993; Kitajima et al. 1994). However, in PBT larvae, even if their ISI success is susceptible to strong aeration, strong aeration during the night to prevent sinking death would not affect the success of ISI in PBT larvae. This is confirmed by the result of this study (Section 3.2 in Chapter 3) which demonstrated that PBT larvae do not achieve ISI during the night.

2-3. Future Research on influence of swimbladder inflation failure, flow control and tank design to improve larval survival

Sinking larvae have been reported in various other aquaculture fish species; amberjack, barfin flounder, seven-band grouper, kelp grouper (Teruya et al. 2009; Kayaba et al. 2003; Shiotani et al. 2003; Sakakura et al. 2006; Hirata et al. 2009; Fui et al. 2012). In these fish species, flow control of rearing water by aeration has also been reported to prevent larval sinking death in barfin flounder and seven-band grouper, kelp

grouper (Kayaba et al. 2003; Shiotani et al. 2003; Sakakura et al. 2006; Fui et al. 2012). However, for all the above mentioned species with the exception of barfin flounder, a species which lacks a swimbladder, the relationship between larval swimbladder inflation and larval survival, vertical distribution within rearing tanks has not yet been investigated. Therefore, influence of swimbladder inflation failure on larval survival should be investigated in those fish species.

On the other hand, the low swimbladder inflation frequency did not affect PBT larval survival in the experimental 1.0 *kl* rearing tanks, in which flow control was employed to prevent larval sinking in this study (Section 3.1 and Section 3.2 in Chapter 3). Sumida et al. (2011) reported that the aspect ratio (the ratio of water depth relative to the half width or radius of tank: AR) of rearing tanks affects the flow pattern generated by aeration in rearing tanks, and they suggested that high AR prevents larval sinking death (Sumida et al. 2011). Furthermore, upwelling current generated by aeration was reportedly faster in the higher AR tanks than in lower AR tanks (Shiotani et al. 2005). Results of these studies suggest that tanks with high AR possess a greater possibility to prevent sinking death of PBT larvae than those with lower AR.

In this study, survival in small experimental tanks tended to be higher (from 43.2% to 48.6% in 500 *l* tanks in Chapter 1; from 22.2% to 57.7% and 28.1% to 63.7% in 1.0 *kl* tanks in Section 3.1 and 3.2 in Chapter 3 respectively) than that in mass-scale tanks (19.3% in 50 *kl* tanks by Tanaka et al. 2009; from 0.8% to 26.0% in 30 and 20 *kl* tanks in Chapter 2). In small experimental tanks (1.0 *kl*: 1.04 and 500 *l*: 1.20), the AR was higher than those in mass-scale tanks (20 *kl* tank: 0.44 and 30 *kl* tank: 0.35). Therefore, the differences in survival observed between experimental and mass-scale tanks could be the result of differences in tank AR. Moreover, the results in Chapter 2,

that ISI failure reduced the larval survival in mass-scale tank, imply that the generation of enough vertical circular current to prevent the larval sinking in mass-scale tank with low AR is more difficult than in small experiment tanks with high AR.

Therefore, suitable rearing tank design on the AR and the shape to mitigate larval sinking should be investigated to improve larval survival via prevention of sinking death. Moreover, the suitable flow control method for the rearing tanks seen to be different with tank AR and shape; therefore, it should be examined in each tank AR and shape. The outcomes of such research would contribute to improve the mass-production technique of other aquaculture fish species with larval sinking death in hatchery.

2-4. The relationship between ISI promotion and occurrence of surface death in PBT larviculture

While, making oil film on the rearing water surface prevents surface death effectively, Section 3.1 in chapter 3 has shown that oil film removal to promote ISI induces a high incidence of surface death, and the high incidence period of surface death overlaps with the window of 3 dph to promote most effectively the ISI identified in this study. Moreover, Section 3.2 in chapter 3 has shown that the number of surface death larvae was largest at 18:00 in 24 hours on 3 dph, and it also corresponded to the optimal timing of the day to promote ISI. These results mean making oil film to prevent surface death conflicts with the surface film removal to promote ISI on the extremely finite term of a few hours before the end of light period on 3 dph in PBT larviculture.

Moreover, PBT larval swim up and activity near the water surface was observed more frequently in a few hours before the end of light period on 3 dph than

other times and dphs in this study and in the mass production tanks in hatchery. Therefore, these behaviors are considered to be for air gulping at the water surface for ISI, and to trigger their surface death simultaneously, consequently, larval surface death will not avoid in ISI promotion by existing SFR using surface skimmer.

Therefore, further study should be conducted on the surface condition of rearing water and the method in which both ISI promotion and surface death prevention can be achieved simultaneously, or on the effective prevention method of surface death other than making oil film to improve the mortality due to both surface death and sinking death, although, the minimized operation of SFR within the optimal timing for ISI promotion during the few hours before the end of light period on 3 dph is only solution to mitigate surface death at present.

2-5. Influence of swimbladder inflation failure on mortality, growth and development of lordotic deformity in postflexion larvae and juveniles PBT

Growth retardation, mortality and development of lordotic deformity in juveniles due to SBI failure has been reported in some aquaculture species. In contrast, this study demonstrated that SBI failure cause neither a significant level of mortality after the transitional period from postflexion larvae to juvenile stage nor growth retardation and lordotic deformity in PBT juveniles. However, SBI failure cause mortality due to sinking death in PBT early larval stage and growth retardation from early larval to early juvenile stage; therefore SBI should be promoted in the early larval stage in PBT fingerling production. Moreover, it is highly possible that SBI failure increases energy consumption in their swimming. Further investigation is required to

clarify the influence of SBI failure and the function of swimbladder including the adult stage in PBT.

Summary

General introduction

Bluefin tuna aquaculture has been developed an economically important industry. Consequently, excessive demand of wild-caught tuna for seedling fish of the tuna aquaculture has resulted in over-fishing. Therefore, development of full-cycle bluefin tuna aquaculture methodology, which does not rely on natural resources, is necessary for sustainable tuna aquaculture.

Recent attempts at developing Bluefin tuna fingerling production technology have been made in many countries. In Pacific bluefin tuna (PBT); however, the survival in fingerling production is still low. Although, the poor PBT survival is due to various cause, mass mortality by both larval surface death and sinking death during the early larval stage have been considered to be seriously affects PBT aquaculture operations. Especially, larval sinking death has been identified as a particularly serious problem causing mass mortality of PBT larviculture.

Swimbladder inflation plays an important role in controlling larval body density and their buoyancy. On the other hand, PBT larval body density is greater than that of rearing sea water, even when larvae possess an inflated swimbladder, and is believed to be the primary cause of sinking death particularly during the night-time on ceasing swimming (Takashi et al. 2006). Therefore, larval swimbladder inflation and flow control of rearing water during the nighttime to suspend larvae within the rearing water column have the possibility to improve larval sinking death in PBT larviculture. However, the definite relationships between swimbladder inflation failure and survival

have not yet been investigated in PBT not to mention the effective promotional method of initial swimbladder inflation (ISI). In addition, the flow control in rearing tanks has also not yet been examined in detail its enhancement effect of survival in larviculture. Furthermore, regarding the swimbladder, its inflation failure induces poor growth and vertebral deformity, and it often negatively affects the production efficiency in other aquaculture fish species. However, the definite relationships between swimbladder inflation failure and growth, vertebral deformity have not yet been elucidated in PBT.

The purpose of this study is to improve PBT fingerling production technology. Firstly, in Chapter 1, the effects of different air supply rate during the nighttime on larval survival and water circulation of rearing tank were evaluated, and larval sinking velocity was also determined to improve larval mortality due to sinking death.In Chapter 2, the influence of ISI failure on larval vertical distribution and survival were investigated. In Chapter 3, the promotional and inhibitory conditions of water surface for ISI and the optimal timing for ISI promotion were investigated to develop the suitable ISI promotion method; additionally, the relationship between ISI promotion and occurrence of surface death was also investigated. In Chapter 4, influence of swimbladder inflation failure on mortality, growth and lordotic deformity in postflexion larvae and juveniles were investigated to obtain the information for the improvement of fingerling production technology in PBT.

Chapter 1: Flow control by aeration to prevent sinking death in PBT larvae

This study evaluated the effect of flow control by aeration in the nighttime on larval survival, and examined sinking velocity of PBT larvae. The experiment was held in 500 *l* tanks, in which larval survival was compared among air supply rate of 0, 300 and 900 *ml*/min during the night. Larval sinking velocity was also measured in the larvae with (WIS) and without inflated swimbladder (WOIS) at night. In addition, the flow field in a 500 *l* tank was measured at air supply rate of 300 and 900 *ml*/min, and estimated danger zone for larval sinking (EDZ), where the upward flow velocity was less than the larval sinking velocity, was calculated as the area within the cross-section of the tank to assess the correlation between larval survival and water circulation patterns in the tank.

Larval survival increased with increasing air supply rate, and it showed highest at air supply rates >900 *ml*/min. Moreover, larval sinking velocities were significantly higher in WOIS than in WIS from 5 days-post-hatch (dph). Water circulation speed increased, and the size of the EDZ reduced at higher air supply rates. These results suggest that higher air supply rate increase larval survival by generation of counteracting upward flow to larval sinking.

Chapter 2: Influence of ISI failure on survival and vertical distribution in PBT larvae

This study investigated the influence of ISI failure on survival and vertical distribution in PBT larvae to prevent mass mortality due to sinking death.

In Experiment 1, swimbladder inflation frequency and survival within an ISI promoted (PS) group, for which surface film of rearing water was removed, and a group without ISI promotion (NPS) were compared in 20 and 30 *kl* tanks. The PS group demonstrated significantly higher swimbladder inflation frequency and increased survival than the NPS group within 20 *kl* tanks at 9 dph. Similar tendencies were observed within 30 *kl* tanks.

In Experiment 2, larval vertical distribution and swimbladder inflation frequency in the nighttime were examined through larval sampling within the upper and middle layers of the water column and tank bottom within 1.6 and 30 *kl* tanks at 5 dph. Larvae at tank bottom had higher distributional density and significantly lower swimbladder inflation frequency than those distributed in the upper and middle layers within 1.6 *kl* tanks. Similar tendencies were observed within 30 *kl* tanks.

Results of this study indicate larval ISI promotion improve larval survival via prevention of sinking death in mass-scale PBT larviculture.

Chapter 3: Promotion of ISI in PBT larvae

3.1 Conditions of water surface and optimal period to promote ISI in PBT larvae

In this study, ISI promotion of PBT larvae was studied by following three experiments to improve the larval survival.

Experiment 1 was conducted to explore promotion and inhibition of ISI under different water surface conditions; including the use of surface skimmer to remove

autogenous surface film (SS), covering the water surface with liquid-paraffin-layer (LP) and oil film (OF), and a control (non-treatment, NT). Significantly higher swimbladder inflation frequency was observed in SS (62.2%) than NT (11.9%), LP (2.7%) and OF (3.9%). This indicates that ISI in PBT larvae can be promoted by removal of surface substances on rearing water (surface film) which inhibit larval air gulping at water surface.

Experiment 2 aimed to elucidate proper day of larval age (days-post-hatch: dph) to start skimming to promote ISI with the following four periods differing by the commencement dphs of removing the artificially formed surface oil film using surface skimmer : from 3 to 8 (SF3D), 4 to 8 (SF4D),5 to 8 (SF5D), 6 to 8 (SF6D) dph. Significant improvement in swimbladder inflation frequency was observed in SF3D (80.2%); however, the frequency was very poor in SF4D, SF5D, and SF6D (17.8–7.5%) at the end of experiment (8 dph).

Experiment 3 aimed to elucidate the optimal period to promote ISI with the following four different periods of removing the artificially formed surface oil film using surface skimmer: 1 day of 3 dph (SF3 group), from 3 to 4 dph (SF3–4 group), from 3 to 5 dph (SF3–5 group) and from 3 to 8 dph (SF3–8 group). Skimming in 1 day of 3 dph (SF3: 57.0 ± 14.6%) gave the similar promotion effect of ISI to skimming on 3 dph and later (SF3–4: 62.0 ± 9.1%, SF3–5: 61.8 ± 4.9%, SF3–8: 71.0 ± 13.5%) without significant difference in swimbladder inflation frequency at the end of experiment (8 dph). These results indicate the need of surface film removal (SFR) without missing a narrow window, 1 day of 3 dph, to promote ISI effectively in practical PBT larviculture.

On the other hand, the removing surface film caused highest incidences of surface death on 3 dph, and this dph with highest incidence of surface death

corresponded with the optimal period to promote ISI on 3 dph in Experiment 2. This result indicates that the promotion of ISI and the prevention of surface death conflict with each other on 3 dph.

3.2 Optimal timing in the day to promote ISI in PBT larvae

This study investigated the optimal timing of day to promote ISI for improved PBT larval survival.

Larval swimbladder inflation frequency was compared based on three experiments using different time schemes of SFR. SFR was conducted from 05:00 to 19:00 (light period: S.5–19), 19:00 to 05:00 (dark period: S.19–5), 08:00 to 19:00 (S.8–19) and the entire day (S.24) in Experiment 1; from 08:00 to19:00 (S.8–19-E2), 08:00 to 13:00 (S.8–13), 13:00 to 19:00 (S.13–19) in Experiment 2; and from 13:00 to 16:00 (S.13–16), 16:00 to 19:00 (S.16–19), 18:00–19:00 (S.18–19) in Experiment 3.

The swimbladder inflation frequency at the end of experiment (9 dph) was significantly higher ($P < 0.001$) in S.24 (91.1 ± 5.7%), S.5–19 (92.2 ± 5.1%) and S.8–19 (93.3 ± 3.4%) than in S.19–5 (11.1 ± 5.1%) in Experiment1, and remarkably higher in S.8–19-E2 (81.7%) and S.13–19 (88.3%) than in S.8–13 (0.0%) in Experiment 2, and significantly higher ($P < 0.001$) in S.16–19 (84.4 ± 5.1%) and S.18–19 (70.0 ± 12.0%) than in S.13–16 (7.8 ± 3.9%) in Experiment 3. These results indicate the SFR during light period is effective; while that during the dark period had no effect to promote larval ISI, moreover, indicate that the optimal timing of the day to promote ISI by SFR is a few hours before the end of light period (16:00–19:00).

Additionally, in S.24 of Experiment 1, incidence of surface death was highest at 18:00 during 24 hours on 3 dph. This time with highest incidence of surface death also corresponded to the optimal timing of the day to promote ISI as well as the corresponding between the dph with highest incidence of surface death and the optimal period to promote ISI on 3 dph observed in Section 3.1 in Chapter 3.

Chapter 4: Influence of swimbladder inflation failure on mortality, growth and development of lordotic deformity in PBT postflexion larvae and juveniles

This study examined the influence of swimbladder inflation failure on mortality (Experiment 1), lordotic deformity (Experiment 2), and growth (Experiment 1, 2) in PBT postflexion larvae and juveniles by generation the cohort failed swimbladder inflation.

In Experiment 1, rearing experiment was conducted for postflexion larvae to juveniles (from 18 to 30 dph). Mortality was not significantly different between the fish with (WIS) and without (WOIS) inflated swimbladders. Standard length (SL) and body weight (BW) were significantly smaller in WOIS than WIS. Moreover, the swimbladder inflation was found in WOIS after postflexion stage. This inflation considered to be due to the so-called late or secondary inflation of the swimbladder independent of air gulping for ISI.

In Experiment 2, two examination trials were conducted on swimbladder inflation, vertebral deformity and growth for juvenile stage. In both trials, lordotic deformities were found neither in WOIS nor in WIS. Although SL and BW were

significantly smaller in WOIS than WIS at 22 dph, no significant differences in SL and BW were found between them after 37 dph.

The results of Experiment 1 and 2 indicate that swimbladder inflation failure in PBT causes growth retardation until juveniles of 30 dph as well as other aquaculture fish species; however, it cause neither significant levels of mortality after the transitional period from postflexion to the juvenile stage nor growth retardation in juveniles after 37 dph and lordotic deformities in juvenile differently from various other aquaculture fish species.

General discussion

1. Achievements in this study

Chapter 1 demonstrated that increased air supply rate to rearing water during the nighttime mitigate larval mortality due to sinking death in PBT larviculture. Chapter 2 demonstrated that larval ISI failure reduces survival via larval sinking to tank bottom in PBT larviculture in mass-scale tanks. Therefore, larval mortality due to sinking death should be improved by mitigation of larval sinking via both promotion of larval ISI and flow control of rearing water during the nighttime in combination. Moreover, Chapter 3 elucidated that PBT larval ISI can effectively promote by SFR, and that the optimal period and timing of the day to effectively promote ISI is extremely finite term of twilight just before the end of light period on 3 dph. Therefore, to promote ISI effectively, SFR should be done without missing extremely finite term of a few hours

before the end of light period on 3 dph in PBT larviculture. Additionally, Chapter 4 elucidated that swimbladder inflation failure cause neither significant levels of mortality after the transitional period from postflexion to the juvenile stage nor growth retardation in juveniles after 37 dph and lordotic deformities in juvenile differently from various other aquaculture fish species reported, although, swimbladder inflation failure causes growth retardation from early larval to early juvenile stage in PBT.

2. Proposal to improve PBT fingerling production efficiency and research for the future

2-1. Prevention of larval sinking death in PBT larviculture

Flow control of rearing water by higher air supply rate during the nighttime improved larval survival due to sinking death via generation the counteracting upward flow to larval sinking in PBT larviculture. Moreover, promotion of larval ISI improved the survival via mitigation of larval sinking to rearing tank bottom in mass production tanks, even when flow field was controlled by aeration to prevent larval sinking. Therefore, the author proposes that larval survival due to sinking death should be improved by mitigation of larval sinking using measure of both flow control of rearing water and promotion of larval ISI in combination in mass-scale PBT larviculture.

2-2. Promotion of ISI in PBT larviculture

The ISI in PBT larvae can be promoted effectively by SFR using cleaning devise such as surface skimmer. Moreover, in PBT larviculture, the ‘window’, when effective promotion of ISI is possible, was extremely narrow 1 day of 3 dph compared with other reported fish species. Furthermore, the optimal timing of the day to promote ISI was extremely finite term of a few hours before the end of light period, and SFR during the dark period and the time from morning to early afternoon produced no effect of ISI promotion. Therefore, SFR should be done without missing this extremely finite term of a few hours before the end of light period on 3 dph to promote ISI effectively in PBT larviculture.

2-3. Future Research on influence of swimbladder inflation failure, flow control and tank design to improve larval survival

Although, larval sinking and the prevention measure by flow control have been reported in other aquaculture fish species with sinking death, the relationship between larval swimbladder inflation and larval survival, vertical distribution within rearing tanks has not yet been investigated. Therefore, study on these topics should be performed to improve larval survival in these fish species other than PBT.

It is suggested that tanks with high AR possess a greater capacity to prevent sinking death (Sumida et al. 2011). Moreover, in this study, PBT larval survival in small experimental tanks with high AR tended to be higher than that in mass-scale tanks with low AR. Therefore, suitable rearing tank design on the AR and the shape to mitigate larval sinking should be investigated to improve mortality due to sinking death. Moreover, the suitable flow control method for the rearing tanks seems to be different

with tank AR and shape; therefore, it might have to be examined in each tank AR and shape. The outcomes of these researches would contribute to improve the mass-production technique of other aquaculture fish species with larval sinking death in hatchery.

2-4. The relationship between the promotion of ISI and occurrence of surface death in PBT larviculture

In this study, both the optimal period and timing of the day to promote ISI corresponded with the time of highest incidence of surface death on the extremely finite term of a few hours before the end of light period on 3 dph in PBT larviculture. Moreover, PBT larval swim up behavior was observed more frequently within a few hours before the end of light period on 3 dph than other times and dphs. These behaviors are considered for air gulping at water surface for ISI, and to trigger their surface death. Consequently, larval surface death will not avoid in ISI promotion by existing SFR using surface skimmer.

Therefore, further study should be performed on the surface condition of rearing water and the method in which both ISI promotion and surface death prevention can be achieved, or on the effective prevention method of surface death other than making oil film, although, the minimized operation of SFR within the optimal timing for ISI promotion during the few hours before the end of light period on 3 dph is only solution to mitigate surface death at present.

2-5. Influence of swimbladder inflation failure on mortality, growth and lordotic deformity in postflexion larvae and juveniles PBT

In this study, swimbladder inflation failure cause neither significant levels of mortality after the transitional period from postflexion larvae to the juvenile stage nor growth retardation in juveniles after 37 dph and lordotic deformity in PBT juveniles differently from other reported fish species. However, swimbladder inflation failure cause mortality due to sinking death in PBT early larval stage and growth retardation from early larval to early juvenile stage, therefore swimbladder inflation should be promoted in PBT fingerling production.

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