Doctoral Thesis

Study on mass mortality of Pacific bluefin tuna juvenile

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（Supervisor: Professor Dr. Yasunori Ishibashi）
HONRYO TOMOKI

Contents

General introduction

The history of challenge to produce artificially hatched Pacific bluefin tuna (PBF; *Thunnus orientalis*) is long since the project was launched in 1970s (Harada *et al.*, 1971, Miyashita 2002). The Aquaculture Research Institute at Kindai University has been attempted to artificially hatch PBF eggs and developing protocols to rear larvae through commercial size. After the half a century, our institute has successfully closed the life cycle of PBF in captivity (Sawada *et al.*, 2005). Cultured PBF hatchlings experience high mortality e.g. early mortality including surface and sinking death, mortality due to aggressive behavior and cannibalism, and a characteristic mortality termed collision death occurred during the larviculture of PBF conducted in land-based tanks (Miyashita 2002). PBF production on a commercial-scale was technically challenging. Many studies have reported potential factor induces mortality (Sakamoto *et al...* 2004; Takashi *et al.*, 2006). Accordingly, several protocols were developed to improve the survival (Kurata *et al.*, 2012; Nakagawa *et al.*, 2011; Nikaidou *et al.*, 2006; Seoka *et al*., 2008), which increased fingerling production to a commercial scale. In such circumstance, tuna populations especially PBF and Atlantic bluefin tuna (*T. thynnus*) have been depleted due to global overfishing and the expansion of aquaculture worldwide (Benetti *et al.*, 2016; Cop 15, 2010; Cyranoski, 2010). Fingerlings that are artificially cultured independently from natural populations can help to satisfy market demand and conserve wild populations. Thus, the demand for artificially hatched juveniles were increased for aquaculture industry and the amount of producing fingerlings were also increased. Therefore, more efficient culture techniques are required and it is important to improve production efficiency in order to replace wild caught juvenile with artificially hatched juvenile to guarantee sustainability (Okada *et al.*, 2021). Several problems occurred both in land-based tanks especially collision death and the considerable mortality occurred in sea cages should be overcame to achieve this.

In the ontogenetic development of PBF, swimming mode changes from intermittent sprinting to continuous cruising when larvae reach 26-34 mm body length (Fukuda *et al.*, 2010a) and morphological functions develop to enhance swimming ability during the juvenile stage (Hattori *et al.*, 2001; Tamura and Takagi 2009). In captive condition such as tank culture of PBF, these physical developments lead to increasing mortality termed collision death. The collision of PBF juveniles with the rearing tank wall and/or net cage can result in physical injuries such as broken and/or dislocation of vertebral column, parasphenoid, frontal bone, and/or skin lesions and in death (Masuma *et al.*, 2001;

Miyashita *et al.*, 2000; Higuchi *et al.*, 2014). This mortality occurs not only during the fingerling production process, but also during broodstock management (Kadota *et al.*, 2016; Yazawa *et al.*, 2011). It is reported that visual disorientation induces burst swimming and collision with tank wall and/or sea net cage (Masuma *et al*., 2001). Even though a factor that induces this mortality has already elucidated, the mechanism is still unclear and the technical countermeasure has not been established. In addition, the cause of death due to collision death has not partly identified. The collision death is the first priority to be solved during land-based nursing period.

In the PBF fingerling production process, the juveniles were transferred from land-based tanks to sea cages at the age of approximately 30 to 35 days-post-hatching (dph) (Okada *et al.*, 2021). Immediately after the transportation, various factors including insufficient feeding leading to poor growth, accidental ingestion of marine litter, and traumatic injuries caused by collision and/or contact with the net cage caused the high mortality in sea cage culture of PBF juvenile (Okada *et al.*, 2014). The poor growth was defined by the statistical comparison of body weight between the survived fish with dead individuals. Takii *et al.*, (2005) reported the low tolerance of PBF for fasting. In spite of fish were fed diet just after the transportation has completed, the dead fish due to poor growth accounts 25.0-45.0 % within total mortality. The factor causes this mortality is unclear and effective countermeasure is required. Thus, we have tried to elucidate the mechanism causes this mortality and develop prevention techniques.

In the present study, we have investigated the mechanism causes poor growth in sea cage. In addition, the aim of this study is to clarify the characteristic of collision death and develop countermeasure against mortality. Finally, technical developments during tank and sea cage culture of PBF are included a purpose of this study. In the Chapter 1, we investigated the stress response following to transportation from land-based tanks to sea cages by taking samples from time course and estimated the time required to recovery from transportation in order to reveal the cause of death due to poor growth. In Chapter 2, we reviewed the definition of collision death from hematological assessment. And in the Chapter 3, we examined the photoenvironment which controllable parameter in land-based tanks to clarify the factor causes collision death and develop protocol mitigate the mortality. The night-time lighting was found as effective countermeasure against collision death and its benefit was conformed in sea cages in Chapter 4. Finally, technical development of night-time lighting in sea cages was completed in Chapter 5.

Chapter 1: Mass mortality during and immediately after transport: Estimated time for recovery from transportation stress and starvation in juvenile PBF

1.1 Introduction

Even though the various strategies were developed to increase the viability of PBF fingerlings in the tank, the survival rate was still lower than those of other cultured marine teleosts, especially fish that were transferred and stocked in large sea cages (30 m in diameter; Ishibashi 2012; Miyashita, 2002; Okada *et al.*, 2014). Okada *et al.*, (2014) reported several factors that reduced survival of PBF juveniles in captivity, including deficient growth that accounted for 25.0–45.0% of mortality. Moreover, a high rate of poor growth occurred during a limited period after transportation i.e., 3–5 days (d) after stocking. This problem should be resolved to establish more efficient culture techniques for PBF fingerlings. Hence, the present study aimed to reveal the causes of mortality using physiological analysis.

Biomarkers, such as heat shock proteins that include a family of 70 kDa proteins (Hsp70-family), are well known as chaperone molecules. Hypoxia-inducible factors (HIF) are widely analyzed in fish, including adult PBF, to assess their physiological condition (Mladineo and Block, 2009). The ubiquitous Hsp70-family responds to thermal fluctuations and is also induced by various stressors (Afonso *et al.*, 2008; Caipang *et al*., 2008; Das *et al*., 2005; Iwama *et al.*, 1999). Since the Hsp70-family functions at a cellular level, such as in the maintenance of protein integrity, its gene expression is used as a potential indicator of stress. Additionally, the oxygen-dependent gene HIF-1, which is a heterodimer composed of α and β subunits, is also a well-known biomarker in fishes, including PBF, especially HIF-1α (Mladineo and Block, 2009; Terova *et al*., 2008). In conjunction with neuroendocrine responses, such as the production of the stress hormone cortisol typically secreted in the hypothalamic-pituitary-interrenal (HPI) system, which is indicative of a general stress response at the cellular level. The combination of physiology and biomarkers provides insight into the physical conditions that cause stress in fishes.

In the PBF fingerling production, it is necessary to transport juveniles at the age of 30–40 dph from

land-based tanks to sea cages since they show rapid growth after metamorphosis and collide with the walls of the rearing tank (Miyashita *et al*., 2000). However, the fish are possibly stressed by multiple factors during transportation, such as handling, capture, chasing, temporal hypoxia, and confinement, and PBF juveniles show skin injuries and low tolerance to handling (Ishibashi, 2012). In addition, a decrease in the free glucose content has also been noted in juvenile PBF after transport; however, it is difficult to detect individual effects in fish during transportation. PBF are vulnerable to stress (Ishibashi, 2012; Masuma, 2008); nevertheless, the time taken by PBF juveniles to recover from transportation stress remains unclear. Okada *et al*., (2014) reported that the mortality caused by deficient growth possibly occurred as a result of insufficient feed intake. Therefore, we hypothesized that the stress response inhibits feed intake immediately after transport. This study estimated the time required to recover from two different transportation distances by sampling the fish at multiple time points during transportation to sea cages (Exp 1 and Exp 2). We aimed to determine the physical stress responses by transportation from land-based tanks to sea cages using physiological and molecular biomarkers to understand the cause of the deficient growth reported by Okada *et al.*, (2014). In addition, simulated-transport was conducted from tank-to-tank and starvation tolerance was examined to determine whether these stress responses are attributable to transportation or the uncontrolled environments in the sea cages (Exp 3).

1. 2 Materials and methods

Fish transportation, starvation, and sample collections

Experiment-1 (Exp 1): Long distance transportation

PBF juveniles (32dph, mean BW: 1.6 ± 0.4 g, mean BL: 57.1 ± 5.0 mm, n = 10) were raised at the Uragami Hatchery, Aquaculture Technology and Production Center, Kindai University, from eggs spawned by captive broodstock and transported to sea cages using vehicles. Fish were individually captured from the land-based nursery tanks using hand-nets and temporarily stocked in 1,000 l carry tanks at a density of approximately 1,000 fish per tank. Fish were transported from the Uragami Hatchery to sea cages located in Kushimoto Bay, which was a distance of 14 km and took about 45

min to drive. Then, the tanks were transferred onto a boat and transported 1.2 km for 10 min (Fig. 1-1). During transportation, pure oxygen was pumped into the carry tanks and the dissolved oxygen level was maintained at 100% saturation without water flow or exchange. Fish were immediately fed an artificial diet (Magokoro, Marubeni Nisshin Feed Co., Ltd. Tokyo, Japan) after being stocked at a density of 12.9 g per m^3 (33,994 individuals) in the sea cage (30 m in diameter, 6 m in depth), but were not fed during transportation to avoid water quality degradation.

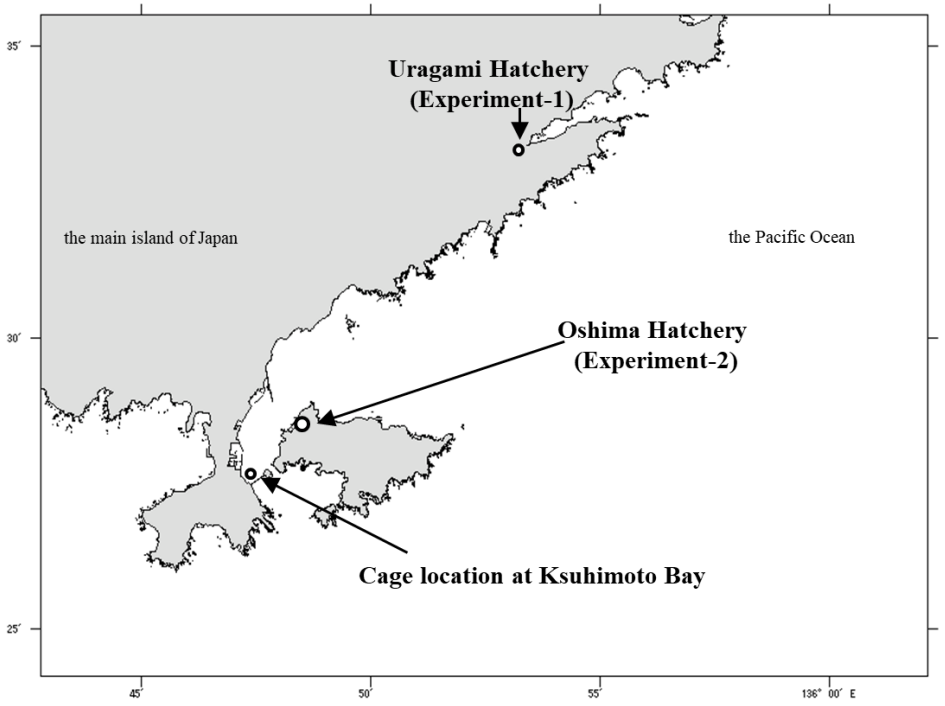

Figure 1-1 Map of the Uragami Hatchery (Experiment-1), Oshima Hatchery (Experiment-2), and sea cage locations. The grey area indicates the main island of Japan and the white area indicates the Pacific Ocean.

Ten fish were captured by hand-net and sacrificed at the land-based nursery tank prior to transportation and frozen immediately using liquid nitrogen. Ten fish were sacrificed at each of the multiple time points during transportation (immediately before release into the sea cage (BR), at 24,

48, 72 h, and 7 d after stocking) and preserved using liquid nitrogen. When sampling was performed from the sea cage, a small amount of diet was fed to gather the fish, and then fish were randomly captured using a hand-net and immediately preserved similarly to the protocol conducted at land-based nursery tank. All fish were stored at −80°C prior to analysis. After stocking the sea cage, fish were fed the artificial diet several times a day to satiation from dusk till dawn and nocturnal lighting was provided using an LED (Marine Tech Co., Ltd. Fukuoka, Japan) to alleviate mortality from collision with the cage perimeter (Ishibashi *et al.*, 2009). Average light intensity from 00:00–01:00 h was measured immediately below the LED hanging at the center of the sea cage using a DEFI-L logger (JFE Advantech Co., Ltd., Hyogo, Japan) and was 14.5 ± 1.3 μmoles m^{-2} s^{-1}. Water temperature (26.4 ± 0.5°C), salinity (32.6 ± 0.6 g L^{-1}), and dissolved oxygen (DO) level (6.75 ± 0.5 mg L^{-1}) at 5 m deep and transparency (10.5 ± 2.5 m) were measured during the 7 d of feeding. Divers collected the dead fish each day, which were counted to calculate survival during the 3 weeks of feeding in the sea cages.

Experiment-2 (Exp 2): Short distance transportation

To compare the effects of transportation distance on the stress response, we sampled PBF juveniles transported from the Oshima Hatchery, Aquaculture Technology and Production Center, Kindai University, which was a direct distance of 2.3 km and took about 20 min to drive, using the same protocols as those in Exp 1 (Figure 1-1). Fish (35 dph, mean BW: 1.9 ± 0.7 g, mean BL: 57.7 ± 7.1 mm, n = 20) were individually captured from land-based nursery tanks using a hand-net and temporarily stocked in 1,000 l carry tanks at a density of approximately 1,500 fish per tank. During transport, pure oxygen was pumped into carry tanks and the dissolved oxygen level was maintained at 100% saturation without water flow or exchange. Fish were released into a separate sea cage (20 m diameter, 6 m depth) at a density of 4.1 g per m^3 (4,000 individuals) and immediately fed an artificial diet after being stocked in the sea cage, but were not fed during transport. Twenty fish were sacrificed at each time point to evaluate stress levels, similarly to Exp 1, using the same capture and preservation methods. After stocking in the sea cage, fish were fed the artificial diet several times a day to satiation from dusk till dawn and nocturnal lighting was provided using an LED. The average light intensity

was 11.8 ± 0.7 μmoles m^{-2} s^{-1}, which was measured using the same method as in Exp 1. Water temperature, salinity, and DO at a 3 m depth, and transparency were 23.8 ± 0.9°C, 32.2 ± 0.9 g L^{-1}, 6.27 ± 0.7 mg L^{-1}, and 8.1 ± 2.4 m, respectively. Divers collected the dead fish each day, which were counted to calculate survival during the 3 weeks of feeding at sea cage.

Experiment-3 (Exp 3): Simulated-transport and starvation trial

PBF juveniles were raised in a 30,000 l tank (6 m in diameter) and captured using the same methods as in Exp 1 and Exp 2. All culture procedures were similar until the fish were transferred to carry tanks. Two carry tanks were prepared and stocked with 300 fish each. Pure oxygen was pumped into both tanks with no water flow or exchange. After 15 min of simulated-transport, which was close to the transportation time required for short distances (Exp 2), fish were re-stocked into two 30,000 l tanks. Fish in the "Fed" group resumed feeding 3 h after stocking and fish in the "Starvation" group were not fed for a further 9 d. To investigate and compare the changes in stress responses and proximate compositions, 20 fish were sacrificed at each of the multiple time points using the same protocols as in Exp 1 and Exp 2. Fish from each group were captured and preserved at the pre-transport point, BR, and 3 h after stocking, then at 6 h, and 1, 2, 3, 5, and 7 d after stocking. All of the sampled fish were immediately frozen using liquid nitrogen and stored at −80°C until analysis. During the experiment, the water temperature and DO level were maintained at 26.5 ± 0.5°C and 12.2 ± 1.2 mg L^{-1}, respectively. Nocturnal lighting of approximately > 2.3 μmoles m^{-2} s^{-1} was provided for both tanks using LED lamps hanging above the center of the tanks (Honryo *et al*., 2013). Dead fish were removed from each tank and counted to calculate survival and daily mortality rates. During the simulated transportation, 11 and 13 PBF juveniles died, and therefore these were subtracted from the initial numbers. Fish in the Fed group were fed an artificial diet from 0600–1800 h at 3-h intervals.

Whole-body cortisol, glucose, and proximate composition analysis

Five (Exp 1), 10 (Exp 2), and 5 (Exp 3) fish from each time point were sacrificed to determine the changes in whole-body cortisol and glucose levels. Frozen fish were homogenized with 5 × PBS using

an ice-cold mixer-mill, and 1.2 ml homogenate was mixed with 6 ml diethyl ether. Cortisol was extracted using protocols described previously (Honryo *et al.*, 2013; Ishibashi *et al.*, 2009). Whole-body cortisol levels were measured using the enzyme immunoassay method and a commercial kit (Cortisol Express Kit, Cayman Chemical Company, MI, US). An absorbance of 420 nm was determined after a 90 min color-reaction in the dark following the manufacturer's protocols. Each sample was replicated three times. The remaining homogenate was immediately centrifuged at 8,000 *g* for 10 min at 4°C, and the supernatant used to analyze the whole-body glucose levels using the glucose-oxidase method (Glucose CII test kit, Wako Pure Chemical Industries, Japan) following the manufacturer protocols. Proximate compositions of whole body carcasses in Exp 3 were analyzed using standard methods (AOAC, 1995). Approximately 5 fish were pooled and sacrificed to determine moisture, ash, crude lipid, and crude protein contents (g per fish). These analyses were repeated three times.

Gene expression of the Hsp70-family in the liver and HIF-1α in the gills

Five (Exp 1), 9–10 (Exp 2), and 4–5 (Exp 3) fish from each time point were sacrificed to determine the relative mRNA gene expression levels of the Hsp70-family in the liver and HIF-1α in the gills. RNA purification and reverse transcription were conducted based on the methods of Mladineo and Block (2009) and Agawa *et al.*, (2012) with minor modifications. Total RNA was extracted from the dissected liver and gills using the Ambion RNAlater solution and standard Phenol-Chloroform method, and mRNA was purified using a commercial kit (Oligotex-dt30, Takara Bio Inc., Japan). cDNA was synthesized in a 20-μl reaction volume containing 1 μg mRNA, 25 pmol of primers, 0.5 mM of each dNTP, 20 units of the RNase inhibitor (TOYOBO, Japan), and 200 units of Prime Script Reverse Transcriptase (TaKaRa Bio Inc., Japan). To quantify the transcript, synthesized cDNA was used as a template for quantitative real-time PCR using a Rotor-Gene 6000 real-time PCR system (Qiagen, Germany). Quantitative real-time PCR assays were conducted using a 20-μl reaction volume containing 10 μl SYBR premix Ex Taq II (TaKaRa Bio Inc., Japan), 8.0 pmol forward (Hsp70-family; 5′ GAC ATG AAG CAC TGG C 3′, HIF-1*α*; 5′ TCG GAG GTG TTC TAC GAG C 3′) and reverse

(Hsp70-family; 5′ AGG ACC ATG GAG GAG 3′, HIF-1α; 5′ TCC GTT TCC TCT TCT GCC AC 3′) primers that were designed by Mladineo and Block (2009). Each sample was replicated 3 times using 4.0 µl of template cDNA. Cycling conditions for each qPCR were as follows: HIF-1α; 2 min at 95°C followed by 35 cycles of 20 s at 95°C, 20 s at 61°C, and 30 s at 72°C; Hsp70-family; 2 min at 95°C followed by 40 cycles of 20 s at 95°C, 20 s at 51°C, and 40 s at 72°C. The *β-actin* gene was used as a housekeeping gene to calculate the relative quantity following the protocols described by Agawa *et al.*, (2012). DNA contamination and primer-dimer effects were checked using no-template cDNA reactions as a negative control. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany) prior to sequencing and we confirmed accordance using the database (Accession number; EU300942) for HIF-1α mRNA partial codes. In contrast, we confirmed only 90% accordance with the database (Accession number; EU300943) for Hsp70 mRNA partial codes as they were mixed with an Hsp70-like gene (Accession number; EU300944); therefore, we considered transcript levels as those from the Hsp70-family.

Statistical analysis

All of the values for whole-body cortisol, glucose, and proximate compositions are represented by means ± standard deviation (SD). The mean values from Exp 1 and Exp 2 were compared over time in individual experiments using a one-way ANOVA followed by a Tukey-HSD test. For Exp 3, a two-way ANOVA with a Tukey-HSD multiple comparison test was used to compare treatments over time. The whole-body proximate compositions of fasting PBF juveniles were compared over time using a one-way ANOVA with a Tukey-HSD test. All differences were considered significant at $P < 0.05$. In Exp 3, the condition factor (CF) was calculated using the formula: $CF = 100 \times (W/L^3)$, where W denotes BW (g) and L denotes BL (cm). The relative quantities of the Hsp70-family in the liver and HIF-1α in the gills were represented using boxplots and the mean values of each time point were compared using an ANOVA followed by Bonferroni's correction at ~~*P*~~*p* < 0.05. Prior to the statistical analysis of relative expression levels of each gene, the changes of *β-actin* gene levels were compared temporally using ANOVA followed by Bonferroni's correction and were found to not be significant.

In Exp 3, the daily survival rate was compared among treatments using the Kaplan-Meier log-rank test. All statistical analyses were conducted using SPSS 16.0 (IBM).

1.3 Results

Survival, changes in whole-body cortisol and glucose levels in Exp 1 and 2

The changes of survival rate are shown in Fig. 1-2. Heavy mortalities occurred in both experiments, where almost 50% of the juveniles died within a week after transportation.

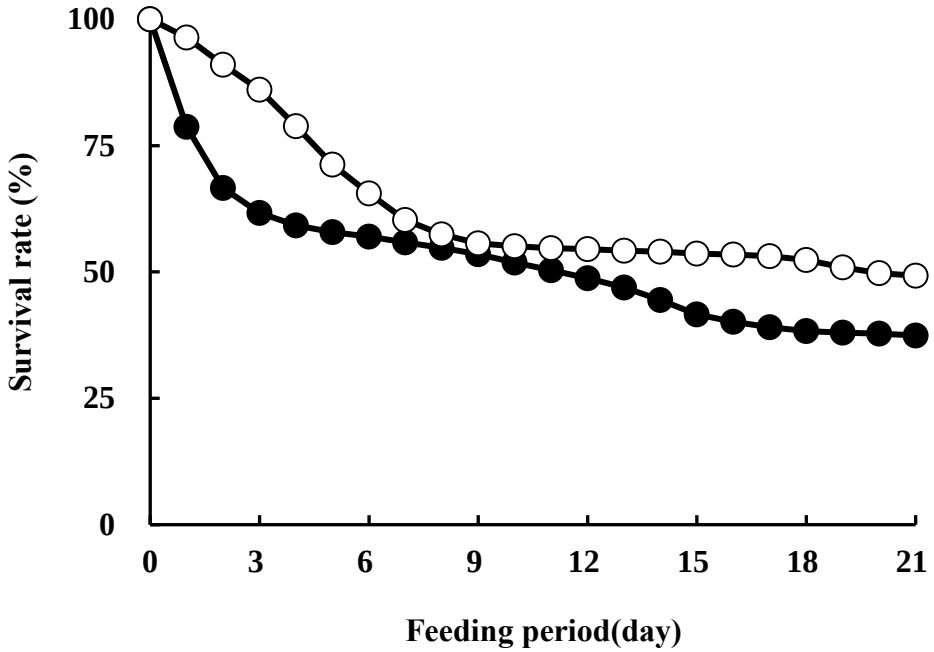

Figure 1-2 The changes of survival rate in Pacific bluefin tuna juveniles following transportation from land-based tanks to sea cages during 3 weeks of feeding. ○; Exp 1 (long distance; 15.2 km) ●; Exp 2 (short distance; 2.3 km)

Figures 1-3 and 1-4 show the changes in whole-body cortisol and glucose levels in PBF juveniles following transportation. There were obvious increases in whole-body cortisol levels, compared with pre-transport (Initial point) levels, at BR in both Exp 1 ($n = 5$) and Exp 2 ($p < 0.05$, ANOVA followed by Tukey-HSD test, $n = 10$) irrespective of the transportation distance. We observed 243 and 220%

increases from initial levels in Exp 1 and Exp 2, respectively, but these levels returned to initial levels within 24 h after stocking in the sea cage, and then returned to similar levels as those during pre-transport. The changes in whole-body glucose levels showed the same trends both in Exp 1 and Exp 2, where they gradually decreased from pre-transport levels over 48 h after fish were stocked in the sea cages and recovered after 72 h. We observed an approximately 0.59-fold decrease at 48 h in Exp 1 ($p < 0.05$, ANOVA followed by Tukey-HSD test, $n = 5$) and a 0.63–0.68 fold decrease at 24–48 h in Exp 2 ($n = 10$) compared to the pre-transport levels. A significant decrease from pre-transport levels was observed only in Exp 1. After 72 h of stocking, glucose levels returned to pre-transport levels.

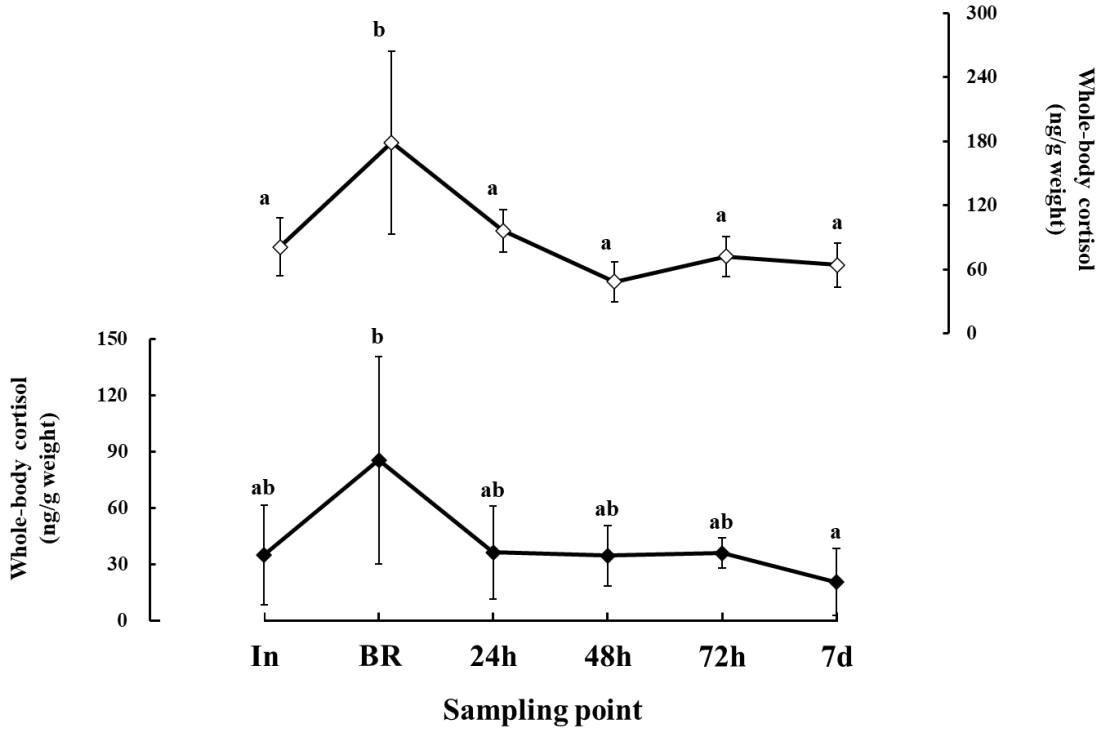

Figure 1-3 Changes in whole-body cortisol levels in Pacific bluefin tuna juveniles following transportation from land-based tanks to sea cages. ◆ Exp 1 (long distance;15.2 km, $n = 5$); ◇ Exp 2 (short distance;2.3 km, $n = 10$). In- initial; BR- before release; 24, 48, 72 h- 24, 48, and 72 h after stocking; 7 d- 7 d after stocking. Whole body cortisol levels having different letters within an experimental response curve indicate significant differences (ANOVA followed by Tukey-HSD test, $p < 0.05$).

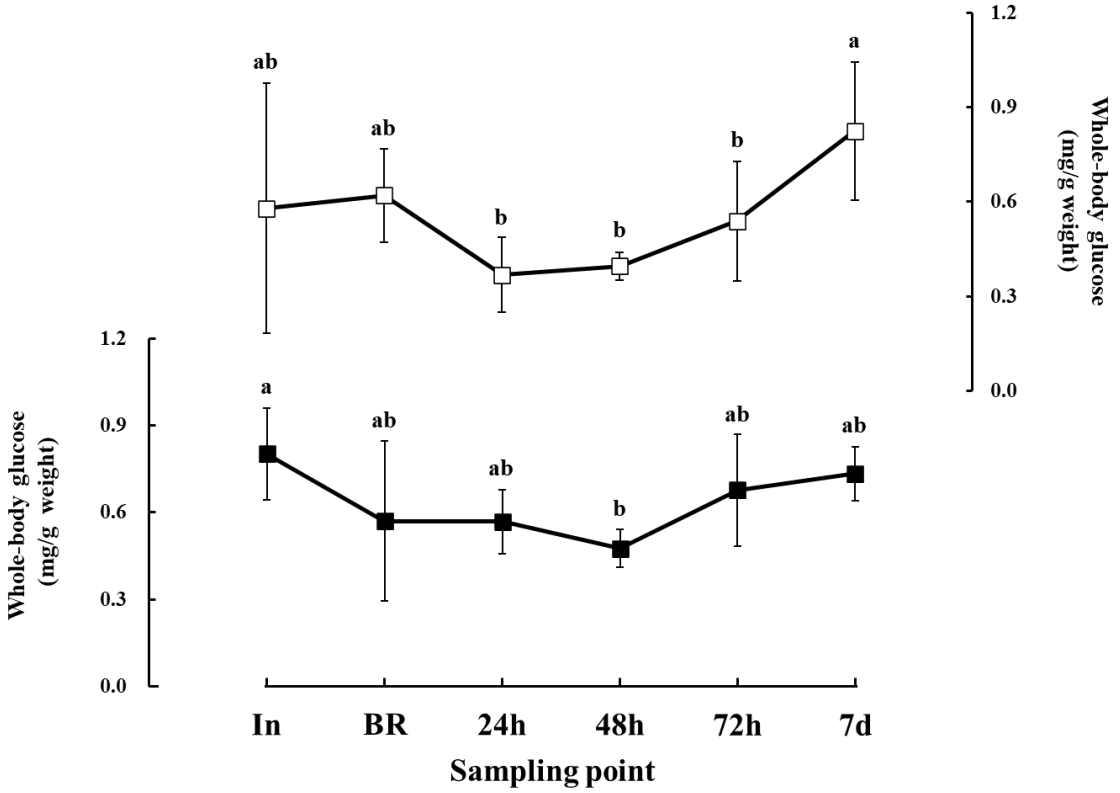

Figure 1-4 Changes in whole-body glucose levels in Pacific bluefin tuna juveniles following transportation from land-based tanks to sea cages. ■ Exp 1 (long distance- 15.2 km, n = 5); □ Exp 2 (short distance- 2.3 km, n = 10). In- initial; BR- before release; 24, 48, 72 h- 24, 48, and 72 h after stocking; 7 d- 7 d after stocking. Whole body glucose levels having different letters within an experimental response curve indicate significant differences (ANOVA followed by Tukey-HSD test, p <0 .05).

Stress-related gene expressions in Exp 1 and Exp 2

The relative quantities of HIF-1*α* and Hsp70-family gene expression in the gills and liver of PBF juveniles are shown in Figures 1-5 and 1-6, respectively. Absolute quantities of the gene expression of each gene were standardized to the housekeeping gene *β-actin*. At the sampling point BR in Exp 1, the relative quantity of HIF-1*α* gene expression in the gills was upregulated but returned to initial levels within 24 h, similarly to the whole-body cortisol levels. However, no obvious changes were observed in Exp 2 as the SD was large over the sampling time. Significant differences were not

detected in Exp 1 or Exp 2. Although there were large SDs in the relative quantity of Hsp70-family gene expressions in the liver, it appeared to be upregulated only after 48 h of stocking in Exp 1. Contrary to this, significant upregulation was detected from 24–48 h after stocking in Exp 2, but returned to initial levels after 72 h ($p < 0.05$, ANOVA followed by Bonferroni correction, $n = 10$).

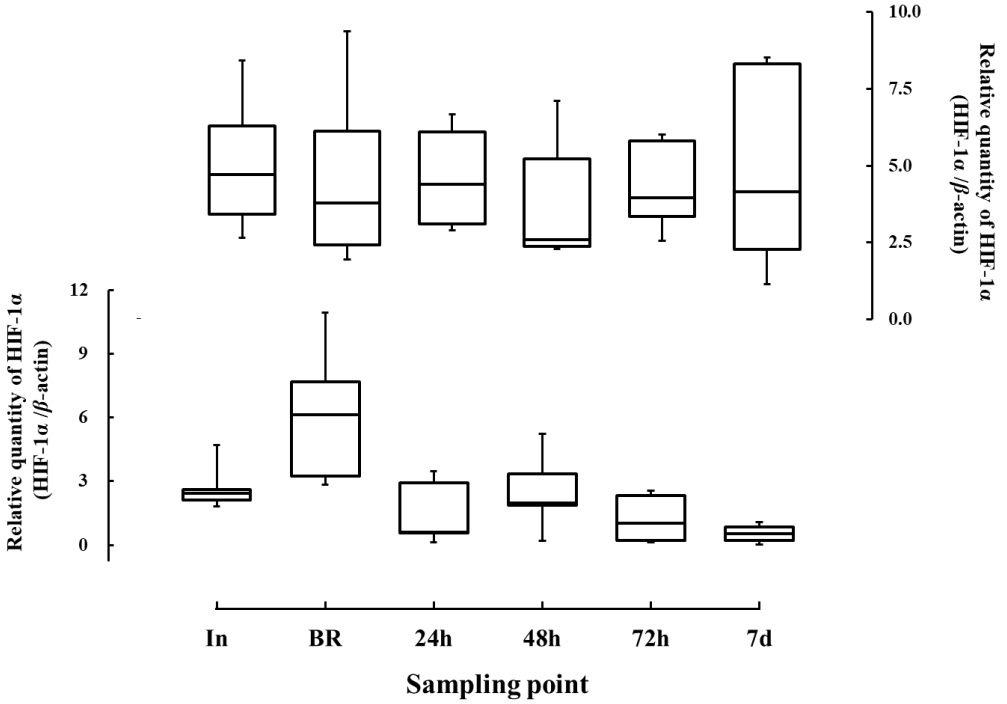

Figure 1-5 Changes in the relative quantity of HIF-1α gene expression levels in the gills of Pacific bluefin tuna juveniles following transportation from land-based tanks to sea cages. The lower figure depicts Exp 1 (long distance; 15.2 km, $n = 5$) and the upper figure depicts Exp 2 (short distance; 2.3 km, $n = 9$–10). In- initial; BR- before release; 24, 48, 72 h- 24, 48, and 72 h after stocking; 7 d- 7 d after stocking. Significant ($p < 0.05$) differences were not detected at any time point.

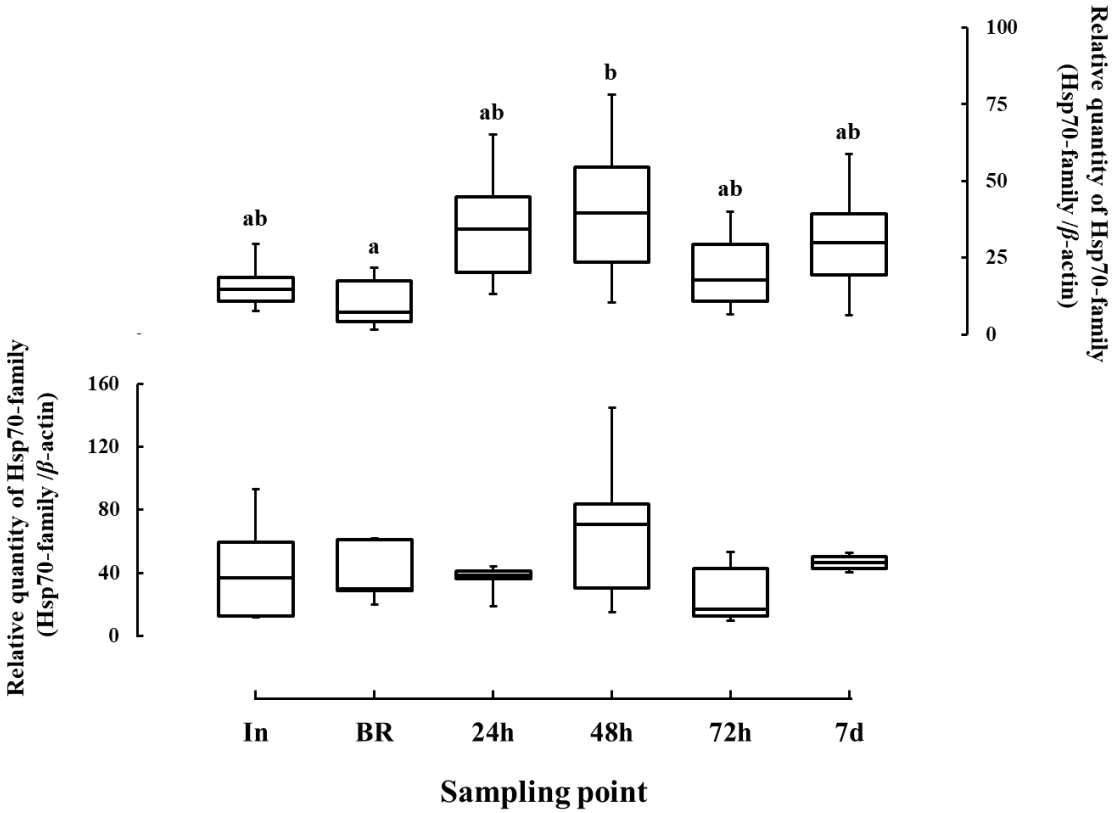

Figure 1-6 Changes in the relative quantity of Hsp70-family gene expression levels in liver of Pacific bluefin tuna juveniles following transportation from land-based tanks to sea cages. The lower figure depicts Exp 1 (long distance; 15.2 km, $n = 10$) and the upper figure depicts Exp 2 (short distance; 2.3 km, $n = 10$). In- initial; BR- before release; 24, 48, 72 h- 24, 48, and 72 h after stocking; 7 d- 7 d after stocking. Values with different letters in Experiment 2 (upper) were significantly different (ANOVA followed by Bonferroni correction, $p < 0.05$).

Simulated-transport and starvation (Exp 3)

Survival rate, daily mortality rate, and condition factor

The changes in survival rate and CF following simulated-transport and starvation of PBF juveniles are shown in Figures 1-7 and 1-8. Owing to daily mortality being higher in the "Fed" group than in the "Starvation" group during the early days of stocking, i.e., 1–2 d, significant differences among

treatment groups were detected from 1 to 4 d and at 8 d after stocking ($p < 0.001$, Kaplan-Meier log-rank test, $n = 289$ and 287, respectively). The Fed group showed significantly lower survival than the Starvation group until 4 d after stocking, and then daily mortality increased in the Starvation group from d 6. Hence, the survival rate was inversed after 5 d of stocking and almost all fish died by d 8 after fasting in the Starvation group.

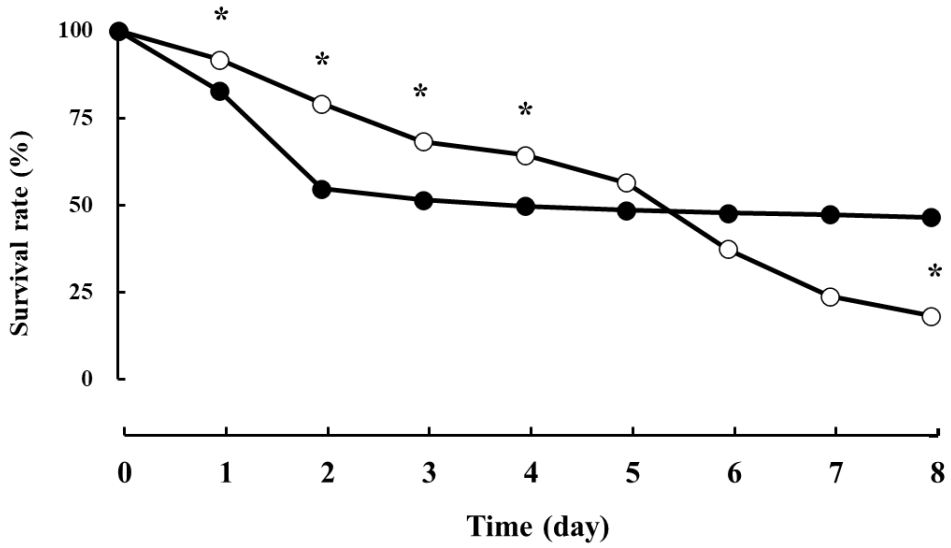

Figure 1-7 Changes in the survival rate following simulated-transport and starvation of Pacific bluefin tuna juveniles (●; Fed group, ○; Starvation group). Asterisks indicate significant differences among treatments (Kaplan-Meier log-rank test, $p < 0.001$, $n = 287–289$). 11 and 13 PBF juveniles were died during simulated-transport, and therefore these numbers were subtracted from data analysis.

The effect of starvation on CF was evident from 2 d after stocking and significant differences among treatments were detected ($p < 0.05$, two-way ANOVA followed by Tukey-HSD test, $n = 5$). In the Fed group, CF values decreased from the pre-transport levels until 6 h after stocking and then gradually increased. Conversely, CF gradually decreased in the Starvation group throughout the experimental period.

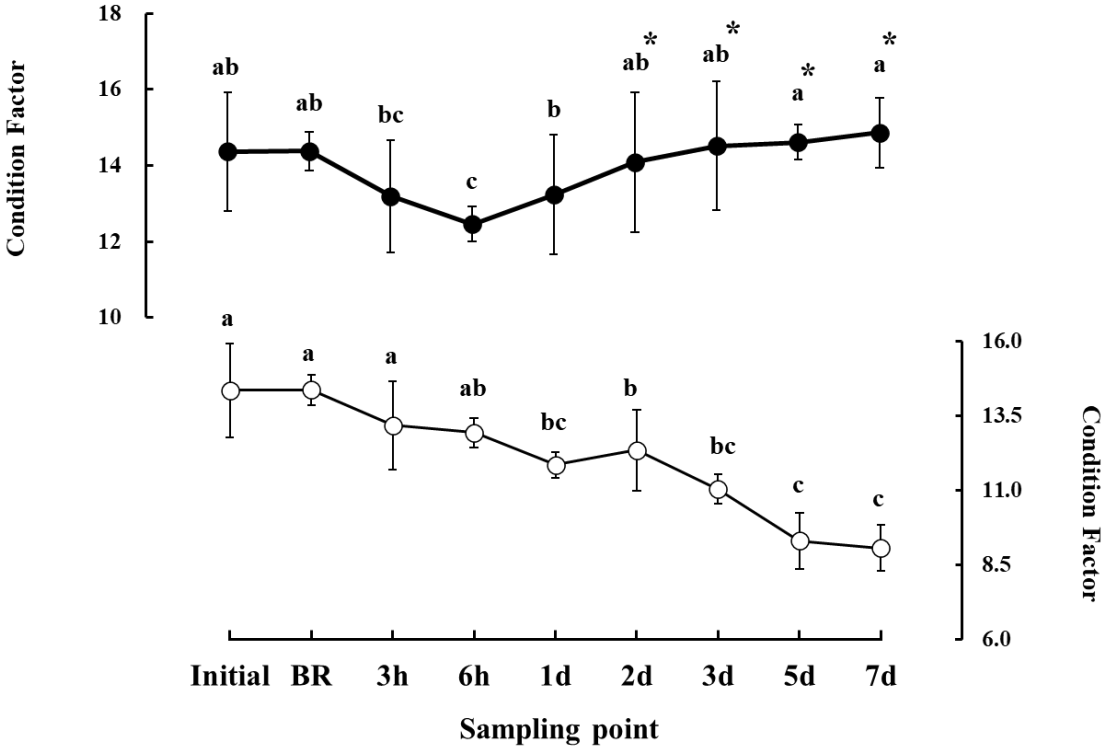

Figure 1-8 Changes in the condition factor (CF) following simulated-transport and starvation of Pacific bluefin tuna juveniles (●; Fed group, ○; Starvation group). BR- Before release; 3, 6 h- 3 and 6 h after release into tanks; 1, 2, 3, 5, 7 d- 1, 2, 3, 5, and 7 days after release into tanks. Asterisks and superscript letters indicate significant differences among treatments and in the same curve, respectively (two-way ANOVA followed by Tukey-HSD test, $p < 0.05$, $n = 5$).

Changes in whole-body cortisol and glucose levels

The changes in whole-body cortisol and glucose levels in PBF juveniles following simulated-transportation and starvation are shown in Figures 1-9 and 1-10. The whole-body cortisol levels reached a peak 3 h after stocking in new tanks and returned to initial levels within 24 h. There were no significant differences among treatments; however, a significant increase compared to the initial level was observed 6 h after stocking in the Fed group. The whole-body glucose levels showed a significant increase in both Fed and Starvation groups at BR. However, the whole-body glucose levels decreased within 48 h after stocking in Exp 1 and Exp 2. In Exp 3, glucose levels remained stable. There were significant differences among treatment groups in whole-body glucose levels, mainly from 2–3 d after stocking ($p < 0.05$, two-way ANOVA followed by Tukey-HSD test, $n = 5$) that coincided with CF changes.

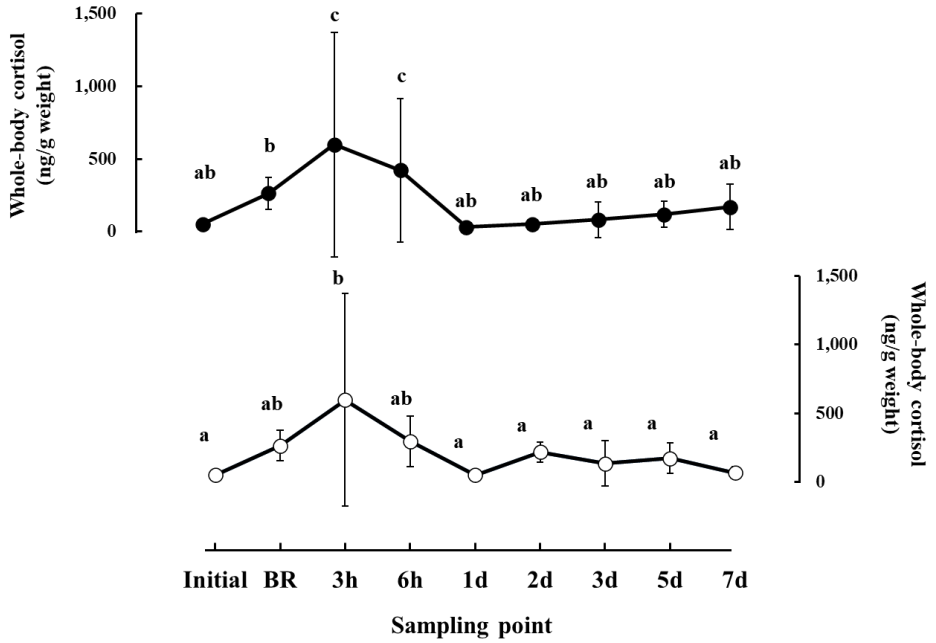

Figure 1-9 Changes in whole-body cortisol levels following simulated-transport and starvation of Pacific bluefin tuna juveniles (●; Fed group, ○; Starvation group). BR- Before release; 3, 6 h- 3 and 6 h after release into tanks; 1, 2, 3, 5, 7 d- 1, 2, 3, 5, and 7 days after release into tanks. Superscript letters indicate significant differences in the same curve (two-way ANOVA followed by Tukey-HSD test, $p < 0.05$, $n = 5$). Significant differences among treatments were not detected.

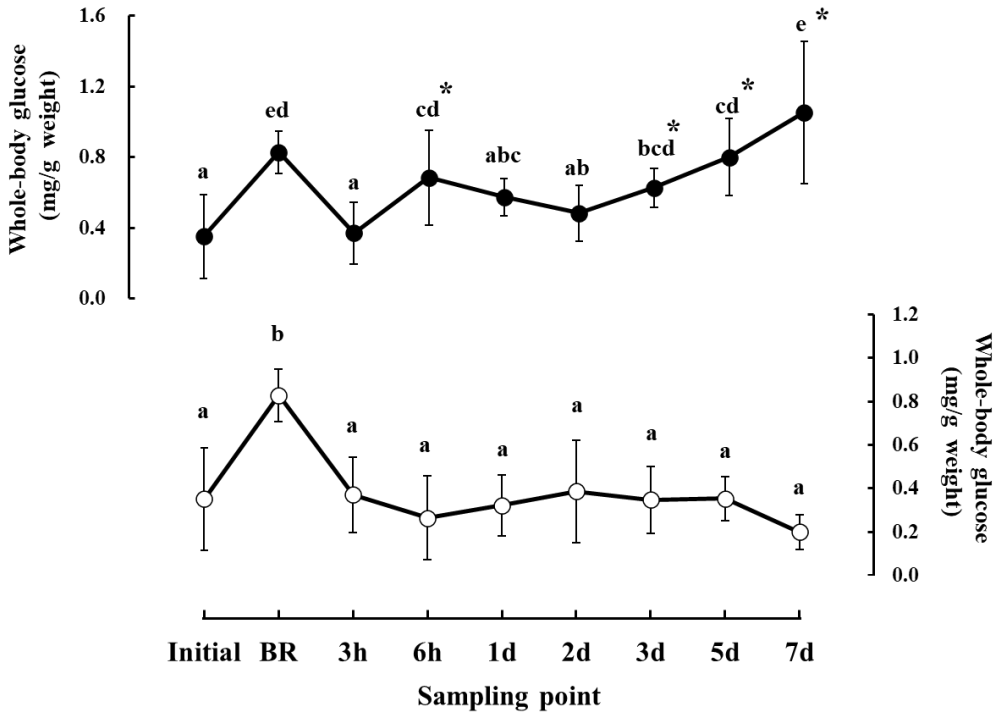

Figure 1-10 Changes in whole-body glucose levels following simulated-transport and starvation of Pacific bluefin tuna juveniles (●; Fed group, ○; Starvation group). BR- Before release; 3, 6 h- 3 and 6 h after release into tanks; 1, 2, 3, 5, 7 d- 1, 2, 3, 5, and 7 days after release into tanks. Asterisks and superscript letters indicate significant differences among treatments and in the same curve, respectively (two-way ANOVA followed by Tukey-HSD test, $p < 0.05$, $n = 5$).

Relative quantities of stress-related gene expressions

The relative quantities of HIF-1α and Hsp70-family gene expression in the gills and liver of PBF juveniles following simulated-transportation and starvation are shown in Figures 1-11 and 1-12, respectively. The relative quantity of HIF-1α gene expression in the gills was upregulated between 3 h and 1 d after stocking and significant differences were detected in the Fed group In addition, there was a significant difference among treatments at 1 d after stocking ($p < 0.05$, two-way ANOVA followed by Bonferroni correction, $n = 4$–5). This returned to initial levels within the next 24 h and

then remained at the initial level. In contrast, the relative expression of the Hsp70-family genes in the liver in the Fed group was low, but slightly upregulated at 3 d after stocking and returned to initial levels within 24 h; however, this upregulation was not statistically significant. Significant differences among treatments were detected at 5 and 7 d after stocking as the levels in the Starvation group were upregulated under fasting conditions ($p < 0.05$, two-way ANOVA followed by Bonferroni correction, $n = 4–5$).

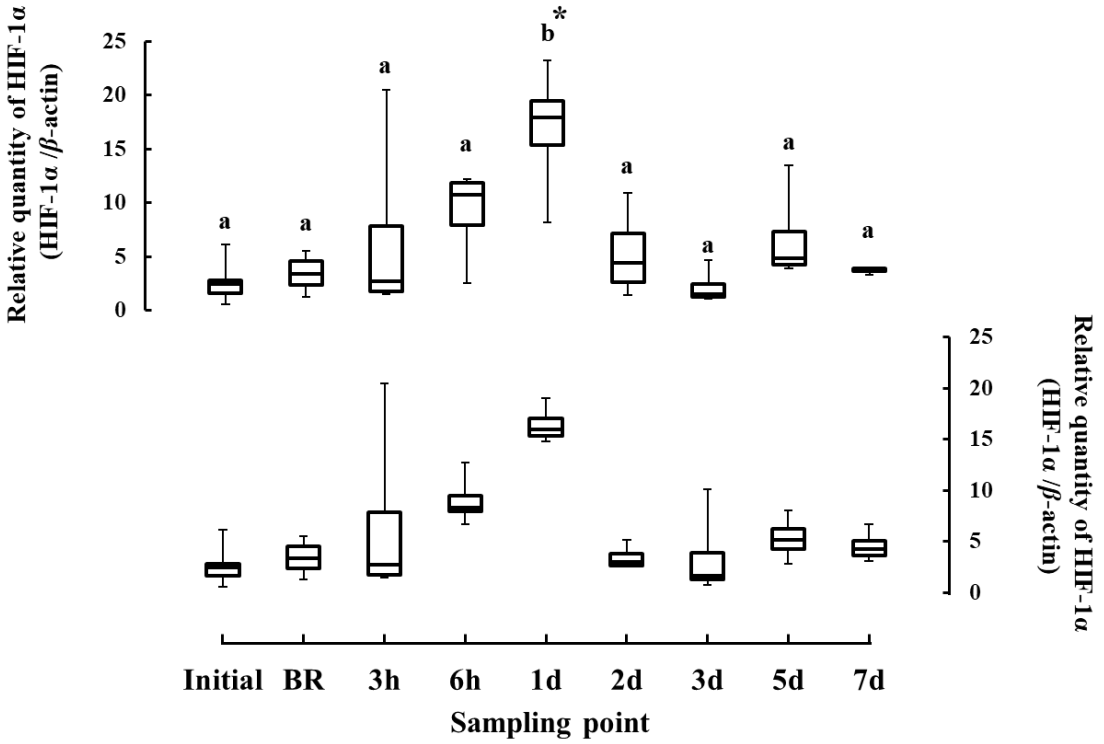

Figure 1-11 Changes in the relative quantity of HIF-1α gene expression levels in gills following simulated-transport and starvation of Pacific bluefin tuna juveniles. The lower figure depicts the Starvation group and the upper figure depicts the Fed group. BR- Before release; 3, 6 h- 3 and 6 h after release into tanks; 1, 2, 3, 5, 7 d- 1, 2, 3, 5, and 7 days after release into tanks. Asterisks and superscript letters indicate significant differences among treatments and in the same curve, respectively (Bonferroni correction, $p < 0.05$, $n = 4–5$).

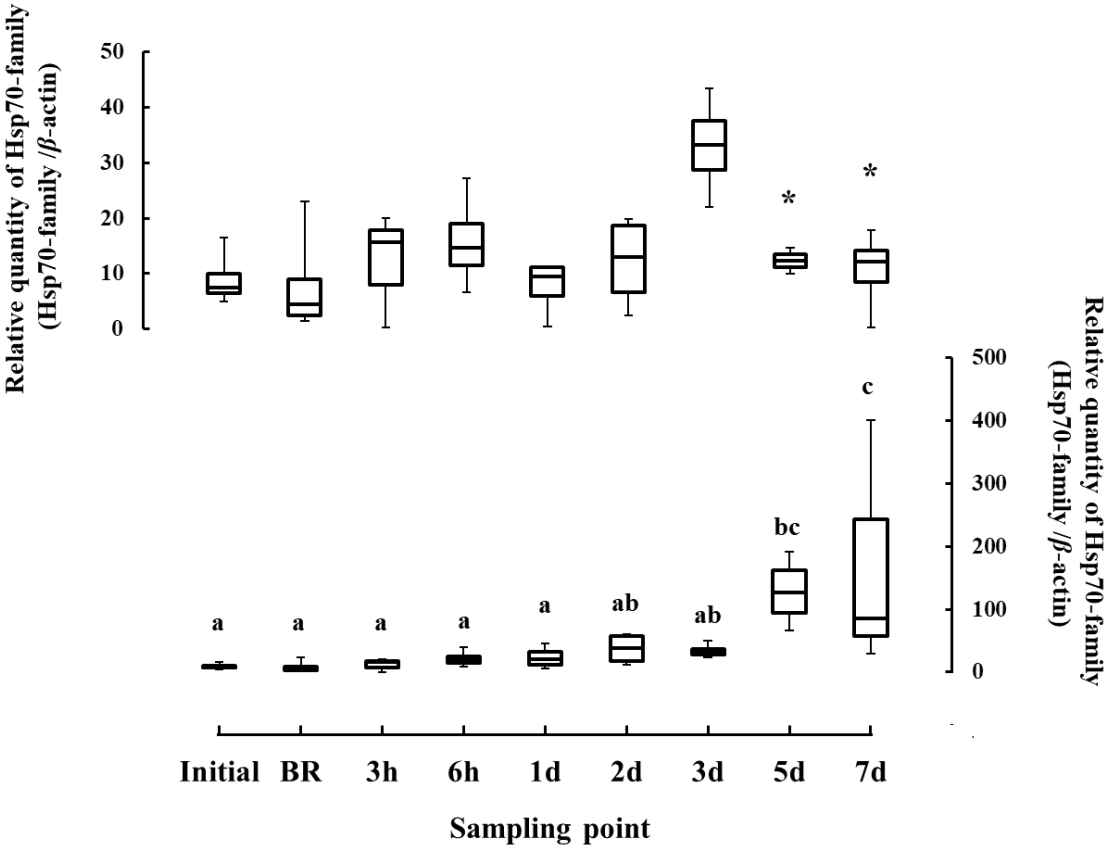

Figure 1-12 Changes in the relative quantity of Hsp70-family gene expression levels in liver following simulated-transport and starvation of Pacific bluefin tuna juveniles. The lower figure depicts the Starvation group and the upper figure depicts the Fed group. BR- Before release; 3, 6 h- 3 and 6 h after release into tanks; 1, 2, 3, 5, 7 d- 1, 2, 3, 5, and 7 days after release into tanks. Asterisks and superscript letters indicate significant differences among treatments and in the same curve, respectively (Bonferroni correction, $p < 0.05$, $n = 4–5$).

Whole-body proximate compositions

The changes in whole-body moisture, ash, crude lipid, and crude protein contents (g per fish) are shown in Figure 1-13. To clearly show these changes of proximate compositions following simulated-transport, the results were represented as grams of matter per fish. All proximate compositions, except

ash content, significantly decreased from initial levels. Despite a slight increase in proximate compositions at BR, 26.4, 43.3, and 63.0% of moisture, crude protein, and crude lipid content, respectively, were lost by the end of the experiment owing to fasting.

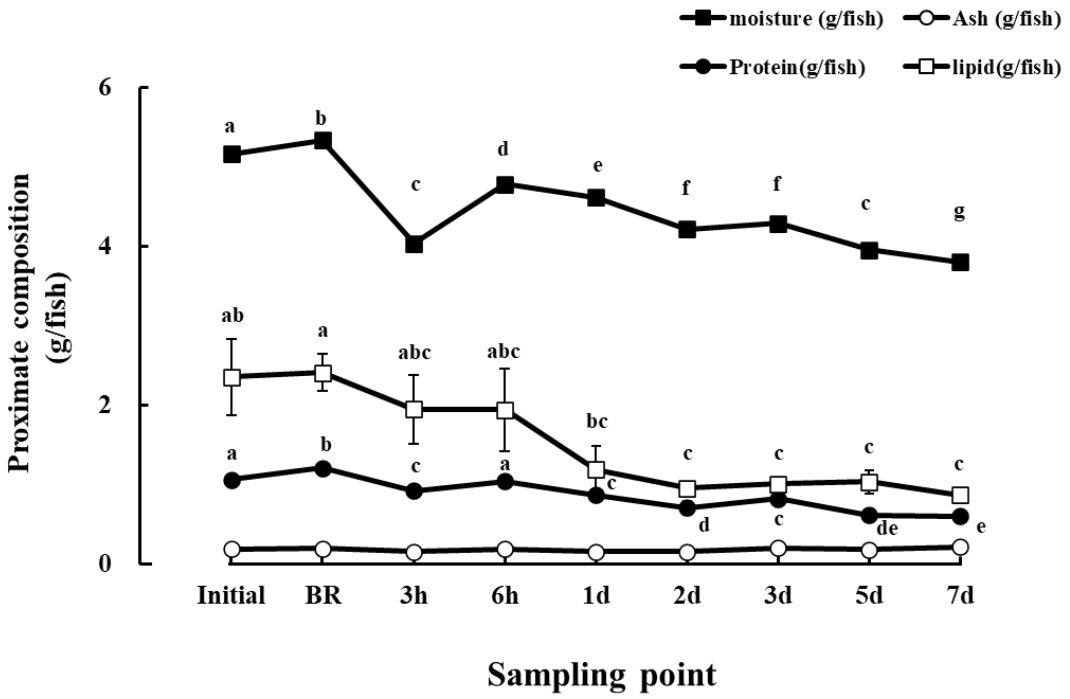

Figure 1-13 Changes in the proximate compositions of Pacific bluefin tuna juveniles following simulated-transport and starvation. BR- Before release; 3, 6 h- 3 and 6 h after release into tanks; 1, 2, 3, 5, 7 d- 1, 2, 3, 5, and 7 days after release into tanks. Superscript letters indicate significant differences in the same curve (ANOVA followed by Tukey-HSD, $p < 0.05$, $n = 3$).

1.4 Discussion

This study aimed to reveal the stress responses in PBF juveniles following two different distances of transportation from nursery tanks to sea cages. In addition, we also aimed to determine whether these stress responses were caused by the transportation itself or the environmental conditions in the sea cages. Results of this study suggested that the stress responses caused by transportation from land-

based tanks to sea cages and the uncontrolled environmental factors in the sea cages were mitigated by the third day, irrespective of transportation distance. In addition, uncontrollable environmental factors reinforced the stress response of transferred PBF juveniles in sea cages. Hence, the first 3 d could be critical periods for transferred PBF juveniles and these stress responses possibly caused insufficient feed intake resulting in poorer growth of PBF juveniles in sea cages.

Estimated time for recovery from transportation stress

Almost 50% of transported juveniles died within a week in both Exp 1 and 2, regardless of transportation distance and stocking density, similarly to the previous investigations; however, such heavy mortality, which occurred just after transport from land-based tanks to sea cages, was not reported in other marine teleosts owing to the low stress tolerance of PBF juveniles (Miyashita 2002; Masuma 2008; Higuchi *et al*., 2014; Okada *et al*., 2014). In addition, the initial stocking density of 2.73–10.72 g per m^3 in sea cages did not influence survival, growth, and hematological indices of PBF juveniles (unpublished results, Aquaculture Research Institute of Kindai University), although the effects of stocking density in sea cages on physiological stress response of PBF juveniles has not been examined yet. In the present study, the stocking densities in each experiment were varied, with Exp 1 having almost three times higher stocking density than that of Exp 2 (Exp 1; 12.9 g per m^3 and Exp 2; 4.1 g per m^3). It has been reported that stocking density is correlated with stress response in fishes (Barcellos *et al*., 1999; Costas *et al*., 2008); however, it has also been found that 10-fold higher rearing densities did not significantly influence the cortisol level in juvenile sea bass, *Dicentrarchus labrax* (Orbcastel *et al*., 2010). Accordingly, the effect of stocking density on stress response varied with species. In the present study, the stress response was similar between Exp 1 and 2, which was commonly represented by cortisol and HIF-1*a* gene expression elevations at the BR (Fig. 1-3), decreasing whole-body glucose levels over 48 h of stocking (Fig. 1-4) and the upregulation of Hsp70-family gene expressions at 48 h after stocking (Fig. 1-6). Thus, it has been predicted that the effect of stocking density on the stress response of transferred PBF juveniles could be much lower than that of transportation stress and uncontrollable environment factors in sea cages. Moreover, we captured the

gathering fish by feeding small amounts of feed in the sea cages. This implies that sacrificed fish had possibly recovered or tended to recover from transportation stress. Therefore, these results could underestimate the overall stress response of fish stocked in sea cages. In fact, the changes of survival rate suggested that the high mortality ended a week after transportation (Fig. 1-2). However, at least 3 d were still required for recovery from transportation stress, even for active fish that gathered at the water surface for feeding and thus, this period was critical for the viability of PBF juveniles. Results of Exp 3 revealed that the decreasing whole-body glucose levels and upregulation of Hsp70-family gene expression in the liver following transportation might be reinforced by the uncontrolled environment in the sea cage, rather than the transportation per se. In general, stress responses, such as cortisol secretion, in the hypothalamic-pituitary-internal (HPI) axis suppresses feed intake, resulting in inhibited growth (Barton and Iwama, 1991; Bonga, 1997; Mommsen, 1999). The deficiency in growth caused by insufficient feed intake (Okada *et al.*, 2014), which resulted in the mass mortality of PBF juveniles immediately after stocking in the sea cage, could be partly induced and explained by the stress responses observed in this study. Hence, this study characterized the possible correspondence between mortality in sea cages and physiological responses to transportation stress.

Stress response of transferred PBF juvenile

The involvement of the hypothalamus and pituitary gland in the control of corticosteroid secretion has been well established in vertebrates, including teleosts, with corticotropin-releasing hormone and adrenocorticotropic hormone as the most important secretagogues, and cortisol or corticosterone as the adrenocortical end product of the HPI axis (Bonga, 1997). An elevation of cortisol levels is one of the most widely used indicators of fish stress, and this hormone has broad activity in fish. Cortisol secretion from the interrenal gland affects the metabolism of carbohydrates, proteins, and lipids (Mommsen *et al*., 1999). In addition to the evaluation of cortisol changes, the glucose level has also been evaluated as another indicator of stress in several fishes, such as chinook salmon (*Oncorhynchus tshawytscha*) (Sharpe *et al.*, 1998), red sea bream (*Pagrus major*) (Ishibashi *et al.*, 2002), cobia (*Rachycentron canadum*) (Cnaani and Mclean, 2009), and brook charr (*Salvelinus fontinalis* (Crespel

et al., 2011), because the rapid rise in plasma glucose concentration following an acute stressor is predominantly caused by the glycogenolytic action of catecholamines (Bonga, 1997). During transportation including capture, chasing, and temporary confinement, induced cortisol secretion, which showed that fish were stressed in Exp 1, 2, and 3. In this study, whole-body glucose levels were significantly increased only at BR coincidently with cortisol increases in Exp 3. In contrast, significant elevations were not confirmed in Exp 1 and 2, suggesting that the different dynamics of stress responses in these experiments were inconclusive, therefore further study is required to elucidate this. Once PBF juveniles were transferred, approximately 40% of whole-body glucose had been reduced within 48 h after fish were stocked in sea cage both in Exp 1 and 2 (Fig. 1-4). This could have been reinforced by the sea cage environment, which was not controllable, since the same tendency was not observed in Exp 3. In previous studies, a decrease in the free glucose content was also observed in juvenile PBF after transport (Ishibashi *et al*., 2009; Ishibashi, 2012). We showed that the reduction in free glucose levels in juvenile PBF were attributable to the rapid consumption of glucose supplies in response to stress. In general, rates of glucose turnover and glucose oxidation in fishes are lower than those in mammals (Hemre *et al*., 2002). However, Weber *et al*., (1986) reported that glucose turnover rates in skipjack tuna (*Katsuwonus pelamis*) were similar to those in mammals, but much higher than those in other teleosts. Further research focusing on glycogen metabolism and catecholamine dynamics should be conducted to clarify these issues using different sizes of PBF and different types of stressors.

Insufficient feed intake after transportation

The results of Exp 3; simulated-transportation indicated that the effects of starvation on PBF juveniles were initiated on d 2 (Fig.1-8). The proximate compositions of PBF juveniles indicated that approximately 50% of crude lipid and crude protein contents were lost after 5 d of fasting and these levels may be fatal for PBF juveniles, which may explain the increased mortality (Fig.1-7 and 1-13). In general, fish stop growing and body weight decreases by consuming energy sources and suppressing protein synthesis under fasting and/or reduced food availability conditions. For example, in Atlantic

cod, *Gadus morhua*, it has been reported that hepatic lipids followed by hepatic glycogen and muscle proteins were mobilized in response to starvation (Guderley *et al.*, 2003). Moreover, starvation significantly reduced the crude lipids of the liver and white muscle in sea bass (Chatzifotis *et al.*, 2011) and hepatic glycogen, RNA/DNA ratio, and crude protein in barfin flounder, *Verasper moseri*, juveniles (Takaya and Kawamata, 2000). Takii *et al.,* (2005) showed that juvenile PBF have low fasting tolerance owing to their high energy requirements (142.7 kJ/kg BW for daily maintenance of 0.85 g fish), which is remarkably higher than those of other aquaculture fish, such as rainbow trout (*O. mykiss*; 42 kJ/kg BW for 300 g fish; Storebakken *et al*., 1991) and yellowtail (*Seriola quinqueradiata*; 82 kJ/kg BW for 700 g fish; Watanabe *et al*., 2000). Hence, similarly to other marine teleosts, fasting influenced PBF juveniles, and even 5 d of fasting resulted in increased mortality. Mortality may occur in less time in sea cages owing to uncontrollable factors, which reinforced the stress response of PBF juvenile. The energy-partitioning rate in juvenile PBF is unlike that of other fishes as the ratio for standard metabolism accounts for 41.3% and only 20.7% is retained as energy for growth (Ohnishi *et al*., 2016). This report indicated that PBF juveniles require large amounts of energy to maintain homeostasis. Therefore, the stress response and acclimation to a new environment requires considerably more energy to maintain PBF juveniles stocked in sea cages after transportation than under normal conditions. Moreover, this study indicated that stress response owing to transportation was considerably reduced by d 3. These results suggested that feed intake was suppressed by stress response from transportation and a low tolerance of fasting also occurred, compounding the high mortality in sea cages. Therefore, it is necessary to improve the feeding strategy to prevent deficient growth during the first 2 d after stocking. However, it should be noted that during the first 4 d of the experiments, the survival rate was significantly higher in the Starvation group, although the CF and proximate compositions indicated an effect of starvation from d 2. From the changes in whole-body cortisol levels, it is evident that significant upregulation was maintained only in the Fed group even 6 h after the stress (3 h after resumed feeding) compared to initial levels (Fig. 1-9). In addition, the HIF-1α expression levels in the gills of the Fed group after one day were significantly higher than those in the Starvation group (Fig. 1-11). Further study is required to determine if the stress response of PBF

juveniles can be alleviated by feed intake. In addition, it is anticipated that limited feed intake caused by commodious sea cages induced an energy turnover within two days after stocking. This might also have resulted in deficient growth and juvenile mortality. From the disproportionate energy intake and turnover rate, PBF juveniles may have lost homeostasis and died. Large sea cages of 20–30 m diameter might be too commodious for PBF juveniles to consume sufficient amounts of food. Therefore, survival could possibly be improved by enhancing the feeding method, frequency, and/or type of artificial diets used in sea cages.

Biomarkers

The relative quantities of HIF-1α gene expressions increased within one hour then returned to initial levels by 24 h, similarly to whole-body cortisol, both in Exp 1 and Exp 2. In addition, upregulation of HIF-1α was observed from 3 h to one day after stocking and returned to initial levels by 48 h in Exp 3. When considering HIF-1α levels alone, PBF juveniles recovered from transportation/simulated transportation stress within one to two days after stocking in a new environment (sea cages or tanks). However, the relative quantities of Hsp70-family gene expressions were upregulated after 48 h of stocking in sea cage, although were not obviously changed during 5 d after simulated transport. Both the HIF-1α gene and Hsp70 gene are known to be upregulated under hypoxia, following exposure to cold and warm temperatures in tuna (Mladineo and Block, 2009). In addition, Terova *et al*., (2008) noted that copies of HIF-1α mRNA significantly increased in response to both acute and chronic hypoxia, whereas they remained unchanged in sea bass liver exposed to hyperoxic conditions. Hypoxia challenge induced a 2-fold increase in whole-body cortisol levels and had a stimulatory effect on Hsp70 gene expression in larval rainbow trout (Fuzzen *et al.*, 2011). This study indicated that both stress-related genes were useful biomarkers for PBF juveniles under stress, similarly to those in other fish and provided insight into the estimated time for recovery from acute transportation stress. In addition, our results showed that HIF-1α gene expression in the gills responded earlier than Hsp70-family gene expression in the liver, especially in Exp 1. To cope with transportation stress, whole-body cortisol and HIF-1α gene expressions in gills may have primarily responded to acute stress and

then Hsp70-family genes acted as chaperone molecules. Hsp70-family genes are known to prevent premature folding and aggregation of proteins and protein translocation, and mediate steroid and receptor binding in cells (Iwama, 1999). Basu *et al*., (2001) suggested that elevated levels of cortisol could suppress and mediate Hsp70 levels in the liver and gills following physiological stress. Hence, the Hsp70-family gene expression that was upregulated between 24–48 h after stocking in the sea cages (Exp 1 and 2) may have indicated damaged cells that were under repair, which supported our conclusion that the stress response to transportation was mitigated by the third day after stocking. In Exp 3, Hsp70-family expression was markedly upregulated in the Fed group on the third day after stocking and 5–7 d after fasting in the Starvation group. This indicated that PBF juveniles also required 3 d to recover from simulated-transport and fasting induced high expression of Hsp70-family genes after 5 d.

In conclusion, this study showed that PBF juveniles were clearly stressed by transportation, including environmental changes, from land-based nursery tanks to sea cages. Results showed that the estimated time required to recover from transportation was at least 72 h. The stress response possibly inhibited the feed intake and induced mass mortality of PBF juveniles in sea cages immediately after stocking. Therefore, the first 3 d after stocking is a critical period for juvenile PBF culture in sea cages.

Chapter 2: Mass mortality after transport;

Blood chemistry of PBF juveniles showing abnormal swimming behavior

2.1 Introduction

Adult scombrid fish, including PBF, are known as ram ventilators, which optimize gas exchange to meet metabolic demands and retain higher respiratory rates than other fish. Both physical and physiological modifications to the oxygen transport system promote high metabolic performance in tuna (Bushnell and Jones, 1994; Wegner *et al*., 2010). Under ram ventilation, oxygen intake is dependent on ambient water passing through the mouth to the branchial arches of the gills (Roberts, 1975). Gills also serve in osmotic and ionic regulation, acid-base regulation, and the excretion of nitrogenous wastes (Evans *et al*., 2005). Because the oxygen demands of tuna are higher than those of other teleosts (Miyashita *et al*., 1999), their gills have an unusually large surface area and very thin blood-water barriers to permit rapid rates of gas transfer almost an order of magnitude greater than those of other teleosts (Bushnell and Brill, 1992). Therefore, continuous swimming is considered necessary for ram ventilators to maintain homeostasis.

The switch to ram ventilation is an ontologenetic change, which is advantageous once fish reach a size where they can maintain sufficient speed to overcome gill flow resistance (Roberts, 1975). In PBF, swimming behavior changes from intermittent sprinting to continuous cruising when individuals reach 26-34 mm body lengths (Fukuda *et al.*, 2010a), and oxygen consumption rapidly increases following their development from larva to juvenile (Miyashita *et al.*, 1999). Morphological functions then develop to enhance swimming ability during the juvenile to young adult stages (Hattori *et al*., 2001; Tamura and Takagi, 2009). Juveniles at 39 days old with 5.0 cm fork length and 1.1 g body weight have been shown to operate as ram ventilators (Ohnishi *et al*., 2016).

In aquaculture situations, these physiological and morphological changes could increase mortality because of collisions causing dislocation or fractures, or both, of the vertebral column, parasphenoid, premaxilla, or frontal bones (Miyashita *et al*., 2000; Higuchi *et al.*, 2014). Several factors, including the poor scotopic vision of PBF juveniles and visual disorientation during ambient light conditions,

have been found to contribute to collision deaths (Masuma *et al*., 2001; Ishibashi *et al*., 2009; Fukuda *et al.*, 2010b). However, this mortality caused by the unique ontogeny of PBF still obstructs the practical production of fingerlings. Reported causes of death in cultured PBF include collision injuries, ingestion of marine litter, and poor growth, as well as mortalities without identifiable causes (Okada *et al*., 2014). Unknown causes of death account for over 60% of PBF mortalities within 1 month of sea cage transfer. Because PBF are highly demanded in the market and there are concerns of potential depletion (Cyranoski, 2010; Benetti *et al.*, 2016), more efficient culture techniques are needed, and unknown causes of mortality need to be elucidated. In addition, it should be noted that since collision death has been considered a major source of mortality, only physical inspection such as bone and/or skin injuries have been used in examinations (Miyashita *et al*., 2000; Ishibashi *et al*., 2009; Higuchi *et al*., 2014), but physiological changes in collision mortalities have not yet been investigated. Disturbance and disability in cruise swimming can be fatal in ram ventilators; as such, even non-fatal collisions may result in eventual mortality in PBF due to a loss of ability to maintain efficient gas exchange. Consequently, we hypothesized that mortality occurs due to collision death without physical injuries and to investigate this, therefore, we evaluated the blood chemistries of PBF after experiencing collisions.

2.2 Materials and methods

Three hundred artificially-hatched PBF juveniles (50 dph) were raised from eggs spawned by cultured broodstock in a 30 m^3 overflow system tank of 6 m in diameter and approximately 1 m deep. These PBF were fed an artificial diet (Magokoro S-3, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) and maintained at 24.9°C under 102.2% dissolved oxygen, and 32.0 salinity. During rearing, PBF juveniles showing abnormal swimming (AS) behaviors owing to bumping into the tank wall (frantic, irregular, and unsteady swimming) were immediately removed for further analysis. Normally swimming (NS) fish engaging in schooling behaviors with constant speed were also collected for analysis at the same time.

A total of 10 AS fish (mean body length (BL) and weight (BW) : 81.4 ± 11.8 mm and 7.6 ± 3.8 g,

respectively) and 12 NS fish (mean BL and BW: 87.6 ± 7.2 mm and 9.8 ± 2.4 g, respectively) were individually captured using a hand net and handled with wet soft fabric. Approximately 0.2 mL of blood was collected from the caudal vessel using a heparinized 1 mL syringe with a 25G×1 needle . Anesthesia was not used due to its significant effects on electrolytes and blood pH (unpublished results included in another study, Aquaculture Research Institute of Kindai University). Immediately after collection, blood samples were analyzed for partial pressure of O_2 (PO_2 mmHg) and CO_2 (PCO_2 mmHg), electrolytes (Na^+ and Cl^- mmol L^{-1}; K^+ nmol L^{-1}), H^+ (nmol L^{-1}), $CHCO_3^-$ (nmol L^{-1}), and pH using a blood gas analyzer (Cobas b121, Roche Diagnostics K.K., Tokyo Japan). In addition, both AS and NS specimens were X-rayed (MA-60; Hitex Co., Osaka, Japan and Naomi-NX04H; RF Co., Nagano, Japan) to detect bone injuries.

All values are expressed as mean ± standard deviation. Data of AS fish were analyzed separately for presence (n = 5) or absence (n = 5) of bone injuries using the Mann-Whitney U-test, with differences at $p < 0.05$ regarded as significant. This analysis was conducted in order to determine the differences within the AS specimens prior to the comparison between the groups. The AS data were then pooled, and the means of the AS and NS specimens were similarly evaluated. Statistical analyses were performed using the statistical package for the social sciences (SPSS) v 16.0j software (IBM, Tokyo, Japan).

2.3 Results

There were no significant differences in BL or BW between the groups or between the AS fish with and without bone injuries. Half of the AS specimens showed bone injuries, including dislocation, fracture, or both, of the vertebral column and parasphenoid, which are typical incident of collision to the tank wall. However, no significant differences between fish with and without injuries were detected in any blood chemistry (Table 1).

Table 1. Blood chemistries of Pacific bluefin tuna juveniles with abnormal swimming behavior

	PO_2 (mmHg)	PCO_2 (mmHg)	H^+ (nmol/L)	$CHCO_3^-$ (mmol/L)	pH	Na^+ (mmol/L)	Cl^- (mmol/L)	K^+ (mmol/L)
With injuries (n = 5)	96.1 ± 47.5	9.6 ± 4.6	39.2 ± 9.5	11.5 ± 2.6	7.42 ± 0.11	166.5 ± 17.1	142.5 ± 19.0	6.3 ± 1.5
Without injuries (n = 5)	73.1 ± 33.1	12.0 ± 3.5	45.7 ± 13.3	11.4 ± 1.8	7.36 ± 0.13	169.9 ± 13.3	143.6 ± 12.7	7.5 ± 2.4

No significant differences were detected between individuals with and without physical injuries (Mann-Whitney *U*-test).

In the comparisons of AS and NS fish (Figure 2-1), significantly higher PO_2 was found in the AS fish ($p = 0.01$). Contrastingly, PCO_2 was significantly lower in the AS specimens ($p = 0.0002$). In addition, the blood pH of the AS fish was significantly higher than that of the NS fish ($p = 0.003$), with concomitantly lower H^+ levels ($p = 0.004$) although $_CHCO_3^-$ levels were similar ($p = 0.339$). Even though Na^+ and Cl^- levels were similar ($p = 0.391$ and 0.947, respectively) between the groups, our data also showed that K^+ levels were significantly higher in the AS fish ($p = 0.04$).

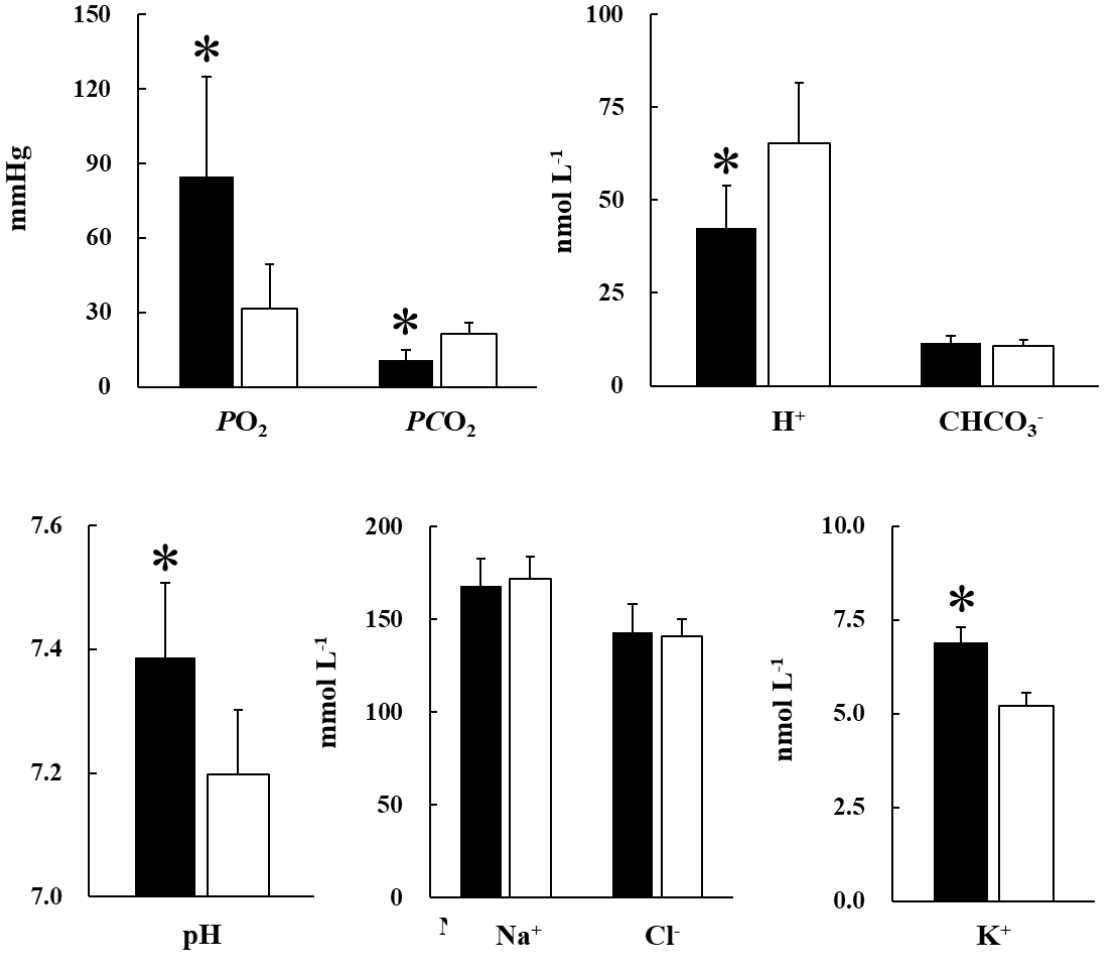

Figure 2-1. Comparative results of blood chemistry analysis between abnormally swimming (AS) and normally swimming (NS) of Pacific bluefin tuna juvenile. Black bars represent AS results (n=10) and white bars represent NS results (n=12). Asterisks indicate significant differences where $p < 0.05$.

2.4 Discussion

Our results from comparisons within the AS specimens suggested that bone injuries are obvious evidence of collision death (Miyashita *et al*., 2000; Higuchi *et al*., 2014; Okada *et al.*, 2014), although the blood chemistries were similar within the AS fish regardless of bone injuries (Table 1). Furthermore, the comparative results of AS and NS specimens revealed that PBF juveniles showing abnormal swimming may undergo hyperventilation and experience metabolic alkalosis, even in the absence of bone injuries (Figure 2-1). Frantic and irregular swimming behavior might constitute an

attempt to increase the amount of water passing through the mouth to the gills after collision. Fish utilize a blood bicarbonate buffer system to maintain pH, and demonstrate considerable ability to maintain internal pH homeostasis, similar to mammals (Claiborne *et al.*, 2002). Although tuna blood has a high Bohr coefficient and high capacity for CO_2 buffering (Bushnell and Jones, 1994), respiratory failure such as that during hyperventilation may be a primary cause of death in ram ventilators. Therefore, this study indicates that, in addition to mortalities due to bone injury after heavy collision impacts, even light collisions may be fatal for ram-ventilating PBF juveniles. Hence, collision death should be defined not only by physical injury, but by physiological injuries as well, particularly due to respiratory failure and imbalances of acid-base regulation.

Our data also indicate that AS specimens lapsed into metabolic alkalosis owing to a significant reduction in H^+ ions. However, both Na^+ and Cl^- levels were statistically similar between AS and NS specimens. Marked depression of blood pH occurs in both freshwater (Jensen *et al*., 1983; Turner *et al.*, 1983; Schwalme and Mackay, 1985) and marine fishes, including some tuna species (Wood *et al*., 1977; Holeton and Heisler, 1983; Perry *et al*., 1985), when fish are stressed, due to a combination of metabolic and respiratory acidosis. Acidosis has generally been attributed to protons generated in association with lactate production and ATP breakdown in white muscle (Wood, 1991). Regardless of fish taxa or the source of the acid-base stress, it is reported that the return of blood toward control pH is of primary importance (Evans *et al*., 2005). Generally, red blood cells play an important role in proton extrusion, and metabolic acid released from white muscle is transiently shuttled to the external environment across the gill epithelium (Lin and Randall 1995; Claiborne *et al*., 2002; Evans *et al*., 2005). However, it appears that H^+-ATPase, with its apical electrogenic excretion of protons, plays a less important role in seawater-adapted teleosts (Lin and Randall, 1993). In the gills, active Na^+ influx (Na^+/acid exchange) is ordinarily stimulated when the intra- and extracellular pH changes; therefore, Na^+ level should be increased to maintain homeostasis of pH. As well as Na^+/acid exchange, Cl^- /HCO_3^- exchanges have been implicated in acid-base transport across the gill epithelium (Ishimatsu and Kita, 1998; Claiborne *et al*., 2002). Therefore, it is thought that the Na^+/acid and Cl^-/HCO_3^- exchange functions of the gills might have been dysfunctional when PBF juveniles were unable to

swim correctly or had physical damage to the gills, or both, leading to excessive excretion of protons and HCO_3^-. Even light impacts from collisions may induce abnormal swimming behavior through the disturbance of cruise swimming. Further studies are required to elucidate this point, however, the present study implies that cruise swimming is essential for the respiratory intake of O_2 and excretion of CO_2, as well as for acid-base maintenance and ion exchange, in ram ventilators.

K^+ levels were significantly higher in AS fish ($p = 0.04$; Figure 2-1), indicating that hyperkalemia was involved in the characteristics of PBF showing abnormal swimming, hyperventilation, and metabolic alkalosis. Because of the need to maintain osmolality, increased K^+ should be paralleled by decreased Na^+ concentration; however, our results did not show this. K^+ is known as a major cytoplasmic electrolyte and it is reported that the shrinkage of red blood cells contributes to plasma hyperkalemia (Jensen, 1990). In addition, large increases in plasma K^+ may interfere with tissue excitability and muscle (e.g. heart) contraction (Jensen, 1990). Hence, our results indicate that it may be difficult for PBF juveniles showing abnormal swimming to recover to normal swimming, which is essential for ram ventilators, due to malfunctions of tissues and muscle. However, the origins of these K^+ influxes are still unclear, and further study is required.

In conclusion, this chapter revealed major blood chemistry changes based on hyperventilation, metabolic alkalosis, and hyperkalemia in PBF juveniles showing abnormal swimming, even in the absence of physical injury. These physiological changes could lead to mortality during fingerling production of PBF.

Chapter 3

Control of the photoenvironment in land-based tank

3.1 Effect of illuminance transition rate on survival and stress response

3.1.1 Introduction

Some studies have suggested that the rapid change of light intensity during the dawn period could be another cause of collision death (Masuma *et al*., 2001; Fukuda *et al.*, 2010; Torisawa *et al*., 2011). Masuma *et al*., (2001) reported that this mortality is caused by visual disorientation due to dim lighting, when the retinomotor response of fish does not match with increasing light intensity. It is supposed that survival of PBF juveniles will increase if dim environments that are a suspected cause of collision death, especially during the dawn period, are controlled. Therefore, in the present chapter, the effects of the rate of increase in light intensity during the dawn period was examined. An experimental group was exposed to artificial illumination that was turned on in series. This group was compared to a control group kept under the natural light environment. We evaluated survival rate, growth performance, and stress response of the juvenile PBF, including whole-body cortisol, glucose levels, and diel changes in plasma cortisol concentration.

3.1.2 Materials and Methods

Fish and Experimental Design

Four experimental tanks were prepared, two for each experimental condition. Each tank was circular, 6 m in diameter, and had a volume of 30 m^3. Two thousand PBF juveniles were hatched from eggs that were spawned naturally from PBF broodstock and were reared for 31 days in a 20 m^3 concrete square tank. At 31 dph, the fish were randomly assigned to experimental tanks. According to Ishibashi *et al.*, (2009), the mortality of juvenile PBF increases for the first 3–5 days immediately following transfer to a new place, because the fish cannot sense the margins of the enclosed space at night. Therefore, fish were acclimated to the experimental tanks for 5 days prior to the start of experiments to eliminate any effects of deaths due to changes in tank shape or size. After 5 days of acclimation, the number of fish in each tank was adjusted to 400 individuals by adding fish. After the acclimation period, 25 and 19 tails of experimental fish were added to the control and test group, respectively. The fish were added prior to start of experiment. Therefore, there was no effect on survival results by adding fish. The average initial size of the experimental fish was as follows: total length (TL) 8.8 ±

0.8 cm, BW 8.4 ± 1.4 g (n = 4). The fish were fed chopped Japanese sand lance (*Ammodytes personatus*) to satiation at suitable times from 07:00 to 18:00 h. During the experiment, the rearing water in the tanks was maintained at 26.7 ± 0.1 ºC with 109 ± 1.5% DO. Rearing water was exchanged eight times each day.

Two different light conditions during the dawn period were investigated: natural light conditions (unlit control group) and reduced rate of increase in light intensity (test group). To control the rate of light intensity increase during the dawn period, 6 automatic timers were connected to 2-W midget incandescent lamps (Maxer-denki, Co Ltd, Nagano, Japan) located above the tank. These automatic timers were controlled in order to increase light intensity gradually from 03:45 to 05:15 h in 15 min intervals and then turned off during the daytime. Both control and test groups were under natural light conditions during the dusk period. The changes in light intensity are displayed in Fig. 3-1.

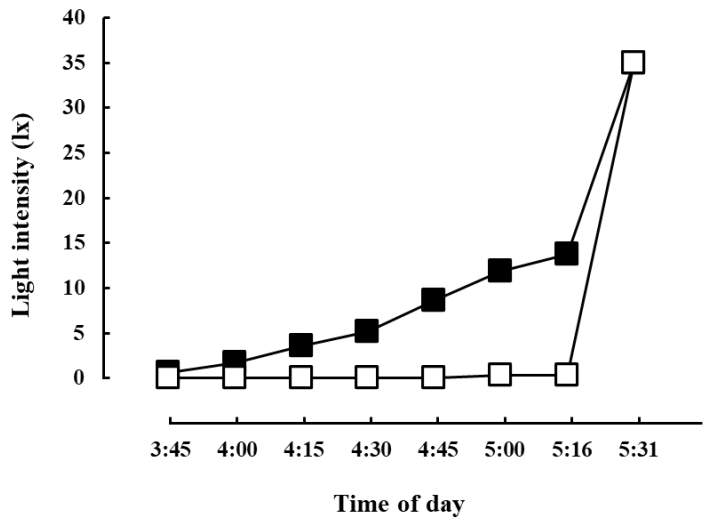

Figure 3-1. Changes in light intensity at the surface of tanks containing Pacific bluefin tuna juvenile during the dawn period. The test group (black rectangles) was exposed to artificially slowed increase in dawn light intensity and the control group (white rectangles) was kept under natural light conditions. All tanks were illuminated by natural sunlight after 05:30 h.

After 05:15 h, test group tanks were illuminated by natural sunlight. The light intensity was measured at the water surface at the center of tank. Double shades were placed between experimental tanks to block light from adjacent tanks. The total experimental period was 9 days. Dead specimens were collected and counted every morning to determine survival rate. In addition, both control and test

groups were observed visually before and after illumination of the tanks during the dawn period. Fish were also observed during the dusk period to confirm whether the artificial lighting influenced fish behaviours.

Sampling and Analysis Methods

During the first few days of the experiment, the fish were too small to collect blood samples; therefore, the whole body was used for analysis. At the beginning of the lighting experiments, four fish were sacrificed to obtain initial (day 0) whole-body cortisol and glucose levels. On the days 1 through 7, three fish were sampled from each experimental tank every morning before feeding at 07:00 h. A total of six fish from test group and six fish from the control group were used to quantify whole-body cortisol and glucose levels. Fish sampled on the 7th day were used to compare the growth performance of the fish in each group. In addition, two fish were sampled from each experimental tank every 3 h on the 7th day. A total of four fish both test and control group were used to quantify diurnal changes in plasma cortisol concentrations. Fish were collected from the experimental tanks using hand-nets with minimum effect on other fish in the tanks. Blood was collected from sampled fish using a sterilized 5-mL syringe and TL (cm), fork length (FL cm), and BW (g) were measured. These measurements were used to calculate growth performance indices, including CF, specific growth rate (SGR %/day), and weight gain (%/day) using the following formulae.

$$CF = (W_f L^{-3}) \times 100$$

$$SGR\ (\%/day) = 100 \times (\ln W_f - \ln W_i)/\text{time (days)}$$

$$\text{Weight Gain}\ (\%/day) = 100 \times (W_f - W_i)/((W_i + W_f)/2)/\text{time (days)}$$

where W_i = initial body weight (g), W_f = final body weight (g), and L = final fork length (cm).

The blood samples were immediately centrifuged at 3000 × g for 15 min to isolate the plasma. Sampled fish were anesthetized and killed within 1 min by using iced physiological saline, and whole fish and plasma were frozen in liquid nitrogen within several seconds of collection. Samples were stored at −80 °C until they were chemically analyzed.

The cortisol and glucose concentrations in whole bodies and plasma were measured using enzyme immunoassay (EIA) and glucose CII test kits (Wako Pure Chemical Industries Ltd., Osaka, Japan) and previously published methods (Ishibashi *et al*., 2009; Honryo *et al.*, 2013).

Statistical Analyses

Whole-body cortisol levels, glucose levels, and diel changes in plasma cortisol concentrations are expressed as mean ± standard deviation (SD) and were statistically analyzed by multivariate two-way analysis of variance (MANOVA) with Bonferroni correction. A multiple comparison that among the day of sampling on the whole-body cortisol and glucose levels and the time of day on the plasma cortisol concentrations between the control and the treatment were carried. Survival was compared between groups using Fisher's exact test. Growth performance was expressed as mean ± standard deviation (SD) and the growth performance of groups was compared using a Mann-Whitney *U*-test. These statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) program for Windows (version 16.0J, IBM Corp., Tokyo, Japan), and $p < 0.05$ was considered to represent a significant difference.

3.1. 3 Results

Survival Rate, Growth Performances, and Visual Observation of Fish Behaviours

The survival rate over the 9-day experiment is shown in Fig. 3-2. The survival rate of the test group was slightly lower than that of control group, although the difference was not statistically significant ($p = 0.064$). Just after the start of the experiment, the survival rate of test group decreased, and this tendency continued throughout the experiment.

Fish body size and weight measurements taken before the experiment and on the 7th day are provided in Table 2. Body weight on the 7th day was 2.5–2.7 times greater than body weight at the start of the experiment. All of the body-size indices tended to be higher for the control group than for the test group. However, none of these differences were significant.

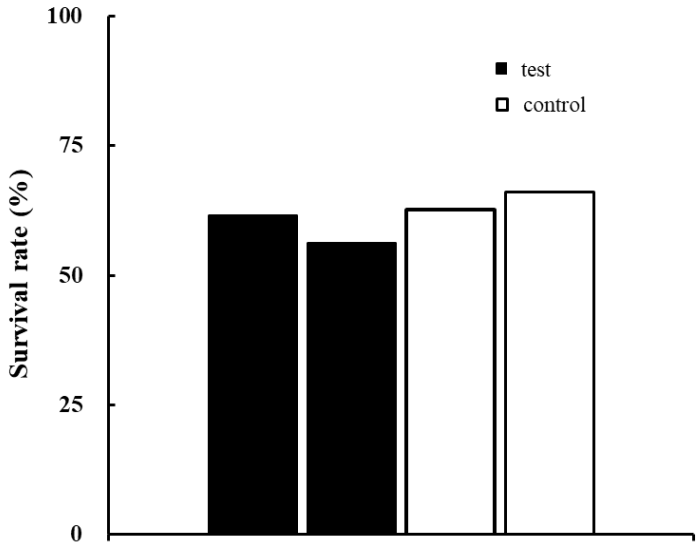

Figure 3-2. The survival of Pacific bluefin tuna juvenile cultured for 9 days under different lighting conditions. The test group (black) was exposed to artificially slowed increase in dawn light intensity and the control group (white) was kept under natural light conditions. Survival for each of two tanks per treatment are shown. No significant differences were detected between the groups (Fisher's exact test, $p = 0.064$).

Table 2. Growth performances of Pacific bluefin tuna juvenile cultured under different light condition.

	group	Total length (cm)	Fork length (cm)	Body weight (g)	CF	SGR (%/day^{-1})	WG (%/day)
Initial		8.8 ± 0.8	8.3 ± 0.7	8.4 ± 1.8	14.4 ± 0.3		
7th day	test	11.7 ± 1.1	10.9 ± 1.0	21.5 ± 6.6	16.1 ± 2.7	12.7 ± 4.6	11.7 ± 3.9
	control	12.0 ± 0.8	11.2 ± 0.8	23.2 ± 5.5	16.5 ± 3.2	14.1 ± 3.7	12.9 ± 3.0

Values represent mean ± SD.(Initial; $n = 4$, Final; $n = 28$ per treatment)

No significant differences were detected between groups.

To confirm the effect of artificial illumination during the dawn period on juvenile behaviours, fish were visually observed before and after the lights turned on during the dawn period and again during the dusk period. When the first light (0.64 lx) was switched on at 03:45 h with 0.64 lx, some of the test-group fish responded by abruptly turning or increasing swimming speed, but immediately resumed normal swimming behaviour. No frantic behaviour, such as bumping into the tank wall was observed. No fish collided with the tank wall between 03:45 h and 04:30 h when the light intensity was between 0.64 lx and 5.21 lx. However, abnormal swimming behaviour that included irregular swimming speed, stampeding, and collision with the tank wall was observed between 04:30 to 04:45 h when the light intensity was between 5.21 lx and 8.59 lx. After 04:45 h, such abnormal behaviour was moderated and the fish became calm. In the unlit control group, we did not observe abnormal swimming behaviour until 05:30 h. After 05:30 h, when sunlight penetrated into the experimental tanks and light intensity increased rapidly from 0.39 lx to 35 lx, the control group exhibited similar abnormal behaviour to that of the test group. However, the frequency and degree of collisions were noticeably milder than those of the test group. No abnormal behaviour was observed during dusk in either group.

Stress Responses

The changes in the whole-body cortisol levels of PBF juveniles during the experiment are shown in Fig. 3-3. The cortisol levels of the three fish sampled every day from each experimental tank were analyzed using a MANOVA with Bonferroni correction (n = 6). Groups did not differ in cortisol level on any of the experimental days.

However, on days 1–6, the control group had significantly lower whole-body glucose than the test group (Fig. 3-4). In the control group, glucose level decreased slightly on day 1, and then tended to increase through day 7.

Diel rhythms of plasma cortisol concentrations are shown in Fig. 3-5. The plasma cortisol concentrations of fish sampled from each experimental tank every 3 h on the 7th day were compared MANOVA with Bonferroni correction (n = 4 for each time point). Cortisol concentration was not significantly different between groups at any time point. Peak cortisol concentration occurred at 03:00–06:00 h in both groups. In the test group, the cortisol concentration at 06:00 h was significantly higher than the concentrations at all other measured times, except 03:00 h. In the control group, there was wide variation in cortisol levels, and there were no significant differences between sampling times.

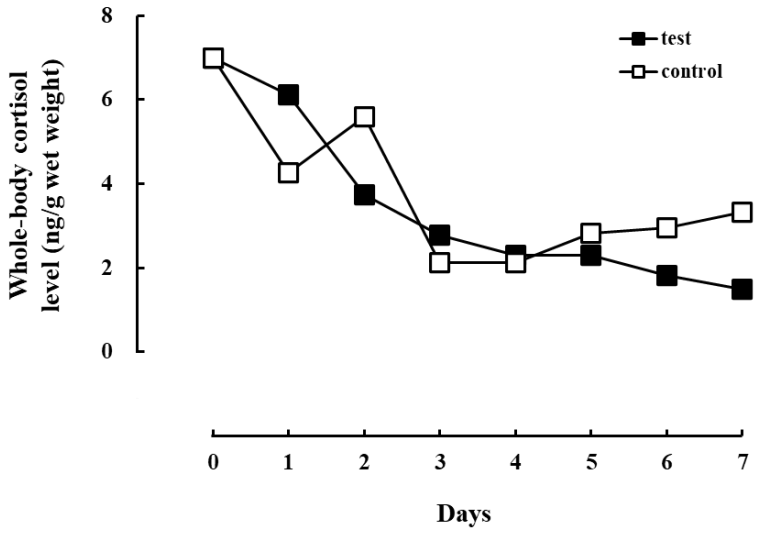

Figure 3-3. Changes in whole-body cortisol level of Pacific bluefin tuna juvenile cultured for 9 days under different lighting conditions. The test group was exposed to artificially slowed increase in dawn light intensity and the control group was kept under natural light conditions. Data are expressed as the mean of two replicate tanks (n = 6, per treatment). Error bars are not shown in order to simplify the figure.

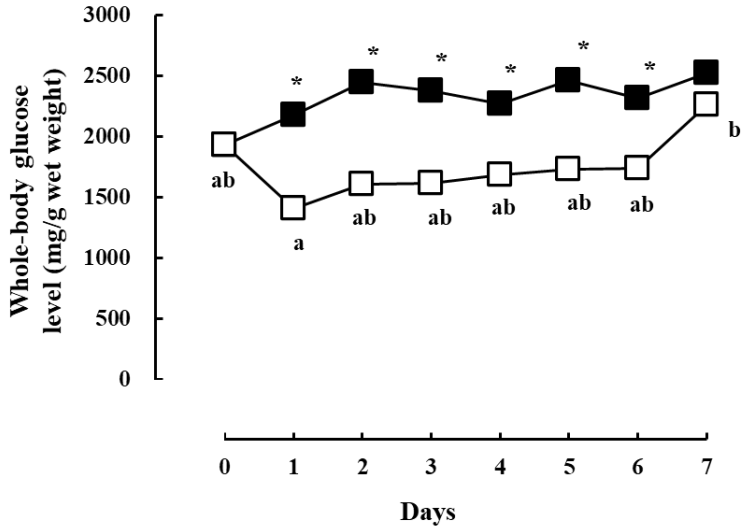

Figure 3-4. Changes of whole-body glucose level of Pacific bluefin tuna juvenile cultured for 9 days under different lighting conditions. The test group was exposed to artificially slowed increase in dawn light intensity and the control group was kept under natural light conditions. Data are expressed as the mean of two replicate tanks (n = 6, per treatment). Error bars are not shown in order to simplify the figure. *Significant differences were detected between groups on days 1–6 (MANOVA with Bonferroni correction). Different letters represent significant differences between sampling dates in the control group.

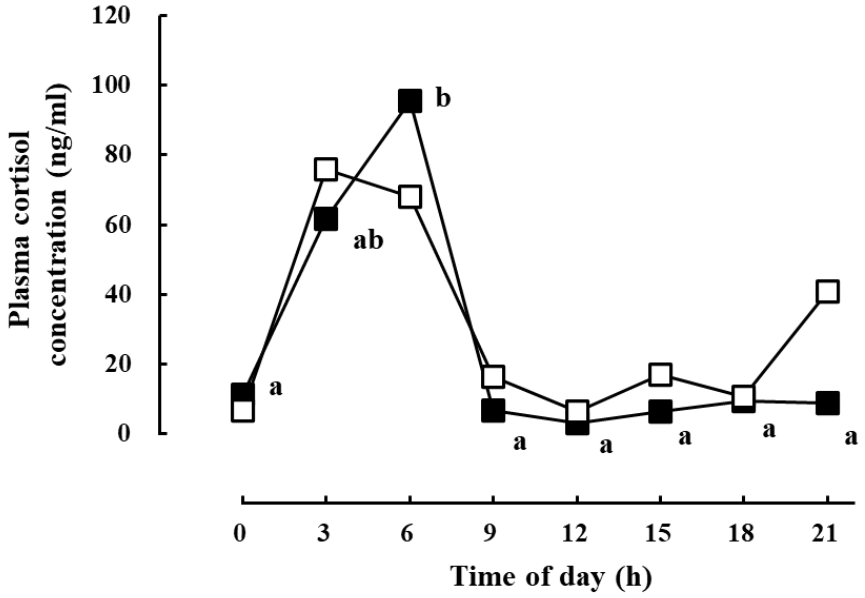

Figure 3-5. Diel rhythms of plasma cortisol concentration of Pacific bluefin tuna juvenile cultured for 9 days under different lighting conditions. The test group was exposed to artificially slowed increase in dawn light intensity and the control group was kept under natural light conditions. Data are expressed as the mean of two replicate tanks (n = 4, per treatment). Error bars are not shown in order to simplify the figure. No significant differences were detected between the groups. Different letters represent significant differences between sampling times within the test group. Cortisol concentration reached a peak at 03:00–06:00 h in the test group (MANOVA with Bonferroni correction).

3.1.4 Discussion

To investigate the causes of mass collision death of juvenile PBF, we compared an artificially slowed increase in dawn light intensity to natural light. The survival rate was not improved by a gradually increasing light regime that provided PBF with artificial illumination from six sets of automatically timed midget lamps during the dawn period. These timer-lamps provided a more gradual increase in light intensity than that experienced by fish in the unlit control condition (Fig. 3-1). However, relative to the control group, survival did not increase and growth performance tended to be poorer in the test group. While there was no significant difference between groups in whole-body cortisol level, whole-body glucose level was significantly higher in the test group than in the control group. Plasma cortisol concentration had an obvious diel rhythm that peaked during the dawn period. We hypothesized that a reduction in rate of light intensity increase during the dawn period might prevent mortality of juvenile

PBF. However, the results of this study indicate that reducing the rate at which light intensity increased using artificial illumination did not prevent mass death of PBF juveniles.

The swimming behaviour of PBF reportedly changes during the twilight period (Torisawa *et al.*, 2011). However, collision with tank walls is rarely observed during the dusk period in practical seedling production at KUFL. In this present study, abnormal behaviour was observed during the dawn period, but not during the dusk period. We suspected that mortality during the dawn period was caused by a rapid change in light intensity (Masuma *et al.*, 2001; Fukuda *et al.*, 2010), in other words, during the transition from scotopic to photopic vision. However, in this study, mortality during dim periods was not prevented by artificial illumination that slowed the increase in light intensity during the dawn period. In fact, the survival rate was lower for fish exposed to this slow transition than for unlit controls and the survival rate of the two groups was not statistically different. The scotopic vision of PBF is reported to be poor (Ishibashi *et al.*, 2009) and the visual-temporal resolution of PBF is inferior to that of chub mackerel, *Scomber japonicas* (Mastumoto *et al.*, 2009; 2011). We previously reported that PBF juveniles tend to die during the night (Honryo *et al*., 2013; Ishibashi *et al*., 2013). Therefore, the main factor influencing juvenile mortality was hypothesized to be the scotophase itself, including the dawn period, rather than the rate of light intensity transition. Nighttime high-intensity lighting (greater than 150 lx) enhanced survival, supporting this hypothesis. By gradually increasing light intensity at dawn in the present study, the dim period was extended and an increase in mortality was induced. It is thought that a low light environment of 5–15 lx is problematic for PBF juveniles (Honryo *et al.*, 2013) because it is at the boundary between scotopic and photopic vision adaptation (Masuma *et al.*, 2001). In fact, we observed the highest frequency of abnormal behaviour in the test group when the tank was illuminated by 5.21–8.59 lx (04:30 h to 04:45 h). Even though the light intensity rapidly increased in the control group from 0.39 to 35 lx in 15 minutes, the degree of abnormal behavior was much milder than in the test group. Abnormal swimming behaviour causes mortality to increase due to collision or contact with the walls of rearing tanks and the observed difference in the degree of abnormal swimming behaviour might directly contribute to any differences in survival rate between groups.

The spectrum of natural sun light differs from that of the incandescent lamps used in this study. However, we reported that the peak spectral sensitivity wavelength of juvenile PBF (SL = 74–223 mm) was 505 nm (Matsumoto *et al.*, 2012). In the present study, the illumination in the test group was provided by incandescent lamps that emit a wide spectrum of visible light that includes 505 nm. Considering this fact, the effect of the different light spectra provided to the test and control groups

were negligible in this study. Furthermore, we have previously reported that there was no notable difference in the survival rate of juvenile PBF exposed to different wavelengths (i.e., 460 nm (blue), 520 nm (green), and 450–680 nm (white)) (Tsutsumi *et al.*, 2014). The negative effect of extended low light intensity on PBF juveniles is therefore attributed to the light intensity itself, rather than to the rate of transition or spectrum.

Plasma cortisol concentration had an obvious diel rhythm that peaked at 03:00–06:00 h (Fig. 3-5). In PBF juveniles, some physiological parameters such as prolactin, somatolactin and growth hormone are known to show diel rhythms under aquacultured condition (Adachi *et al.*, 2008; Adachi *et al.*, 2009). Cortisol concentration was significantly higher during this peak period than at other sampling times in the test group. No such differences were detected in the control group because cortisol concentration was highly variable in this group. However, there was no significant difference in cortisol concentration between the groups and both groups showed same tendency. Plasma or serum cortisol concentration has a diel rhythm in many species, such as gulf killfish, *Fundulus grandis* (Garcia and Meier, 1973), brown trout, *Salmo trutta* L. (Pickering and Pottinger, 1983), rainbow trout, *Salmo gairdneri* Richardson (Laidley and Leatherland, 1988), *Oncorhynchus mykiss* (Holloway *et al*., 1994; Gregory and Wood, 1999), and common dentex, *Dentex dentex* (Pavilidis *et al.*, 1999). Boujard and Leatherland (1992) found that rainbow trout, *Oncorhynchus mykiss,* held under long photoperiods had plasma cortisol concentrations that had an obvious diel rhythm that might have been entrained by feeding activity and that were similar to those observed in the present study. Reddy and Leatherland (1994) reported that there was a significant interaction between the time of feeding and plasma cortisol concentration in immature rainbow trout. They further reported that the diel rhythm of plasma cortisol concentration reached a peak at 24:00 h and was only evident in fish fed during the post-dawn period. The timing of the peak plasma cortisol concentration at 03:00–06:00 h in the present study may be related to the feeding time. The fact that whole-body cortisol levels were not significantly different between the groups indicates that the increase of plasma cortisol concentration was not reflecting a stress response.

However, significant elevations of whole-body glucose levels were detected in the test group. As we observed abnormal behaviour during the dawn period, artificial illumination might have influenced the whole-body glucose stress responses only in the fish of the test group. It is unclear why whole-body glucose level was elevated without any effect on cortisol, Further study is required to understand the differential response of these two stress indices in this species.

Torisawa *et al.*, (2011) reported that schooling behaviours corresponded strongly to vision development. Kitagawa *et al.*, (2004) used archival tags to determine that immature PBF engage in diving behavior in response to both sunrise and sunset. The PBF juveniles that were used in the present study and the immature fish used by Kitagawa *et al.*, (2004) differed in body size and probably also in swimming pattern. PBF juveniles may not dive during sunset but sunrise. This hypothesis is consistent with our observation of abnormal behaviours that occurred before sunrise and lack of any abnormal behaviours during sunset. Feeding activity is considered characteristic of the species and constant throughout the developmental stages of PBF. Direct correlations between behavioral rhythms and endocrinological rhythms are still unclear. Nevertheless, the diel rhythm of plasma cortisol concentration that peaked during the dawn period in this study is similar to that observed by Boujard and Leatherland (1994), suggesting that responses to photophase are associated with feeding times. Plasma cortisol concentration peaked at the transition between scotophase and photophase and pre-feeding diving behaviours caused mortality in the limited space of the rearing tank.

In conclusion, survival rate was slightly lower in tanks in which light intensity changes were artificially slowed during the dawn period than in tanks illuminated only by natural light. This study suggested that the scotophase itself results in an elevated incidence of collision death, rather than the transition rate between scotophase and photophase. Consequently, nighttime lighting is likely the most effective method for preventing mass death in juvenile PBF cultures.

3.2 Effects of night-time light intensity on the survival rate and stress response

3.2.1 Introduction

Miyashita *et al.*, (2000) demonstrated that juvenile PBF that die due to collisions sustain 2 main types of injuries: dislocation of the vertebral column and fracture of the parasphenoid. Even when collisions or contacts are not fatal, injured fish can suffer debility and emaciation, leading to death within few days. It has also been reported that juvenile PBF are unable to gyrate rapidly or remain adequately suspended (Miyashita 2002). Although collision-related mortality may be due to a sudden environmental change, the exact cause is not known. Therefore, a practical solution for reducing collision-related mortality in juvenile PBF is needed to allow for mass production of PBF juveniles. Therefore, this chapter focused on both the light environment, especially during the night, and light intensity in order to determine the effect of night-time lighting on stress responses. The effect of different intensities of night-time lighting on the survival rate and stress response in juvenile PBF was exmained.

3.2.2 Materials and Methods

Fish

Five thousand PBF juveniles hatched from eggs that were spawned naturally from PBF broodstock were reared in a 20-m^3 concrete square tank in July 2004. At 31 dph, the fish were randomly placed in 8 experimental tanks of 30-m^3 volume (6 m in diameter). Two replicate tanks were set up for each experimental group. The mortality of juvenile PBF is high during the 3- to 5-day period immediately following transferring, because the fish cannot sense the margins of the enclosed space at night (Ishibashi *et al.*, 2009). Therefore, after transfer, the fish were acclimated for 5 days to allow for any deaths due to changes in shape or size of habitat. After 5 days of acclimation, the numbers of the experimental fish were controlled by adding or subtracting up to 400 individuals in each tank. The initial average sizes of the experimental fish were as follows: body length 8.8 ± 0.8 cm; body weight 8.4 ± 1.4g (n = 4). The fish were fed chopped Japanese sand lance (*Ammodytes personatus*) up to satiation at suitable times.

Experimental design

Four different night-time light intensities were examined: 0 lx (unlit control), 5 lx (low intensity), 15 lx (medium intensity) and 150 lx (high intensity). Light was provided by a 12-W incandescent lamp placed above the center of the tank. The light intensity was measured at the water surface at the center of tank. Experimental tanks were sited at the indoor ridge, and natural sunlight was provided during daytime. The artificial lighting was turned on from 18:00 h to 06:00 h, and double shades were placed between tanks to block out the light from adjacent tanks. The total experimental period was 12 days, which included 9 days to test the effect of night-time lighting and 3 days for post-experiment observation. During the experiment, the water in the tanks was maintained at 26.7 ± 0.1°C and 109 ± 1.5% of dissolved oxygen. The rearing water was exchanged 8 times each day.

Sampling and analysis methods

To estimate the rate of mortality that occurred during the night-time, dead fish were collected at 0600 h and 1800 h during the lighting phase of the experiment and visually inspected to determine the cause of death. During the night-time lighting, we observed the differences in fish behavior between the experimental groups. Before the start of the lighting experiments, 4 fish were sacrificed to obtain initial (day 0) values for both whole-body cortisol and glucose levels. On days 1 through 7, 6 fish were sampled from each experimental group every morning at 07:00 h to quantify their whole-body cortisol and glucose levels. During the first few days of the experiment, the fish were too small to collect blood samples from; therefore, the whole body was used for analysis. In addition, 4 fish were sampled from each experimental group every 3 h on the 7th day to quantify diurnal changes in their plasma cortisol concentrations. After collecting fish from the experimental tanks using hand-nets, blood was collected using a sterilised 5-mL syringe. The blood samples were immediately centrifuged at 3000 × g for 15 min to isolate the plasma. The sampled fish were anesthetised and killed within several minutes using iced physiological saline, and whole fish or plasma was frozen in liquid nitrogen within several seconds. The samples were stored at −80°C until they were chemically analysed.

Frozen fish were homogenized with 5 volumes of phosphate-buffered saline using a glass homogenizer. Ether-soluble material was extracted from the homogenate. Impurities were then removed using carbon tetrachloride as described previously (Hiroi *et al.*, 1997). Whole-body cortisol levels were measured using the enzyme immunoassay (EIA) of Asahina *et al*., 1995 with some modifications (Ishibashi *et al.*, 2009). The EIA was conducted using anti-rabbit cortisol-3-carboxymethoxime bovine serum albumin (cortisol-3-CMO-BSA), immunoglobulin G (IgG) (FKA404E; Cosmo Bio. Ltd., Tokyo Japan), goat anti-rabbit IgG (Cappel Research Reagents, ICN, Temecula, CA, USA), and cortisol-3-CMO-horseradish peroxidase (HRP) (FKA403; Cosmo Bio, Japan). The reacted solution was colored using the o-phenylenediamine (OPD) kit enzyme-linked immunosorbent assay (ELISA) (Nacalai Tesque, Inc., Tokyo, Japan), and the absorbance at 450 nm was determined using a microplate reader (Model 550; Bio-Rad Laboratories, Inc., Richmond, CA, USA).

The remaining homogenate was centrifuged at 8000 × g for 10 min at 4°C, and the supernatant glucose concentration measured using the glucose CII test kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). The absorbance at 490 nm was determined using a microplate reader (model 550; Bio-Rad Laboratories, Inc.).

Statistical Analyses

Whole-body cortisol levels, glucose levels, and changes in plasma cortisol levels are expressed as mean ± standard deviation (SD) and were analysed by multivariate two-way analysis of variance (MANOVA) with Bonferroni correction. The survival data are displayed using Kaplan-Meier plots (Ilhan, 2004), and the survival rates in different groups were compared using the log-rank test. Statistical analyses were performed using the Statistical Package for the Social Science (SPSS) program for Windows (version 16.0J).

3.2.3 Results

Survival rates

The survival rates of PBF juveniles under different night-time light intensities are shown in Fig. 3-6. On day 9, the survival rate of the high-intensity group (75.8%) was significantly higher than that of the control group (64.3%) ($p < 0.001$, $n = 400$). In contrast, the survival rates of the low- and medium-intensity groups (60.9% and 57.2%, respectively) were less than that of the control group. After the night-time lighting ended, the survival rate in the high-intensity group decreased.

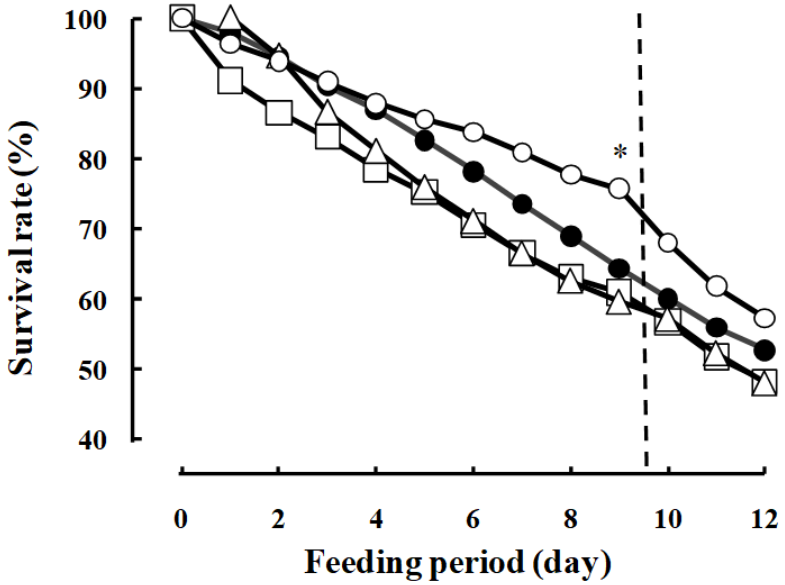

Figure 3-6. Kaplan-Meier plot of the cumulative survival of Pacific bluefin tuna juvenile under different night-time light intensities for 9 days followed by 3 days of observation to confirm the effect of ending the light experiment. ● No light (0 lx); □ Low light intensity (5 lx); △ Medium light intensity (15 lx); ○: High light intensity (150 lx). *The survival rate in the high-intensity group was significantly higher than that in the other 3 groups (Kaplan-Meier analysis, log-rank test; $p < 0.001$, $n = 400$).

Diurnal and nocturnal mortality

The relative mortality rates of each experimental group during daytime and night-time are compared in Fig. 3-7. In all experimental groups, the nocturnal mortality was higher than the diurnal mortality. Moreover, the nocturnal mortality of the control (0 lx) and high-intensity (150 lx) groups was significantly lower than that of the low- (5 lx) and medium-intensity (15 lx) groups. Furthermore, in

the low-intensity (5 lx) group, the diurnal mortality was significantly lower than the nocturnal mortality ($p < 0.05$). These results indicate that PBF juveniles tend to die in environments with less than 15 lx of ambient illumination.

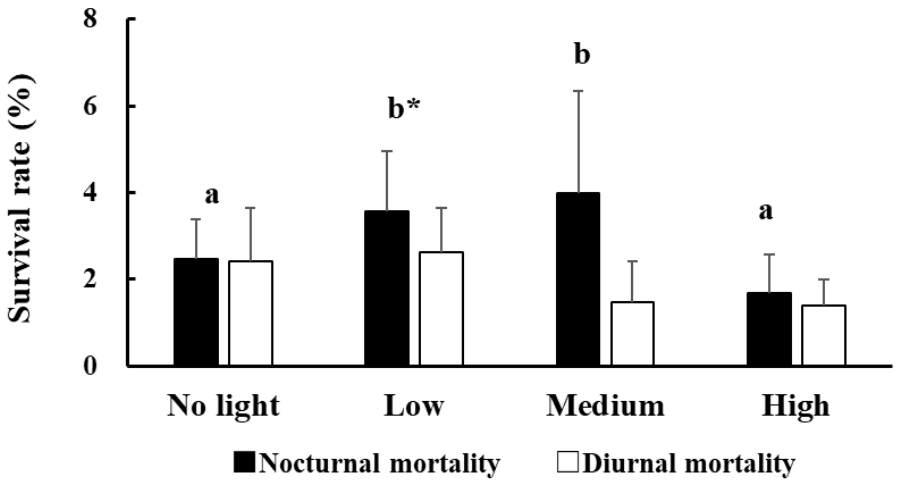

Figure 3-7. Comparison of diurnal and nocturnal mortality rates in Pacific bluefin tuna juvenile under the different light intensities. Data are expressed as the mean ± SD of 9 days in 2 tanks. Different superscript letters represent statistically significant differences (MANOVA with Bonferroni correction, $p < 0.05$). *The diurnal mortality rate was significantly lower than the nocturnal mortality rate ($p < 0.05$) in the low-intensity group.

Changes in whole-body cortisol and glucose levels

Changes in whole-body cortisol levels of PBF juveniles under different night-time light intensities are shown in Fig. 3-8. The 12-day experiment included 9 days of night-time lighting and a 3-day post-observation period. There were no significant differences between the 4 groups. This result revealed that night-time lighting did not cause stress in PBF juveniles.

Changes in whole-body glucose levels are shown in Fig. 3-9. In all 4 groups, glucose levels remained similar to the initial levels. However, on day 7, significant differences were found between the control (0 lx) and low-intensity (5 lx) groups compared with the medium-intensity (15 lx) groups ($p < 0.05$, $n = 6$). On the other hand, no significant differences were detected between the initial value and the all experimental groups.

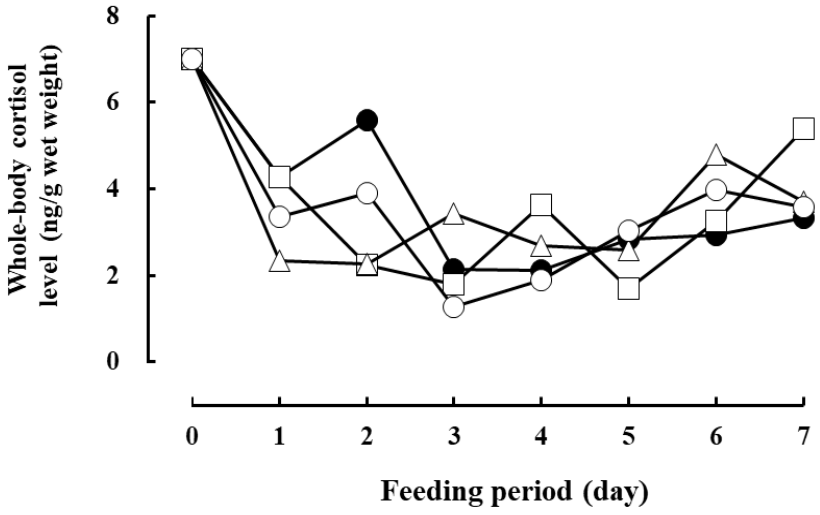

Figure 3-8. Changes in the whole-body cortisol level of Pacific bluefin tuna juveniles under different night-time light intensities. ● No light (0 lx); □ Low light intensity (5 lx); △ Medium light intensity (15 lx); ○ High light intensity (150 lx). Data are expressed as the mean ± SD of 2 tanks (n = 6). Error bars are not shown in order to simplify the figures. No statistically significant differences were detected.

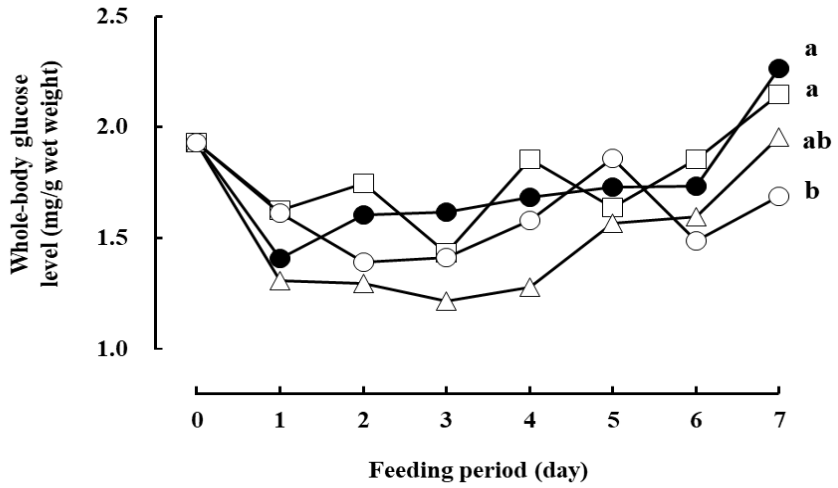

Figure 3-9. Changes in whole-body glucose level of Pacific bluefin tuna juveniles under different night-time light intensities. ● No light (0 lx); □ Low light intensity (5 lx); △ Medium light intensity (15 lx); ○ High light intensity (150 lx). Data are expressed as the mean ± SD of 2 tanks (n = 6). Error bars are not shown in order to simplify the figures. Different superscript letters represent statistically significant differences (MANOVA with Bonferroni correction, $p < 0.05$).

Diel changes in plasma cortisol levels

Diel changes in plasma cortisol levels of PBF juveniles aged 44 dph under different night-time light intensities are shown in Fig. 3-10. In all 4 groups, the plasma cortisol level peaked between 03:00 and 06:00 h. In the high-intensity group, the plasma cortisol level decreased between 03:00 and 06:00 h more than it decreased in other groups. These results indicate that night-time light did not affect diel changes in cortisol levels in juvenile PBF.

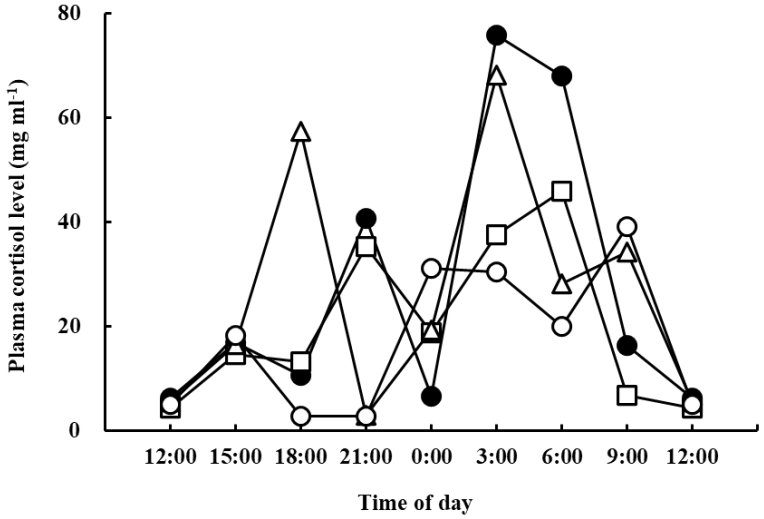

Figure 3-10. Diel changes in the plasma cortisol level of Pacific bluefin tuna juveniles (44 dph) at 3 h intervals under different night-time light intensities. ● No light (0 lx); □ Low light intensity (5 lx); △ Medium light intensity (15 lx); ○ High light intensity (150 lx). Data are expressed as the mean ± SD of 2 tanks (n = 4). Error bars are not shown in order to simplify the figures. No statistically significant differences were detected.

3.2.4 Discussion

Effect of photoperiods

There have been many studies on artificial photoperiods in cultured fish. Results of these studies indicate that the effect of light or darkness on fish growth and survival rates varies among species. For example, Barahona-Fernandes (1979) reported that high-intensity light promoted the growth of sea bass (*Dicentrarchus labrax*) larvae but did not affect their survival rates. In addition, this study confirmed the hypothesis that high-intensity light is lethal to newly hatched larvae, which have no

pigmentation. On the other hand, constant darkness reduced the rate of deformities in the dental bone of Atlantic halibut (*Hippoglossus hippoglossus*) larvae but did not affect their survival (Bolla and Holmefjotd, 1988). In contrast, Duray and Kohno (1988) showed that constant light exposure encouraged the growth and survival rate of rabbit fish (*Siganus guttatus*) larvae. Similarly, the effects of light or darkness on the stress response of cultured fish also vary among species. For example, the growth performance of juvenile red sea bream (*Pagrus major*) during rearing from 1 to 30 g can be enhanced significantly by providing a continuous photoperiod, without causing any significant stress response (Biswas *et al.*, 2006). On the other hand, it has been reported that an artificial photoperiod suppresses the immune system of juvenile rainbow trout (*Oncorhynchus mykiss*; Leonardi and Klempau, 2003).

These results indicate that despite their potential advantages, artificial photoperiods can be an environmental stress for several varieties of cultured fish. Studies suggest that there may be an optimum duration of artificial photoperiods that minimizes their disadvantages. For instance, Puvanendran and Brown (2002) showed that constant light reduced mortality in Atlantic cod (*Gadus morhua*; Grand Banks origin) larvae up to 28 dph. Similarly, Yoseda *et al.*, (2003) suggested that controlled photoperiods in coral trout (*Plectropomus leopardus*) larvae should be stopped after 5 dph to avoid disruption of their internal biological rhythms. For the reasons stated above, artificial lighting has both negative and positive effects that are dependent on the fish species and their developmental stage.

Positive effect of high-intensity night-time lighting

In this study, we showed that high-intensity night-time lighting (150 lx) enhanced the survival rate of PBF juveniles without increasing their stress response, as determined by comparison of whole-body plasma cortisol and glucose levels compared to unlit controls. If the fish under high-intensity light were stressed, whole-body cortisol levels and diurnal changes in plasma cortisol levels would have differed from that of controls. Since there was no significant difference in either whole-body cortisol levels or glucose levels between the control and high-intensity light groups, we conclude that high-

intensity night-time lighting does not seem to affect the health of juvenile PBF. Even though the significant differences were detected in the glucose levels at day 7, there was no difference between the initial value and experimental groups. This indicates that the differences confirmed on day 7 were included within the deviation of fish. Further study is required to prove that no correlation exists between the cortisol and glucose content in this species because these indices were shown to be associated in many fish (Ishibashi *et al.*, 2002a; Ishibashi *et al.*, 2002b; Ishibashi *et al.*, 2002c).

We previously reported that an evident stress response was detected in cultured juvenile PBF in sea cages, and mass death occurred within few days after transport as a result of no-night-time lighting (Ishibashi *et al.*, 2009). This phenomenon proved our hypothesis that juvenile PBF cannot recognize an enclosed space especially during the night after transportation. Therefore, juvenile PBF showed obvious stress responses and huge mortality. As observed from the previous studies (Ishibashi *et al*., 2009) and the present study, the surrounding environments are considerably different between open-sea and land-based tanks, and the acclimated periods have not been specified in previous studies. In the present study, the daily mortality rate and the number of colliding fish were much fewer than that in the previous studies which counts over 15 % because the experimental fish may have been habituated to the experimental tanks as a result of the 5-day acclimated period. The collided fish died within a few days, showing abnormal behavior, and the survival rate reduced. We randomly collected samples, but the abnormal fish were excluded from sampling, and thus, the survival rate was significantly different even though there was no significant stress response. In addition, the high deviation in each stress index indicated that some fish in the experimental group probably showed stress responses. Further investigation is required to explain this phenomenon and to obtain further clarity on the topic.

Negative effect of low- and medium-intensity night-time lighting

We observed that lower night-time light intensity negatively affected the behavior of juvenile PBF. For example, in the low- and medium-intensity groups, juveniles exhibited discordant behavior such as irregularities in schooling, swimming speeds, and distances between fish. In addition, those in the

low-intensity group were much more agitated when we approached their tanks. Furthermore, the survival rate decreased in fish that were reared in environments with less than 15 lx lighting (Fig. 3-6). The observed abnormal swimming behavior may be linked to lower survival rates. Specifically, juvenile PBF tended to die during the night rather than during the day (Fig. 3-7). A possible explanation for this pattern of deaths is that tunas are visual predators because they do not have well-developed olfactory lobes (Kawamura *et al*., 1981). PBF do not dive on cloudy days when light does not penetrate deep areas and visibility is insufficient for feeding (Kitagawa *et al*., 2004). Although Masuma *et al*., (2001) determined that the transition between scotopic and photopic vision in PBF juveniles occurs at a light intensity of 7.52 lx, our results indicated that less than 15 lx ambient illumination was not sufficient for the survival of cultured juvenile PBF (Fig. 3-6). Juvenile PBF may have difficulty recognizing an object at light intensities near the visual threshold at which light adaptation takes place. Light of less than 15 lx in the surroundings, which is approximately the threshold of retinomotor responses for juvenile PBF (Masuma *et al.*, 2001), affects their swimming and schooling behavior because it makes their sight unclear. This may be why lower survival was observed in the low-intensity (5 lx) and medium-intensity (15 lx) groups as compared with the unlit group. Despite their tendency to rely on vision, the light-intensity threshold for schooling in juvenile PBF is much higher than that of other species (Torisawa *et al.*, 2007). The scotopic visual threshold of cultured juvenile PBF for optomotor reactions is at least 40-fold less than that of 4 marine teleosts, namely, grouper (*Epinephelus septemfasciatus*), purplish amberjack (*Seriola dumerili*), ocellate puffer (*Takifugu rubripes*), and red sea bream (*Pagrus major*) (Ishibashi *et al.*, 2009). Furthermore, juvenile PBF have lower temporal resolution and light sensitivity than juvenile chub mackerel (*Scomber japonicus*) and striped jack (*Pseudocaranx dentex*) (Matsumoto *et al*., 2009). Thus, poor scotopic vision in PBF probably explains their high mortality due to collisions with the tank walls when cultured; high-intensity night-time light (150 lx) prevents collision deaths in an otherwise low light intensity environment.

Aquaculture facility

The high-intensity night-time lighting may be a simple and effective way to reduce collision deaths

during seedling production of PBF. However, this study also shows that power outages are a caveat to using this method because sudden environmental changes such as the disappearance of light can result in mass collisions and significant economic loss. Consequently, we suggest that facilities be equipped with uninterruptible power systems if artificial lighting is used in the aquaculture of PBF.

In conclusion, high-intensity (≥ 150 lx) night-time lighting can reduce mass deaths in cultured juvenile PBF during the period between 30 and 50 dph, without eliciting a stress response.

Furthermore, Ishibashi *et al.*, (2013) reported that contact or collision with tank walls was reduced when wall visibility of rearing tank was improved by creating patterns. This technique could be concomitantly used with nighttime lighting. Further study is required to develop more efficient technique to reduce mortality.

Chapter 4

Effectiveness of night-time lighting in sea cage culture of PBF juveniles

4.1 Artificial lighting prevents high night-time mortality caused by poor scotopic vision

4.1.1 Introduction

Previous chapter suggested that night-time lighting is an effective countermeasure against mortality occurred during low light intensity environment including scotphase in land-based tank. This protocol can potentially be applied also for the sea cage culture of PBF. It has been reported that high mortality occurred following to transfer of juvenile PBF from hatchery tanks to sea net cage is a major problem obstructs mass production of fingerlings. Thus, in Chapter 4-1, the effect of the night-time lighting in sea cage was examined. In addition to the rearing experiment, the mechanism in which induce mortality during low light intensity environment including scotophase was investigated by comparison of scotopic visual threshold of PBF juvenile.

4.1.2 Materials and Methods

Feeding experiment

To exmain the effectiveness of night-time lighting in the sea cage, total of 2,018 PBF juveniles (38 dph, mean TL: 8.1±1.9 cm, mean BW: 9.1±3.4 g) were used. These juveniles were transferred from land-based tanks to two experimental sea cages (octagonal shape on a side of 4 m and 6 m in depth) by the similar methods with Chapter 2. A cage was exposed to natural light environment which served as a negative control (treatment; Control). To compare growth and survival with Control cage, another cage was equipped with night-time lighting provided by four sets of fluorescent lights mounted 40 cm above the water surface (treatment; LMS). These lights were switched on from before dusk to after down (17:00 to 08:00 h). In addition, an underwater metal halide light was set up in the upper central part of the net sea cage, and vertical strips of white tape, 5 cm wide and 4 m long, were placed at intervals of about 0.3 m on the sides of the sea cage net. The light intensity during night time under LMS cage was about 3,000 lx and 0-0.07 lx in Control cage. Fish were fed chopped sand lance up to saturation during daylight hours for 30days. The lighting experiment was lasted 23 days, and the influence of switching off the night-time light was determined during 1 week. All the dead fish were collected every morning by scuba divers in each cage and counted to calculate survival rate. During

the feeding period, fish from both cages were sampled to evaluate growth performances on day 1, 3, 5, 8, and 20.

Statistical analysis

All values are expressed as mean ± standard deviation. In the feeding experiment, differences in survival rate between treatments were evaluated with Kaplan-Meier log-rank test at a significance level of $p < 0.001$. In addition, growth indices were evaluated between treatment in each day by the independent t-test at a significance level of $p < 0.05$. Statistical analyses were performed using the statistical package for the social sciences (SPSS) v 23.0 software (IBM, Tokyo, Japan).

4.1.3 Results

Feeding experiment

Survival rate of PBF juveniles reared under the different light condition was shown in Figure 4-1. In the Control cage, more than 75 % of fish died within a week of feeding and resulted in 12 % on the day 23rd. In contrast, the survival rate after 3days of stocking was still high (88%) in LMS cage and resulted in 73 % when night-time lighting was terminated on the day 23rd which was significantly higher than that of Control cage (Kaplan-Meier, log-rank test; $p < 0.001$, $n = 1{,}014$). The effect of terminating night-time lighting was limited. At the end of feeding experiment, survival rates were as follows; 10.9 % in Control cage and 60.0 % in LMS cage.

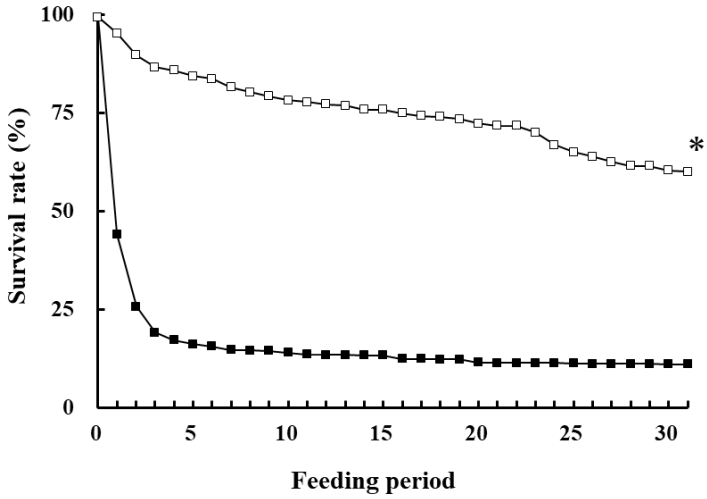

Figure 4-1. Changes in the survival rate of Pacific bluefin tuna juveniles cultured under the different lighting condition in sea cage (□; LMS, ■; Control). Asterisks indicate significant differences between treatments (Kaplan-Meier log-rank test, $p < 0.001$, $n = 1{,}014$).

Figure 4-2 expressed that the final TL and BW were 2 times longer and 6.7-8.6 times greater than the beginning of experiment, and the fish under the LMS cage was significantly greater than the Control cage at the end of experiment (independent t-test, $p < 0.05$, $n = 6$).

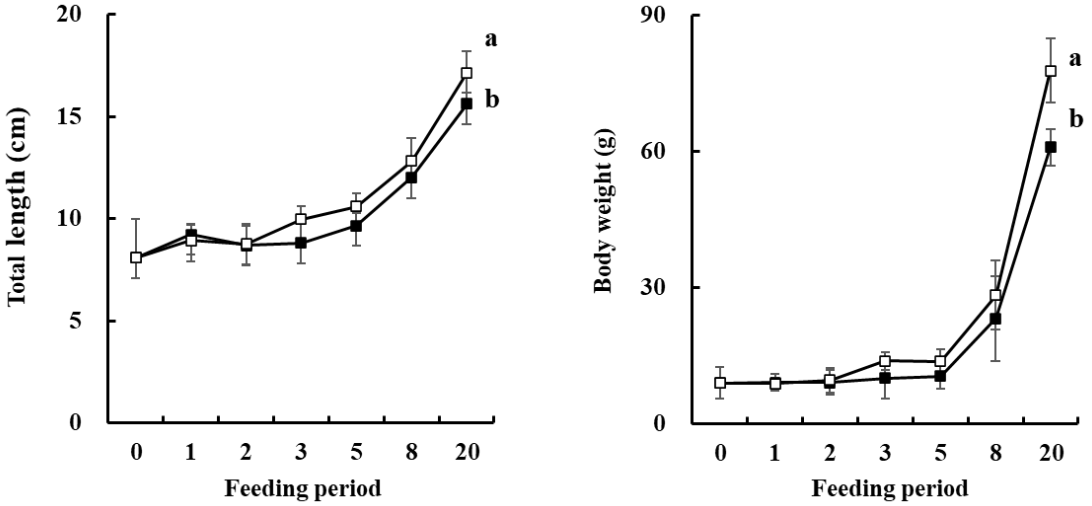

Figure 4-2. The comparison of total length (left panel) and body weight (right panel) during the feeding experiment (□; LMS, ■; Control). Different superscript letters represent statistically significant differences (independent t-test, $p < 0.05$).

4.1.4 Discussion

Our results clearly suggested that mass mortality occurred just after the transportation could be prevented by providing night-time lighting. PBF juveniles were died under the low light intensity environment both in tank and sea cage culture and this was explained by visual character of PBF. The optomotor reaction is considered to be an excellent index for evaluating the visual characteristics of fish larvae and juveniles (Carvalho *et al.*, 2002). Ishibashi *et al.*, (2009) showed that early juvenile PBT have a significantly lower optomotor reaction under twilight conditions, compared with four coastal teleosts at similar developmental stages, and provides new knowledge relevant to vision characteristics in marine fish. Although adult tunas may have specific visual characteristics, the visual capability of juveniles is inferior to that of other juvenile teleosts. Since the visual threshold of juvenile PBT is 0.09 lx under white illuminant, juvenile PBT cannot see enough of the net enclosure at night (Ishibashi *et al.*, 2009). It is considered that when PBF juveniles are transferred to an open-sea net

cage, they cannot sense the margins of the enclosed space in the nighttime and they touch or collide with the net wall, resulting in mass mortality. Thus, providing night-time lighting which eliminate the low light-intensity environment both in tank and sea cage could be an effective countermeasure against mortality in PBF fingerling production process.

The growth under LMS treatment were significantly promoted at the end of experiment, however, this was not confirmed at the previous chapter in which examined the effect of nigh-time lighting in the tank culture of PBF. Therefore, further study is required to elucidate this advantageous effect of night-time lighting in the sea cage.

4.2 Advantages of nighttime lighting for growth

4.2.1 Introduction

Ishibashi *et al.*, (2009) reported that nighttime lighting effectively prevents mass mortality of PBF juveniles, especially when fish are transferred from land-based tanks to open sea cages, owing to the low scotopic vision of PBF juveniles. Because of their low scotopic vision, PBF juveniles cannot recognize the wall of sea cage nets and collide with it, resulting in trauma injuries and death. Nighttime lighting is also known to attract various zooplankton, positive-phototaxis larvae, and other biota. PBF juveniles ingest such gathered feed items under nighttime lighting in sea cages (unpublished, Fig. 4-3); however, the effect of such feed on the growth and viability of PBF juveniles has not been investigated yet. In this investigation, we hypothesized that the growth and viability of PBF juveniles can be improved by the intake of feed items gathered under nighttime lighting when they are transferred to sea cages. Rearing environments such as water temperature, tide, and other factors in cages placed in the sea can be subject to irregular changes. We anticipated that the type and quantity of gathered feed items under nighttime lighting would differ depending on the season and location of the cage. Thus, it would be impossible to guarantee repeatability and certainty of the experiment using a sea cage. Hence, we carried out a study on the transfer of juvenile PBF using land-based tanks that were not influenced by variations in environmental conditions, such as tide and temperature.

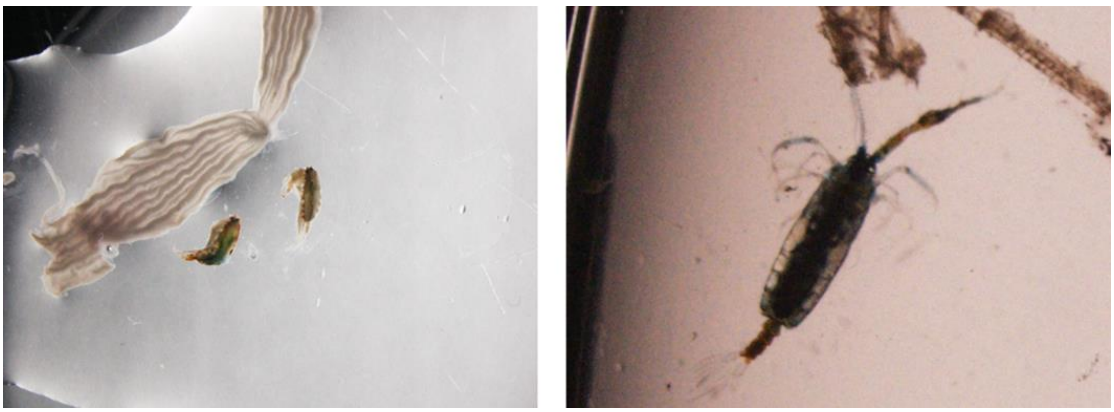

Figure 4-3. The gut contents of Pacific bluefin tuna juveniles captured under the night-time lighting in sea cages. The photos were taken by Dr. Masato Kawahara (Aquaculture Technology and Production Center of Kindai University on 14th, August 2016).

4.2.2 Materials and Methods

This study met the ethical standards prescribed by the Animal Experimentation Committee of the Aquaculture Research Institute of Kindai University (ARIKU-AEC-2022-67).

Experimental fish used in this study were artificially hatched PBF juveniles collected 24 days post-hatching [mean total length (TL) was 3.4 cm; mean body weight (BW) was 0.36 g, n = 10] reared in a 60 kl-volume circular concrete tank. PBF juvenile individuals (n = 47–55) were randomly distributed and stocked in eight 1 kl-volume tanks. An automatic feeder was deployed in each tank during the day (06:00–18:00 h), through which the fish were fed an appropriate-size (0.91–1.41 mm) artificial diet (Magokoro Diet Size C, Marubeni Nisshin Feed). In addition, fish were fed live prey, such as that used in fingerling production (e.g., *Artemia*, yolk-sac larvae of *Oplegnathus fasciatus,* and fertilized eggs of *O. fasciatus*), from 09:00–16:00 h during the experiment.

The experimental treatment group was given nighttime feed, in which fish were fed live prey at 19:00 and 22:00 h (positive treatment; Night-feed, n = 4). The control group (negative treatment; Control, n = 4) were not fed during the night. Experimental duration was 1 week. Because a high incidence of mortality while transferring fish from nursery tanks to sea cages has been reported (Okada *et al.*, 2014), the day of transportation was considered as day 1 and the feeding experiment was terminated on day 7. The appropriate intensity (147.8 ± 6.3 l×; Honryo *et al.*, 2013) of nighttime lighting was provided to each experimental tank. Rearing conditions such as water temperature (°C), dissolved oxygen level (%), and salinity (mg L^{-1}) were constant, as given in Table 4. UV-treated sea water was provided to each tank at a rate of 2.0 L min^{-1}. Dead fish were counted every day when the tank bottom was siphoned for cleaning.

Table 4. Rearing conditions of the Chapter 4-2.

treatment	replications	temperature (°C)	dissolved oxygen (%)	pH	Salinity (mg L^{-1})
Night-feed	4	27.0±0.1	114.8±6.8	8.16±0.04	32.2±0.2
Control	4	27.0±0.1	118.9±5.5	8.20±0.02	32.1±0.3

In this study, growth was compared between the two treatments. Ten individuals were randomly selected from among the surviving fish and measured for TL and BW at the end of experiment. In addition, gut contents were anatomically examined during the night (22:00–23:00 h) on days 1, 3, and 5 in both treatments. PBF juveniles were captured from each tank (n = 3–5) using a hand net and then immediately euthanized using ice-cold sea water, similar to previous methods (Honryo *et al.*, 2013). The TL and BW of captured individuals were measured, and then the abdomen was cut open using scissors and the gut was removed. The stomach and intestine were cut open and their contents were inspected. When the experiment was terminated on day 7, all the surviving fish were captured and counted to calculate the survival rate. Survival rate was calculated using the number of stocked individuals after subtracting the number of sampled fish that were taken to investigate gut contents and the daily dead fish count. Significant differences between the treatments were compared using an independent t-test at $p < 0.05$.

4.2.3 Results

The survival rate was found to be 77.6 ± 6.4 % in the treatment group with night-feeding, and 76.0 ± 6.2 % in the control group. These values were found to be statistically similar (p = 0.755, n = 4). On the other hand, comparison of TL (p = 0.028, n = 40) and BW (p = 0.018, n = 40) between the two treatments showed significant differences. Treatment of providing night-feed resulted in higher TL and BW than in those of control group (Fig. 4-4). Additionally, 69.2–77.8 % of fish in the night-feed treatment group showed evidence of having ingested live prey, which was in contrast to the control group whose stomachs were found to be empty at night.

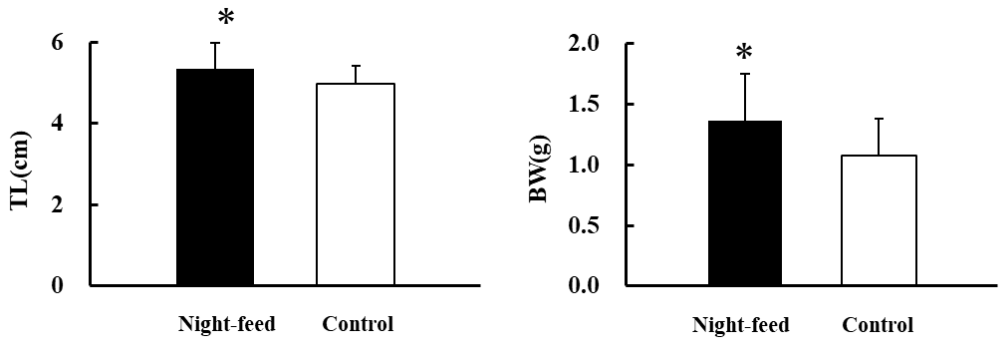

Figure 4-4. The comparison of total length (left panel) and body weight (right panel) at the end of rearing experiment. Asterisks indicates the significant differences between the treatments at p <0.05.

4.2.4 Discussion

Collision death is a typical factor associated with mortality occurring during PBF fingerling production (Miyashita *et al.*, 2000), and is known to be caused by visual disorientation due to dim lighting, when the retinomotor response of fish does not match with increasing light intensity (Masuma *et al.*, 2001). It has also been reported that scotophase itself induces death in PBF juveniles (Honryo *et al.*, 2014b). PBF juveniles can die from hyperventilation and an imbalance in acid-base regulation when their cruise swimming is disturbed (Honryo *et al.*, 2019). Thus, nighttime lighting plays an important role in preventing collision death, especially when specific techniques like finding a suitable light intensity (116 μmol $\cdot m^{-2} \cdot s^{-1}$) are established (Honryo *et al.*, 2020). When bright conditions are provided during nighttime, PBF juveniles adapt to the light (Masuma *et al.*, 2001) and can ingest feed in sea cages. For instance, Fig. 4-3 shows the gut contents of PBF juveniles captured from sea cage cultures during the night.

These results suggest that feeding during the night did not influence the viability of PBF juveniles; however, growth had significantly improved. In our past visual observation of sea cages located at Kushimoto Bay, Wakayama Pref., Japan, the presence of various prey such as zooplankton (mainly copepods) was confirmed under nighttime lighting. Some positive-phototaxic larvae had also possibly gathered. The presence of this biota may be subject to constant changes because of tides, season, and location. Hence, the exact effect of nighttime live feeding cannot be determined using sea cages. This validation study demonstrated that nighttime lighting is a useful technique that has direct consequences on improving survival by preventing collision death, and implies a subsidiary benefit of inducing nighttime feeding of live prey, which results in improved growth of PBF juveniles. Crucial factors, which support this subsidiary benefit of nighttime lighting, at sea cages are suitable light intensity, nutritional values of the gathered live prey, and amount of feed ingested by PBF juveniles. Further sustained investigations need to be conducted on sea cages at different locations and seasons to fine-tune techniques associated with culture of PBF juveniles.

Chapter 5

Technological development of night-time lighting in the sea cage

5-1 Optimal period of night-time lighting in the sea cage

5.1.1 Introduction

We have previously reported that night-time lighting prevents the mass death of PBF juvenile immediately after transport to sea cages as part of the process of fingerling production, which has expanded in recent years. In addition, high-intensity night-time lighting (> 150 lx) could serve as a countermeasure against mortality in land-based tanks (Honryo *et al.*, 2013). These studies also confirmed that night-time lighting does not negatively impact fish stress levels, as shown by the evaluation of stress responses, such as changed cortisol and glucose levels, as well as growth performance. High mortality, termed 'collision death', because of collision and/or contact with the walls of sea cages or land-based tanks has been recorded and attributed to the biological characteristics of PBF. The cause and correlates of this type of mortality have been examined, and dislocation of the vertebral column and parasphenoid fractures have been found to be the principal causes of death (Miyashita *et al.*, 2000). Although collision and/or contact with the tank or the walls of sea cages are not immediately fatal, it may result in injury to the body (i.e. scratches) or abnormal behaviour. These external injuries and abnormal behaviours may impact feed intake or lead to debilitation and may result in mortality within a few days. Moreover, it is thought that collision injuries caused by morphological imbalanced development of the lateral muscle and fins (Hattori *et al.*, 2001; Miyashita 2002; Tamura and Takagi 2009); this characteristic disproportion may be an important contributor to mortality. Furthermore, PBF juvenile have lower scotopic vision (Ishibashi *et al.*, 2009), scotopic temporal resolution and light sensitivity than juvenile chub mackerel (*Scomber japonicus*) or striped jack (*Pseudocaranx dentex*; Matsumoto *et al.*, 2009). In addition, previous studies have shown that ambient surroundings of less than 15 lx, which is approximately the threshold of retinomotor responses for PBF juvenile (Masuma *et al.*, 2001), are unsuitable for PBF survival. Hence, high-intensity night-

time lighting helps PBF juvenile identify the tank wall or the walls of sea cage and avoid collisions, which might otherwise occur because of their poor scotopic vision. Therefore, night-time lighting may serve as a highly effective countermeasure against mass death in PBF.

However, artificial night-time lighting, particularly in open sea cages, influences wildlife inhabiting the surrounding environment. Furthermore, the period of night-time lighting should be minimized because of management considerations, including cost, safety, and maintenance of electrical power. Therefore, we conducted a survey to determine the optimal period for night-time lighting that allows PBF to acclimate to the sea cages. This study investigated the effects of different periods of night-time lighting in different net cages on the stress response, growth performance, and survival of PBF juvenile.

5.1.2 Materials and Methods

Fish and experimental design

Eight hundred PBF juvenile were hatched from eggs that were spawned naturally from PBF broodstock and reared in a 30-m^3 fiberglass-reinforced plastic circular tank during July 2006. The initial average sizes of experimental fish at 34 days post-hatching were as follows: total body length, 6.4 ± 0.5 cm; body weight, 3.0 ± 0.8 g (n = 5). Acclimation periods were not set because mass death would occur immediately following transfer from the land-based tank to the open sea cage (Ishibashi *et al.*, 2009). Therefore, fish were immediately distributed into 4 cages that had different periods of night-time lighting: unlit, 4-day, 8-day, and 12-day lighting (control). In this survey, the 12-day-lighting group, which was exposed to night-time lighting throughout the experimental periods, was considered as control. The cages were 6 × 6 × 6 m (length × width × depth) in dimension and were exposed to lighting from before dusk until after dawn (i.e. from about 17:00 to 08:00 h). Lighting was provided by 2 sets of dual-filament fluorescent lights mounted 40 cm above the water surface. Night-time light intensity just above the water surface in the center of each sea cage was about 3300 lx. During the day, natural sun light penetrated the cages, with a maximum lighting intensity at the water surface of more than 100,000 lx. Following the completion of the lighting period for each group, the cage was towed by a boat to a distance of about 600 m from the area of night-time lighting to ensure

the light did not reach the cage (Fig. 5-1). The experimental fish were fed chopped Japanese sand lance (*Ammodytes personatus*) several times a day until satiated. To estimate the survival rate, dead fish were collected every day at 10:00 h by conducting a 5-min diving operation at each of the experimental cages, and the collected fish were counted. In addition, X-ray images (HB-50, HITEX Co., Ltd., Osaka, Japan, settings: 30 kV, 50 mA, 50 s) of the death fish, excluding decayed specimens, were obtained to infer the frequency of bone injuries.

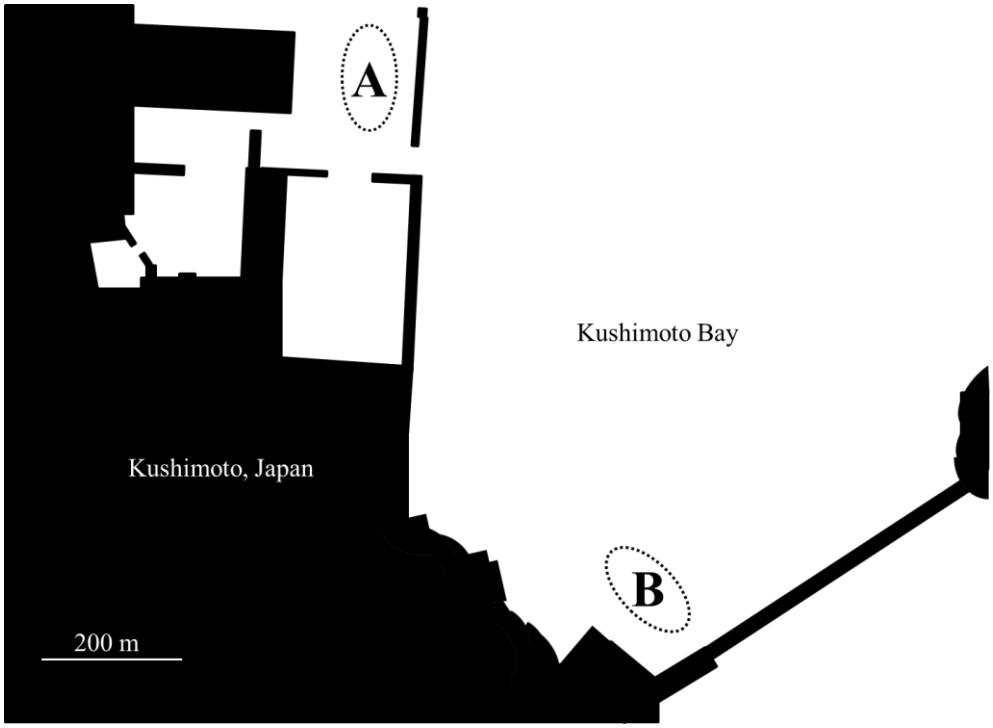

Figure 5-1. The modification map of the cage location at Kushimoto bay, Wakayama, Japan (33°27’32.45”N, 135°47’21.17”E). The A area denotes the location of cage exposed to nigh-time lighting; after termination of night-time lighting, the cages were towed by boat to area B which is at a direct distance of about 600 m. Black and white areas represent the land of Japan and sea, respectively. Original map from Geospatial Information authority of Japan.

Sampling and analysis methods

After the day when the nigh-time lighting was terminated in each cage, whole-body cortisol and

glucose levels of fish were analysed. Hence, on day 1, juveniles reared in the unlit cage were sampled (n = 5). On days 5, 9, and 12, juveniles from the 4-day-lighting cage were sampled to determine the effect of termination of night-time lighting (n = 4–5). Moreover, on days 9 and 12, juveniles from the 8-day-lighting cage were sampled (n = 5). Meanwhile, the 12-day-lighting cage was lighted throughout the experimental period, and was considered the control; hence, fish from this cage were sampled on days 1, 5, 9, and 12 (n = 5) in order to compare with other groups exposed to different lighting periods. Whole-body cortisol levels were measured using the enzyme immunoassay method, and glucose levels were measured using the glucose CII test kit (Wako Pure Chemical Industries Ltd., Osaka, Japan), according to previously described procedures. Fish from the experimental cages were collected by using hand-nets and/or a fishing rod in each experimental group, and sampling was conducted at the same frequency; the sampled fish were anesthetized and sacrificed within 1 min by using iced physiological saline, and were then frozen in liquid nitrogen. This sampling process was carried out within 1 min in each fish to avoid the effect of different handling methods on stress parameters. The samples were stored at −80°C until chemical analysis. At the end of the experiment, the total body length, fork body length (cm) and body weight (g) of the sampled fish were measured. These measurements were used to calculate growth performance indices, including the Condition factor (CF), specific growth rate (SGR % day^{-1}) and weight gain (%) by using the following formulae.

$CF = (Wf\, L^{-3}) \times 100$

SGR (% day^{-1}) = $100 \times (\ln W_f - \ln W_i)$ / time (days)

Weight Gain (% day^{-1}) = $100 \times (W_f - W_i) / ((W_i + W_f)/2)$ / time (days)

where W_i = initial body weight (g), W_f = final body weight (g), and L = final fork length (cm).

Statistical analyses

The survival rate of fish in the different groups was compared using the Kaplan–Meier log-rank test (Altinok, 2004). The values of whole-body cortisol and glucose levels were expressed as the mean ± standard deviation (SD), and were analysed by the t-test on day 1 (unlit group versus 12-day-lighting

group), and on day 5 (4-day-lighting group versus 12-day-lighting group). The stress parameters were also analysed on days 9 and 12 and the growth performance indices were compared using ANOVA with Bonferroni correction at 95% level. Statistical analyses were performed by using the Statistical Package for the Social Science (SPSS) program for Windows v16.0J (IBM Corporation, Tokyo, Japan).

5.1.3 Results

Survival rates and growth performance

Figure 5-2 shows the survival rate of PBF juvenile cultured under different periods of night-time lighting for 12 days.

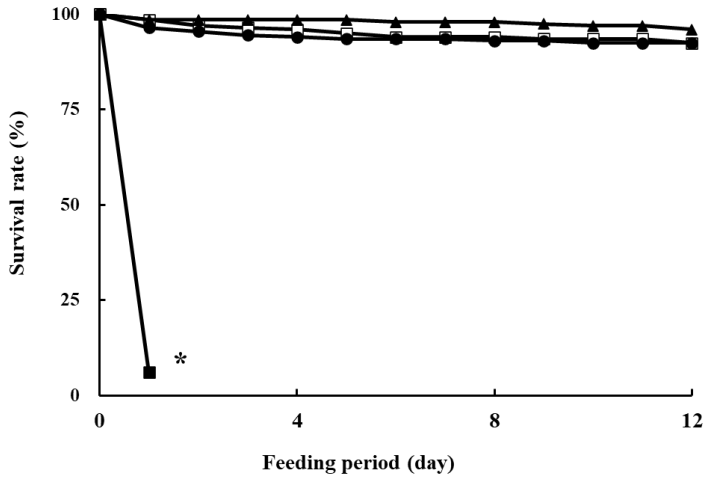

Figure 5-2. The survival rate of Pacific bluefin tuna juvenile cultured under different periods of night-time lighting for 12 days. ■ unlit; □ 4-day lighting; ▲ 8-day lighting ; ● 12-day lighting. *The survival rate in the 0-day group was significantly lower than that in the other 3 groups (Kaplan–Meier analysis, log-rank test; $p < 0.001$, $n = 200$). The unlit group experiment finished on the 1st day.

In the unlit group, mass death occurred just after transfer, with 94% of fish dying on day 1. A significant difference was confirmed between this group and the other groups ($p < 0.001$, $n = 200$). Because of the high mortality, the experiment was discontinued after the 1st day in the unlit group. In

contrast, the 4-day-, 8-day-, and 12-day-lighting groups showed continuously high survival during the period of lighting, with almost identical survival rates (92.5–96.0%). Significant differences were not detected among these 3 groups during the experimental period.

Growth performance, which is measured and calculated from the fish taken at the start and end of the experiment, is shown in Table 5. The final body weight was 5–9 times greater than the initial value. Compared to the 8-day- and 12-day-lighting groups, the 4-day-lighting group showed a slightly lower, although still significant, difference in all indices, except the CF value. However, no significant difference was observed between the 8-day- and 12-day-lighting groups.

Assessed bone injury

To infer the cause of death in the 4 experimental groups, death fish were subject to X-rays; the results are shown in Table 6. Although approximately 50% of the death fish had been decayed or damaged because of being eaten by fish outside of the cages, enough specimens were collected for investigation (n = 91 in the unlit group, n = 6 in the 4-day-lighting group, n = 3 in the 8-day-lighting group, n = 3 in the12-day-lighting group). The types of injury on different body parts were classified according to the classification of Gregory K. W. (2002). Injuries included (a) abnormal shape of skull, obvious fractures, and premaxilla injuries in the cephalic area; (b) dislocation and fractures in the vertebral column; (c) fractures of the parasphenoid; and (d) multiple injuries, including combinations of (a)–(c). Depressed fracture of the frontal bone (Fig. 5-5), which is not described by Miyashita *et al.*, (2000), was also observed. In total 71.4% of the samples collected from the unlit group showed bone injuries.

Table 5 Growth performance of Pacific bluefin tuna juvenile cultured in sea cages under different periods of night-time lighting

		Total length (mm)	Fork length (mm)	Body weight (g)	Condition factor (K)	SGR (% day^{-1})	Weight gain (% day^{-1})
Initial data		64.2 ± 5.4	62.2 ± 4.8	3.0 ± 0.8	1.2 ± 0.1	-	-
Final data in experimental group	4-day lighting	105.2 ± 4.0^{a}	102.4 ± 6.3^{a}	14.8 ± 1.1^{a}	1.4 ± 0.2	13.3 ± 0.6^{a}	11.0 ± 0.3^{a}
	8-day lighting	125.8 ± 4.7^{b}	120.6 ± 3.8^{b}	27.0 ± 2.2^{b}	1.5 ± 0.1	18.3 ± 0.7^{b}	13.3 ± 0.2^{b}
	12-day lighting	121.6 ± 6.3^{b}	116.2 ± 5.6^{b}	26.7 ± 4.5^{b}	1.7 ± 0.3	18.1 ± 1.5^{b}	13.2 ± 0.6^{b}

Values represent mean ± standard deviation (n = 5 per experimental group).

Different superscript letters in the parameter for final data show significant differences at $P < 0.05$.

Table 6 Numbers of specimens subjected to soft-x ray, and estimated cause of death

experimental group	Objects in total dead fish	Injury type(%)					
		a; cephalic part	b; vertebral column	c; parasphenoid	complex(a,b,c)	none	injury rate(%)
un-lit	91/188	26.4	19.8	13.2	12.1	28.6	71.4
4-day lighting	6/13		33.3		33.3	33.3	66.7
8-day lighting	3/6	66.7	33.3				100
12-day lighting	3/15		33.3	66.7			100

Setting value; 30kv, 50mA, 50sec.

Decayed specimens was excluded to taking soft-x ray photograph

Stress response

Changes in whole-body cortisol levels on days 1, 5, 9, and 12 in PBF juvenile cultured under different night-time lighting periods are shown in Fig. 5-3. On day 1, a noticeable and significant increase in cortisol levels was recorded in the unlit group compared with the 12-day-lighting group (*t*-test; $p < 0.05$; $n = 5$). Similarly, a major increase was recorded for the 4-day-lighting group on day 5 (i.e. when lighting was stopped). However, the fish in the 4-day-lighting group recovered from stress on day 9. A significant increase in stress was also recorded for the 8-day-lighting group on day 9 (i.e. after lighting was stopped). On the other hand, this increase was much lower than that observed in the unlit and 4-day-lighting groups. At the end of the experiment, there was no significant difference in stress levels among the groups.

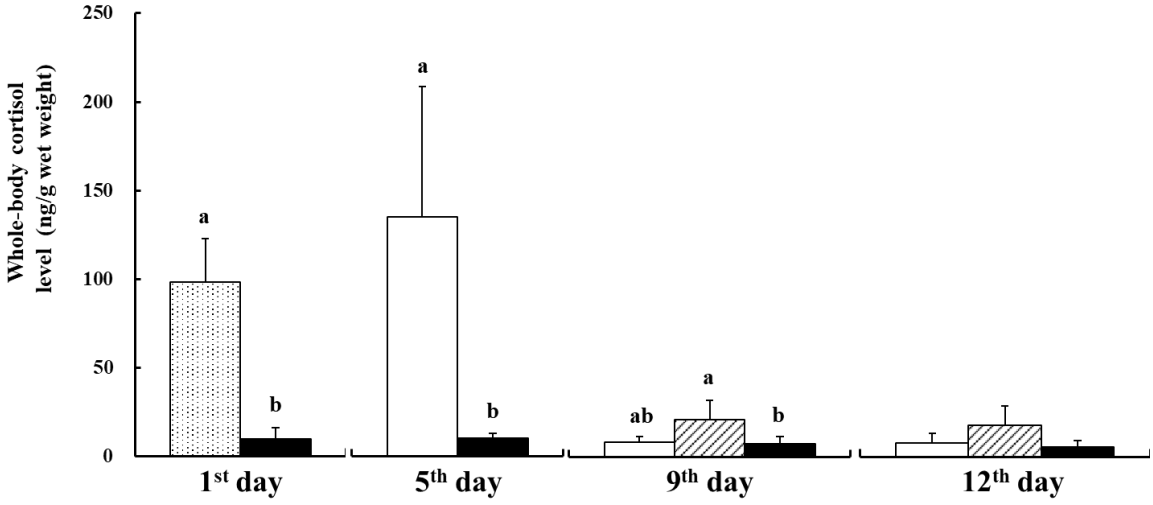

Figure 5-3. Comparison of whole-body cortisol levels of Pacific bluefin tuna juvenile on days 1, 5, 9 and 12 cultured under different night-time lighting periods. Dot, unlit; Black, 12-day lighting; White, 4-day lighting; Diagonal, 8-day lighting. Different letters against the day represent significant differences at $p < 0.05$. $n = 4–5$.

Changes in whole-body glucose levels on days 1, 5, 9, and 12 in PBF juvenile cultured under different night-time lighting periods are shown in Fig. 5-4. The changes in whole-body cortisol levels showed an obvious response to the period when night-time lighting was stopped. In comparison, changes in whole-body glucose levels showed no trend or variation among groups.

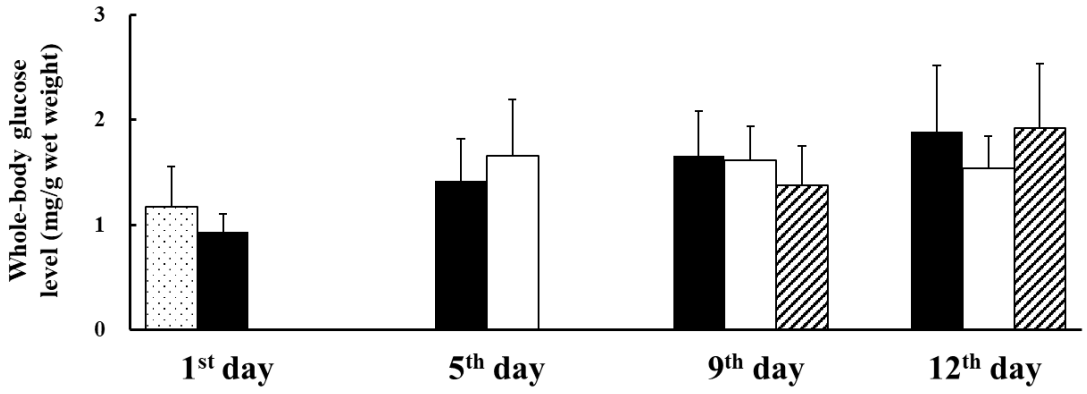

Figure 5-4. Comparison of whole-body glucose levels of Pacific bluefin tuna juvenile on days 1, 5, 9, and 12 cultured under different night-time lighting periods. Dot, unlit; Black, 12-day lighting; White, 4-day lighting; Diagonal, 8-day lighting. Significant differences were not detected at any sampling point. n = 4–5.

5.1.4 Discussion

Optimal period of night-time lighting

Since trials on culturing PBF were first initiated in the 1970s, numerous studies have been conducted on methods to enhance their survival. For example, the early timing of fingerling transport proved advantageous (Harada *et al*., 1971; Harada *et al.*, 1976; Miyashita 2002). Furthermore, survival was enhanced by culturing fingerlings in larger net cages. Accordingly, the Fisheries Laboratory of Kindai University has introduced circular sea cages (diameter, 30 m) for intermediate breeding in recent years (Kumai, 1998). However, the current study showed that survival rates of > 90% could be achieved by providing lighting for 12 days after transport and transfer to small cages (length × width × depth, 6 × 6 × 6 m) (Fig. 5-2). When considering survival rate alone, the necessary period of night-time lighting

is 4 days. However, evaluation of growth performance indicated that longer periods of lighting are required (Table 5), as indicated by the noticeable increase in whole-body cortisol in the 4-day-lighting group (Fig. 5-3) on the day after terminating lighting. Although fish subjected to 8-day night-time lighting also exhibited a significant increase in whole-body cortisol levels the day after lighting was stopped, this rise was less extreme compared to the fish in the 4-day-lighting group, with recovery from stress occurring within 4 days. In addition, similar growth performances were recorded between the 8-day- and 12-day-lighting groups. Furthermore, there was no trend or variation in whole-body glucose levels among the 8-day- and 12-day-lighting groups during the experimental period (Fig. 5-4). Thus, 8-day to 12-day night-time lighting is recommended as the ideal period for acclimation of fish during the intermediate breeding stage of PBF culture.

The results of the bone-injury-type assessment based on X-ray image analysis indicated that specimens from the unlit group primarily expressed a depressed fracture of the frontal bone (Fig. 5-5), and the majority of fish died. Ishibashi *et al.*, (2009) reported similar levels of mass death in an unlit control group within the first few days of being placed in cages. However, the mortality rate was much higher in this study, showing that night-time lighting is essential when using small cages.

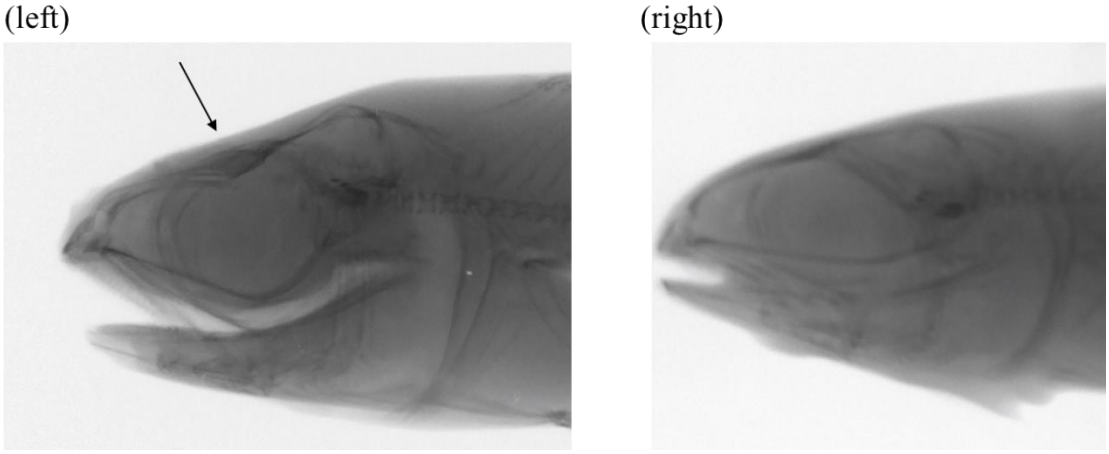

Figure 5-5 X-ray photographs of a dead Pacific bluefin tuna specimen. Black arrow indicates the injury to the frontal bone (left) versus a normal fish (right).

Stress response

Changes in whole-body cortisol levels were highly evident, with major elevations being recorded in the unlit and 4-day-lighting groups after the termination of lighting. In comparison, cortisol levels were much lower in the 8-day-lighting group than in the unlit and 4-day-lighting groups. This difference probably indicates that the acclimation period of PBF juvenile to a new environment might require 8 days or more.

In contrast, the free glucose levels in fish cause a general increase in stress response, particularly when energy levels are low (Arends *et al.*, 1999; Biron *et al.*, 1994; Grutter and Pankhurst, 2000~~,~~; Ishibashi *et al.*, 2002). However, a previous study by our group (Ishibashi *et al*., 2009) showed that the whole-body glucose levels decreased after 18 h of stocking the fish into sea cages compare to the fish just before transporting; nevertheless, the cortisol levels were comparatively increased. A tendency to decrease in plasma free glucose level has also been noted in juvenile PBF reared untreated tank compare to the fish reared in lighting and patterned wall tank. In this present study, a direct correlation between increased whole-body cortisol levels and glucose levels was not obtained. For instance, no change in the free glucose content of PBF juvenile, which have a higher energy demand than adults, was hypothetically suggested to be caused by the rapid consumption of glucose due to the stress response. Korsmeyer *et al.*, (1996) found that yellowfin tuna (~~*Thunnus*~~ *T. albacares*) exhibit moderate swimming velocity in response to hypoxia and reduced ambient temperature during natural movement patterns. In addition, the high metabolic rate (Korsmeyer and Dewar, 2001) and high cardiac function (Blank *et al.*, 2004) of tuna should result in high oxygen consumption rates (Miyashita *et al.*, 1999). Therefore, juvenile tuna might need to keep swimming for survival and respiration, like adult tuna; however, collision and/or contact with the walls of sea cages might result in bone fractures, dislocations and debilitating injuries that interrupt the natural swimming behaviour, resulting in fatality. In conclusion, for the intermediate breeding stage of PBF production, the optimal recommended night-time lighting period is during the first 8–12 days after transport and transfer into cages. Although 8-day night-time lighting might cause some stress, fish recover within 4 days, with no negative impact on growth performance, as compared to when 4-day night-time lighting is used. The 8- to 12-day

durations are significantly shorter than the 3-week acclimation period recommended in a previous investigation (Ishibashi *et al.*, 2009), which contributes toward curtailing costs and additional management requirements, making this technique more practical and economically viable. This duration might prevent mass death occurrence during PBF seedling production, especially death immediately after transportation to the sea cage.

5.2 Essential light intensity of night-time lighting and area of illumination

5.2.1 Introduction

The collision of PBF juveniles with the net cage and/or rearing tank wall can result in vertebral column, parasphenoid, frontal bone, and/or skin lesions and in death (Masuma *et al.*, 2001; Miyashita *et al.*, 2000). This occurs not only during the fingerling production process, but also during broodstock management (Kadota *et al.*, 2016; Yazawa *et al*., 2011). Use of night-time lighting has proven effective in lowering collision rates both in net cages and rearing tanks (Ishibashi *et al.*, 2009; 2013). In fact, light intensity > 150 lx is required in rearing tanks because PBF juveniles have low scotopic vision (Honryo *et al.*, 2013; Ishibashi *et al.*, 2009). However, there is still insufficient information regarding light intensity and area of illumination in sea cages (Higuchi *et al.*, 2014; Okada *et al.*, 2014; Tsuda *et al*., 2012). Optimal light intensity for a sea cage may differ from that for a rearing tank because of its larger surface area and depth. Furthermore, lighting conditions have been improved by the development of the light-emitting diode (LED), which has the advantages of low cost and higher luminance than the previously used fluorescent bulbs. Under these circumstances, the effects of night-time lighting intensity and area of illumination using LED light have yet to be examined. Both issues are addressed in the present study using PBF juveniles transported from a land-based tank to a sea cage. Our findings can provide a basis for improving current sea cage culture methods for industrial-scale production of PBF.

5.2.2. Materials and methods

Experimental design, measurements, and observations

In Experiment 1, we investigated the effect of seven different night-time lighting intensities—i.e., 0 as negative control (NC), 1.2, 4.5, 10.9, 38.0, 115.7, and 329.4 μmol m^{-2} s^{-1} in single rectangular sea cages (13 length × 13 width × 6 m depth; 1014 m3) with the same light coverage. Experiment 2 was conducted to examine the effects of different night-time areas of illumination including spot lighting

and consisted of two trials. Trial 1 was carried out with three replicate cages per treatment; 12 LED devices were installed at the center of the sea cages at 50 × 50-cm intervals (3 × 4 matrix; 12-light treatment) and compared to cages in which a single LED device was installed (1-light treatment). Trial 2 was conducted with two replicate cages per treatment; nine LED devices were installed at the center of the sea cage at 50 × 50-cm intervals (3 × 3 matrix; 9-concentrated-lights treatment) or at 3 × 3-m intervals (3 × 3 matrix; 9-spread-lights treatment) and compared to cages in which a single LED was installed (1-light treatment).

The LEDs were switched on from 16:30 to 08:30 h with power supplied from a small generator. The wavelength of the LEDs used in this study was measured with a photonic multichannel analyzer (PMA-12; Hamamatsu Photonics K. K., Shizuoka, Japan) and are shown in Fig. 5-6. Experiments 1 and 2 were performed for 14 and 10 days, respectively, within the optimal period of night-time lighting (Honryo *et al.*., 2014a). Fish in both experiments were fed similar amounts of an artificial diet (Magokoro; Marubeni Nisshin Feed Co., Tokyo, Japan) until satiation from sunrise to sunset, either by hand or with an automatic feeder.

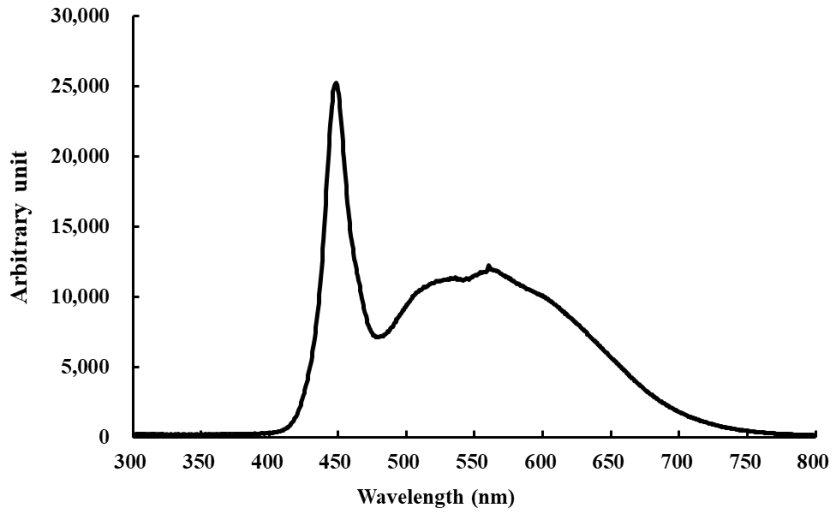

Figure 5-6. Wavelength of the LEDs used for night-time illumination.

Survival rate was calculated by subtracting the number of dead fish from the initial number of stocking collected daily by a scuba diver. Growth indices were compared by collecting surviving fish from each

experimental sea cage after 14 days (n = 15 per treatment, Experiment 1) and 10 days (n = 15-16 per treatment, Experiment 2) of feeding. When sampling was performed from the sea cage in both experiments, a small amount of diet was fed to gather the fish, and then fish were randomly captured using a hand-net and immediately sacrificed by ice-cold seawater. Body length (BL mm) and body weight (BW g) were measured for all the sacrificed fish. The CF was calculated with the following formula: BW (g) / BL^3 (cm) × 1000.

To determine the cause of death, collected dead specimens were subjected to X-ray analysis (MA-60; Hitex Co., Osaka, Japan) and the frequency of bone injury was noted. Then the stomach contents were checked to determine if the cause of death was due to accidental ingestion of marine debris as describes in Okada *et al.*, (2014). In addition, the swimming diameter from the center of night-time illumination was measured at 22:00 h in each treatment.

Fish and LEDs

Experiment 1

PBF juveniles (mean BL: 69.3 ± 7.9 mm and mean BW: 4.7 ± 1.4 g, n = 20) were hatched from eggs that were naturally spawned from PBF broodstock, and fed both the commercial formula diets and hatched larvae of *Oplegnathus fasciatus* in 50-m^3 concrete square tanks at the Aquaculture Technology and Production Center of Kindai University.

A total of 11,900 PBF juveniles used in Experiment 1 were distributed in seven cages (1,700 juveniles per treatment) with different intensities of night-time lighting provided by LED devices (Marintec Co., Fukuoka, Japan) installed at center of each sea cage, with an unlit cage serving as NC. Stocking density in this experiment was 7.88 g m^{-3}. The LEDs were hung about 50 cm above the water surface. The LEDs were mounted inside a triptych water-resistant stainless steel frame (24 × 40 cm) and light intensity was adjusted by changing the number of LED elements so as to uniformly cover the illumination area—e.g., 12 elements were mounted in the frame and used for the 1.2 μmol m^{-2} s^{-1} treatment. The number of elements was proportionally increased according to the experimental design—i.e., 3, 10, 30, 100, and 300 fold. The actual light intensity penetrating to depth in the sea cage

was measured at 22:00 h with a DEFI-L logger (JFE Advantech Co., Hyogo, Japan) when the transparency was 7 m during the daytime. Points at the water surface (0 m) and below the LEDs installed in the sea cage at depths of 0.5, 1, 2, 3, 4, 5, and 6 m were monitored. Approximately 90% of the treatment light intensities were attenuated at 1 m below the water surface and almost demised between 1–2 m. During the experiment, water temperature, salinity, dissolved oxygen level, and transparency were 22.1 ± 0.4 °C, 31.1 ± 0.8 g L^{-1}, 6.86 ± 0.22 mg L^{-1}, and 5.88 ± 1.99 m, respectively.

Experiment 2

PBF juveniles were hatched from eggs that were naturally spawned from PBF broodstock, and fed both the commercial formula diets and hatched larvae of *O. fasciatus* at the Aquaculture Technology and Production Center of Kindai University. The LED element used in Experiment 2 was modified according to the results of Experiment 1, with a much higher illumination at the same wavelength (Marintec Co.). One hundred and eighty LED elements were mounted in a water-resistant frame (14 × 33 cm). The NC treatment in Experiment 1 resulted in a low survival rate; therefore, in Experiment 2, lighting with a single LED device served as the control.

In Trial 1, 12,000 PBF juveniles (36 dph) were distributed in six cages (2,000 juveniles per cage) with different night-time lighting coverage: 1- vs. 12-lights with 0.12% and 1.78% coverage, respectively, across a surface area > 116.7 μmol m^{-2} s^{-1} per cage. At the onset of the trial, fish BL was 58.9 ± 4.7 mm and fish BW was 2.4 ± 0.7 g (n = 27). The stocking density in Trial 1 was 4.7 g m^3. The LEDs were hung 40 cm above the water surface. The light intensity measured at the center of the sea cage under a 12-light treatment was 475.7 μmol m^{-2} s^{-1}. The measured light intensity for the 1-light treatment was 181.2 μmol m^{-2} s^{-1} at the water surface. The actual light intensity penetrating into the depth of sea cage was measured at 22:00 h with the DEFI-L logger when transparency was 7 m during the daytime. The monitored points in the sea cages were as follows: 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, and 6 m in vertical distance from the water surface and 0, 0.5, 1, 2, 3, 4, 5, and 6 m in horizontal distance from the center of the cage (Fig. 5-7). During the experiment, water temperature, salinity, dissolved oxygen level, and transparency were 27.3 ± 1.1 °C, 30.4 ± 1.1 g L^{-1}, 6.56 ± 0.39 mg L^{-1},

and 6.00 ± 1.04 m, respectively.

Trial 2 was conducted to examine the effects of lighting spot with the same night-time lighting coverage. Nine thousand PBF juveniles (33 dph) were distributed in six cages (1,500 juveniles per cage) with different night-time lighting spots: 1- vs. 9-concentrated or –spread-lights, which covered 0.12% and 1.34% of cage surface area with > 116.7 μmol m^{-2} s^{-1}. At the onset of the trial, fish BL was 51.5 ± 4.3 mm and fish BW was 1.6±0.4 g (n = 30). The stocking density was 2.3 g m^{-3}. The LED devices were hung at the same level as in Trial 1 and the light intensity measured at the center of the sea cage under the 9-concentrated lights treatment was 348.8 μmol m^{-2} s^{-1}. The actual light intensity penetrating into the depth of sea cage was also measured at 22:00 h with the DEFI-L logger when transparency was 10 m during the daytime, and it was monitored at the same points as in Trial 1 (Fig. 5-7). During the experiment, water temperature, salinity, dissolved oxygen level, and transparency were as follows; 28.3 ± 0.7 °C, 33.4 ± 0.2 g L^{-1}, 6.49 ± 0.23 mg L^{-1}, and 6.7 ± 1.7 m, respectively.

Statistical analyses

Values are expressed as mean ± standard deviation. In Experiment 1, the relationship between survival rate at the end of the experiment and log-transformed light intensity was analyzed with Pearson's correlation coefficient at a significance level of $p < 0.05$. Differences in survival rate among treatments were evaluated with Kaplan-Meier log-rank test at a significance level of $p < 0.001$. Growth indices were evaluated by the one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) multiple comparison tests. In Experiment 2, survival rate at the end of experiment and growth indices were compared among treatments using the t-test for Trial 1 and ANOVA followed by Tukey's HSD test for Trial 2. Statistical analyses were performed in SPSS v.16.0j software (IBM, Tokyo, Japan).

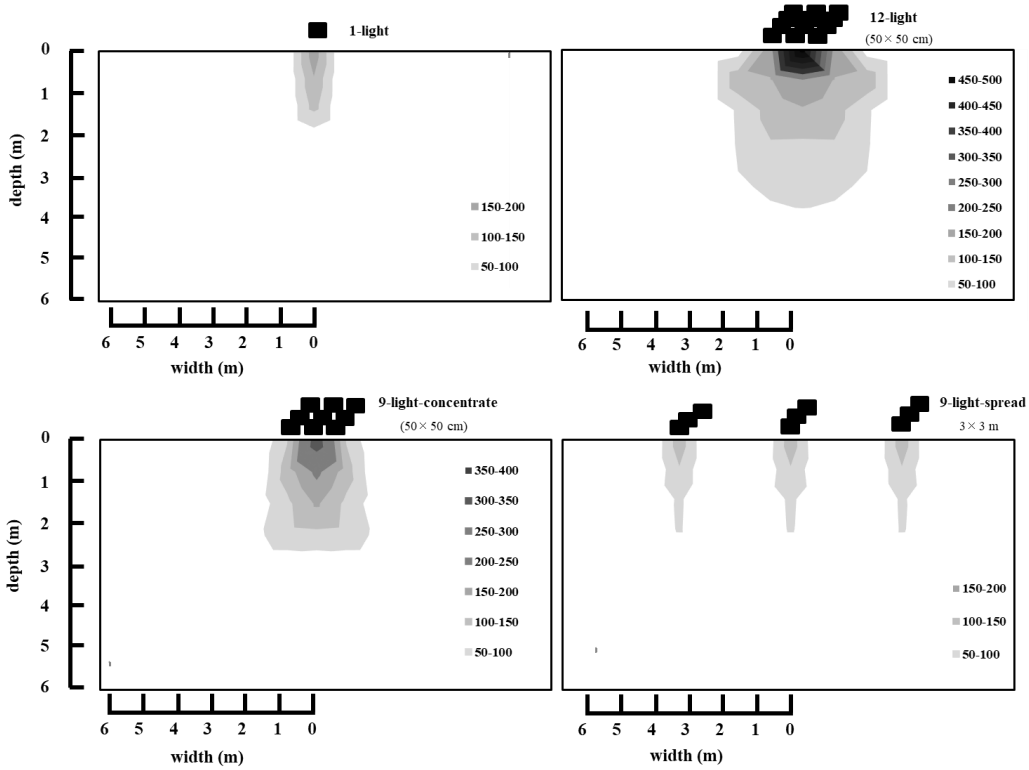

Figure 5-7. Actual light intensity (μmol m^{-2} s^{-1}) penetrating into the sea cage (Experiment 2; contour plot). Upper left: 1-light (Control); upper right: 12-light (Trial 1); lower left: 9-concentrated-lights treatment (Trial 2); and lower right: 9-spread-lights treatment (Trial 2). White areas indicate light intensity < 50 μmol m^{-2} s^{-1}.

5.2.3. Results

Experiment 1

At the end of the experiment, the NC treatment yielded a survival rate of 34.7% only. Survival increased as a function of light intensity, with the highest intensity (329.4 μmol m^{-2} s^{-1}) resulting in 58.4% survival. The relationship between survival at the end of experiment and log-transformed light intensity is shown in Fig. 5-8. The Pearson's correlation analysis revealed that survival and light intensity at the water surface were positively correlated (r = 0.9643, p < 0.01). The treatments could

be statistically divided into three groups, with survival increasing in the rank order of 0–1.2 < 4.5–38.0 < 116.7–329.4 μmol m^{-2} s^{-1} (Kaplan-Meier log-rank test, $p < 0.001$), although there was no significant difference between groups under 116.7 and 329.4 μmol m^{-2} s^{-1} lighting.

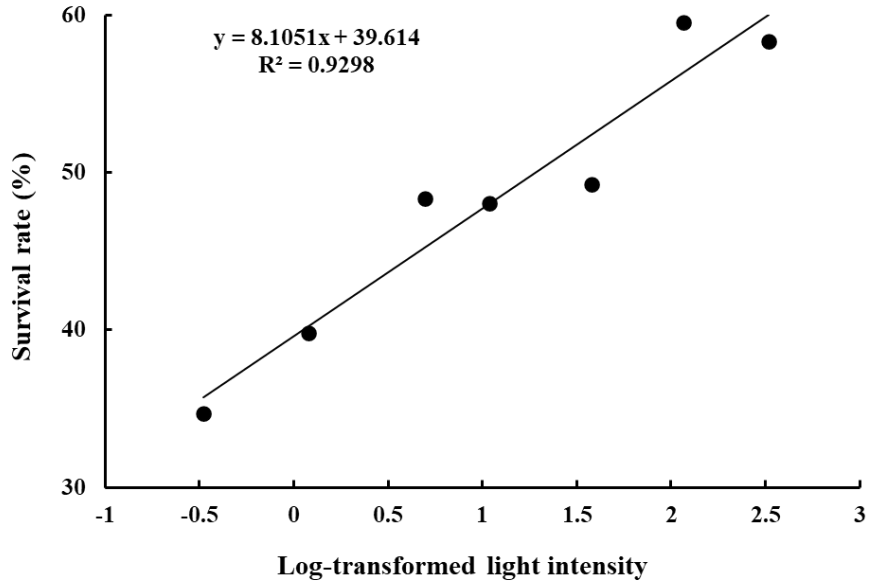

Figure 5-8. Relationship between survival at the end of the experiment and log-transformed light intensity (μmol m^{-2} s^{-1}).

Average BL was 104.8–123.6 mm, average BW was 11.5–18.0 g, and average CF was 13.3–14.0 after 14 days of feeding; however, there were no significant differences among treatment groups (n = 15 per treatment). The number of dead fish with bone injuries collected on days 3, 6, and 14 are shown in Fig. 5-9. In the NC group, 32.4% (22/ 68) and 31.6% (25/79) of dead specimens collected on days 3 and 6 showed evidence of bone injury, whereas these percentages were much lower in the 329.4 μmol m^{-2} s^{-1} lighting group —17.4% (4/23) and 14.6% (7/48) , respectively.

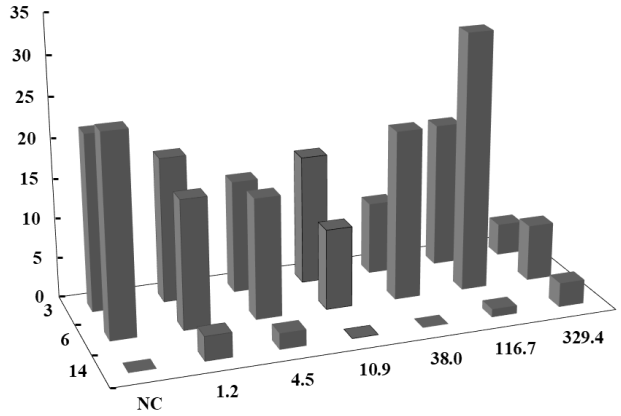

Figure 5-9. Number of dead fish with bone injury on days 3, 6, and 14 under different intensities of night-time light in sea cage cultures of Pacific bluefin tuna juvenile.

Experiment 2

Survival rates of PBF juveniles reared under different night-time lighting areas (Trial 1) and spots (Trial 2) for 10 days are statistically similar and shown in Table 7. The area of illumination had no effect on growth (n = 15-16 per treatment) or the frequency of bone injury.

Table 7 The effect of night-time light area of illumination on the growth and the survival rate of Pacific bluefin tuna juvenile for 10days.

	Treatment	BL (mm)	BW (g)	CF	Survival rate (%)
Trial-1	Initial	58.9 ± 4.7	2.4 ± 0.7	11.5 ± 0.9	-
	1-Light	100.0 ± 6.6	15.1 ± 3.3	14.9 ± 1.1	72.9 ± 8.1
	12-Light	101.5 ± 8.0	15.6 ± 4.3	14.5 ± 1.4	75.4 ± 3.4
Trial-2	Initial	59.4 ± 4.7	1.6 ± 0.4	11.4 ± 1.1	-
	1-Light	89.3 ± 8.8	11.6 ± 2.7	14.5 ± 1.2	60.2 - 73.3
	9-Light-concentrate	88.4 ± 7.4	10.5 ± 3.6	14.5 ± 1.0	63.9 - 76.8
	9-Light-spread	86.6 ± 8.0	9.7 ± 2.6	14.6 ± 1.0	70.9 - 74.7

The significant differences among treatment were not detected (n = 15-16).

BL; body length, BW; body weight, CF; condition factor.

5.2.4. Discussion

The swimming behavior of PBF switches from intermittent sprinting to continuous cruising when total length reaches 26.2–33.8 mm (Fukuda *et al*., 2010); accordingly, red and white muscle volumes increase exponentially to enhance swimming ability (Hattori *et al.*, 2001). This can also lead to increased mortality due to collision death, especially after fish are transferred to a sea cage (Miyashita *et al.*, 2000; Okada *et al.*, 2014). In yellowfin tuna (*T. albacares*), incidental contact with tank walls was found to result in the secondary bacterial or fungal infections on the head and trunk, which could lead to death (Margulies *et al*., 2015). In *T. thynnus* juveniles with a BW of 3–4 g and in southern bluefin tuna (*T. maccoyii*) reared in sea cages, collision death was the main cause of mortality (Chen *et al*., 2015; De la Gandara *et al*., 2015). Thus, collision death is common among tunas reared in captivity, which is a major barrier to mass production. As previously reported (Ishibashi *et al.*, 2009), the present study demonstrated that night-time lighting in sea cage cultures is necessary to ensure maximum survival of PBF juveniles. We demonstrated that the optimal light intensity in the sea cage to reduce mortality from collision was > 116.7 μmol m^{-2} s^{-1} (Fig. 5-8). However, survival and growth were not improved by altering night-time lighting coverage or area.

Mortality due to collision death can increase as a result of a sudden change in surrounding light intensity due to the poor scotopic vision of PBF juveniles (Honryo *et al*., 2013; Ishibashi *et al*., 2009) and visual disorientation around dawn (Masuma *et al.*, 2001). Various protocols have been investigated to enhance the survival of fish in captivity. For example, bubble curtains and strong currents have been used to prevent juvenile yellowfin tuna from contacting tank walls, albeit with limited success (Margulies *et al*., 2015). In another study, a buffer net was settled in front of the tank wall to prevent the collision of Southern Bluefin Tuna, although these investigators recommended transferring juveniles to a larger tank or sea cage to improve survival (Chen *et al.*, 2015). We previously suggested that incandescent illumination in the nursery tank with the light intensity greater than 150 lx at the water surface should be used to counter the low-intensity light environment at night-time or during the twilight period. This is a critical time of the day for collision death (Higuchi *et al.*, 2014; Honryo

et al., 2013; 2014b; Ishibashi *et al.*, 2009). Although it is difficult to convert this lx into μmol m^{-2} s^{-1}, we could reduce the mortality due to collision death by applying LED illumination that provided night-time light at an intensity of > 116.7 μmol m^{-2} s^{-1} at the water surface in the sea cage. We also attempted to enhance the survival of PBF juveniles by applying light over a broader area; however, the lighting provided by 12 LED devices covered 1.78% of the sea cage surface area as compared to 0.12% coverage with a single LED device, and did not increase survival and growth (Table 7). We also examined the effect of lighting different spots in the sea cage (nine vs. one spot at the center of the cage), but this also had no influence on growth and survival (Table 7). Thus, light intensity rather than area of illumination at the water surface is the critical factor for preventing collision death in the sea cage. In fact, we observed that the fish swam towards the center of the light. The nominal school diameter of PBF juveniles was approximately 3.8 m in the 1-light treatment as compared to 6 m in the 12-light treatment. In the 9-spread-lights treatment, fish swam deeper than in the other treatment groups, making it impossible to estimate school diameter. A swimming circuit that centers around the source light may help to prevent contact between fish and the net wall. We hypothesize that fish swim around the night-time lighting in order to avoid visual disorientation. Tunas have a highly developed optic tectum, indicating that vision is an important modality (Kawamura *et al.*, 1981); indeed, schooling behavior in PBF juveniles mainly depends on vision (Torisawa *et al*., 2007). In addition, in wild immature (< 1 year old) PBF, twice-daily diving patterns occurred in response to changes in ambient light intensity at sunrise and sunset (Kitagawa *et al.*, 2004). Accordingly, under captivity such as in a sea cage, juveniles frequently collide with the net wall or bottom of the sea cage. Night-time lighting could prevent this diving behavior as the fish swim around the center of the lit area during twilight and at night, although further study based on behavioral experiment is required to test this hypothesis. It is also possible that lighting with limited coverage (such as 0.12% of the cage surface area) is sufficient to attract PBF juveniles to the center of the sea cage provided, if a suitable light intensity is used (> 116.7 μmol m^{-2} s^{-1}). Similarly, in the nursery tank, fish exhibited behavioral abnormalities such as irregularities in schooling and swimming speed under a light intensity of 5 and 15 lx (Honryo *et al*., 2013). The same phenomenon could occur in sea cages, thereby reducing survival

rates. Light penetration decreased drastically in the water even just below the LED device. However, considering the cost of continuously operating the LED device as well as the effects of night-time lighting on the surrounding environment, night-time lighting should be minimized as much as possible. Although night-time lighting has the advantage of reducing collision death in sea cage cultures of PBF as described above, both negative and positive effects of constant light have been reported in cultured marine teleost and these effects varied according to species (Villamizar *et al*., 2011). The optimal period of night-time lighting in sea cages without obvious stress response in PBF juveniles has already been elucidated (Honryo *et al.*, 2014a). We could update this protocol in which previously used fluorescent bulbs by using LEDs. Although the present study was carried out within the optimal night-time lighting period, further studies are required under the > 116.7 μmol m^{-2} s^{-1} condition provided by LEDs to determine the effects on fish welfare. Furthermore, it should be noted that night-time lighting might also play a role in preventing poor growth as it may attract phototactic zooplanktons and/or fish larvae that serve as prey for PBF juveniles during nighttime. In fact, 8.8% of the dead fish collected on day 3 at the NC treatment of Experiment 1 had poorly grown, as defined by Okada *et al.*, (2004), but poorly growth dead fish were not observed in the treatment using high light intensity, indicating that growth increase also contributed to enhance PBF survival. In addition, the stomachs of fish collected from the unlit cage were empty when these fish were sampled just after sunrise, but the fish taken from the illuminated cages had contents in their stomachs even during the night-time (unpublished results included in another study developed at the Aquaculture Institute of Kindai University). Thus, night-time lighting might enhance the viability of PBF juveniles especially when they are not completely adapted to artificial diet. However, further studies are required to elucidate this effect, although it supported our conclusion that night-time lighting should be provided at > 116.7 μmol m^{-2} s^{-1}. Another common cause of death of juvenile PBF reared in sea cage was the accidental ingestion of marine debris such as wood, styrofoam waste and plastics (Okada *et al.*, 2014). The frequency of debris encounter ranged from 0 – 44 % during the trials and was influenced by the water inflow into the sea cage, its location and the direction of wind and tides. Further study is required to investigate the link between night time lighting and the accidental ingestion of marine debris.

In conclusion, the results of the present study suggest that night-time light intensity > 116.7 μmol m^{-2} s^{-1} is essential for attracting PBF juveniles to the center of the sea cage, thereby reducing the mortality caused by collision with cage walls. These findings are relevant not only to PBF, but also to other species of tuna cultured in sea cages in order to enhance their survival and production.

Summary

Tuna populations, especially the Pacific bluefin tuna (PBF; *Thunnus orientalis*), have been depleted owing to global overfishing. Fingerlings that are artificially cultured independently of natural populations can help satisfy market demand and conserve wild populations. Therefore, more efficient culture techniques are required, and it is important to improve production efficiency to replace wild-caught juveniles with artificially hatched juveniles to guarantee sustainability. However, various mass mortalities occur during the production process of PBF juveniles.

Among mass mortality, collision mortality, which is a characteristic cause of death in juvenile PBFs, occurs frequently during the juvenile stage. During the ontogenetic development of PBF, swimming behavior changes from intermittent sprinting to continuous cruising, and morphological functions develop to enhance swimming ability during the juvenile stage. Collisions with the rearing tank wall and/or net cage can result in physical injury, skin lesions, and death. Preventing collision death is of first priority during the land-based nursing period; however, the factors that induce this mortality are unclear, and effective countermeasures have been developed.

During fingerling production in PBF, juveniles are transferred from land-based tanks to sea cages. Immediately after transportation, various factors, including insufficient feeding leading to poor growth, accidental ingestion of marine litter, traumatic injuries caused by collision and/or contact with the net cage, and unknown causes of death, cause high mortality in sea cage culture. Even though mortality caused by poor growth accounts for 25.0–45.0% of total deaths, the mechanism behind this is unclear.

The aim of this study was to investigate the mechanism that causes high mortality during fingerling production in PBF and to develop countermeasures against mortality. In Chapter 1, the mechanism underlying mortality due to poor growth in sea cages is investigated. Chapter 2 discusses the characteristics of juveniles that died from unknown causes. In addition, the factors that induce mortality in PBF tank cultures were examined, and a technical protocol was developed by controlling the photoenvironment, as described in Chapter 3. Chapter 4 clarifies the effects of night-time lighting on sea cages. Finally, technical developments during the sea cage culture of the PBF are discussed in Chapter 5.

Chapter 1. Mass mortality during and immediately after transport: Estimated time for recovery from transportation stress and starvation

The objective of Chapter 1 was to determine the stress responses to transportation from land-based tanks to sea cages using physiological and molecular biomarkers to understand the cause of poor growth (Exp 1 and Exp 2). In addition, simulated transport was conducted from tank to tank, and starvation tolerance was examined to reveal whether these stress responses are attributable to transportation or uncontrolled environments in the sea cages (Exp 3). Consequently, the estimated time required to recover from transportation stress in PBF juveniles transported over two different distances (Exp 1: long distance, Exp 2: short distance) was investigated. Results showed that whole-body cortisol levels and HIF-1α expression levels in gills increased from pre-transport levels shortly after juveniles were released into the sea cage, but recovered within 24 h. Simultaneously, whole-body glucose levels decreased 48 h after transport. Gene expression of the Hsp70 family in the liver was upregulated 48 h after fish were released into the sea cages in Exp 1 and was significantly upregulated after 48 h in Exp 2. These results indicate that 72 h are required for PBF juveniles to recover from transportation to sea cages. The results of Exp 3 suggested that PBF juveniles were significantly affected after two days of fasting and that the environmental conditions in the sea cage were the main factors resulting in a decrease in whole-body glucose levels and upregulation of Hsp70-family gene expression. Furthermore, changes in proximate composition indicated that PBF juveniles consumed crude lipids and proteins, and were significantly affected by fasting for 1–2 days. This chapter reveals that mortality due to poor growth is induced by inhibiting feeding activity owing to the stress response of environmental alternation in sea cages, rather than transportation per se. We suggest that the first three days after stocking are a critical period for recovery from transportation stress, and it is necessary to develop appropriate management techniques to alleviate the high mortality caused by these stress responses.

Chapter 2. Mass mortality after transport; Blood chemistry of PBF juveniles showing abnormal swimming behavior

Only physical inspections, such as bone and/or skin injuries, have been used in examinations since collision death has been considered a major cause of mortality; however, physiological changes in collision mortalities have not yet been investigated. Disturbance and disability in cruise swimming can be fatal in ram ventilators; as such, even non-fatal collisions may result in eventual mortality in PBF, owing to a loss of the ability to maintain efficient gas exchange. Consequently, we hypothesized that mortality occurs due to collision with the tank wall without evidence of physical injuries and

evaluated the blood chemistry of PBF post collisions. We conducted a chemical analysis of the blood of juvenile PBF showing abnormal swimming (AS) behavior, such as irregular and/or frantic patterns, and compared them to normal swimming (NS) fish to elucidate the causes of death. This chapter identifies three abnormal characteristics in the blood chemistry of AS specimens: 1) hyperventilation accompanied by significantly higher PO_2 and lower PCO_2, 2) metabolic alkalosis due to excessive excretion of protons, and 3) hyperkalemia. Hence, PBF juveniles are subject to mortality due to respiratory failure and homeostatic imbalances in acid-base regulation, even in the absence of physical injury. This indicates that, because PBF juveniles are ram ventilators, even light collision impacts can be fatal if swimming activity is affected. These findings provide insights into the definition of collision deaths.

Chapter 3. Control of the photoenvironment in land-based tanks

Collision death reportedly occurs because of the visual disorientation of PBF juveniles at dawn period. Thus, in Chapter 3, we focus on the photoenvironment, in which controllable factors exist during the land-based culture of PBF. In Chapter 3-1, the transition rate of light intensity at dawn is investigated, and the photoperiod examined for different light intensities of night-time lighting in Chapter 3-2.

3-1 Effect of illuminance transition rate on survival and stress response

To investigate the high mortality of cultured juvenile PBF, especially during land-based culture, we examined the effect of the rate of light intensity change at dawn. Juvenile PBF maintained in a natural light environment (control) were compared with those exposed to an artificially slowed change in light intensity at dawn (test group). For the test group, lamps connected to automatic timers and placed above the rearing tank were switched on from 03:45 to 05:15 h at 15-min intervals. After nine days, the survival rates of the test and control groups were identical. Between days 1 and 6, the whole-body cortisol levels did not differ between the groups. Both groups displayed a diel rhythm in plasma cortisol concentrations, which peaked at 03:00–06:00 h. These results suggest that slowing the increase in light intensity at dawn did not improve survival, and that low light intensity, such as during the scotophase itself, induces high mortality in cultured PBT juveniles.

3-2 Effects of night-time light intensity on the survival rate and stress responses

To determine the effect of nighttime lighting on survival, juvenile PBF were reared under four different nighttime light intensities (0, 5, 15, and 150 lx) for 9 days, followed by a 3-day observation

period without nighttime lighting. The high-intensity nighttime lighting (150 lx) group significantly improved in survival rate (75.8%; $p < 0.001$) compared with the unlit control group (0 lx; 64.3%). Lighting did not influence whole-body cortisol or glucose levels, or diel changes in plasma cortisol levels. In contrast, the survival rates of fish exposed to light intensities of 5 and 15 lx were slightly lower than those in the control group. These results suggest that providing night-time lighting of 150 lx or higher is an effective method for reducing mortality in low-light-intensity environments.

The results presented in Chapter 3 suggest that even though collision death could be induced by changes in light intensity at dawn, artificially controlling its transition rate did not effectively prevent mortality. This Chapter revealed that the existence of a low light intensity environment, typically scotophase, induces mortality of PBF juveniles in land-based tanks; thus, appropriate intensity (greater than 150 lx) of nighttime light, which eliminates the scotophase, contributes to mitigating mortality. In addition, this study suggests that nighttime lighting does not influence the physiological status of PBF juveniles.

Chapter 4. Effectiveness of night-time lighting in the sea cage PBF culture

The previous chapter suggested that night-time lighting is an effective countermeasure against mortality occurring during the scotophase in land-based tanks. This protocol can be applied to sea cage PBF culture because high mortality following the transfer of juvenile PBF from hatchery tanks to sea net cages is a major problem obstructing the mass production of fingerlings. Thus, in Chapter 4, the effect of night-time lighting on sea cages is examined. In addition, we investigated the visual characteristics of juvenile PBF to elucidate the mechanism of mortality during scotophase.

4-1 Artificial lighting prevents high night-time mortality caused by poor scotopic vision

The results showed that nighttime lighting of the sea cages led to reduced collisions with the net wall by juvenile PBF and almost eliminated mass mortality following transfer to sea cages. We discovered that PBF in the early juvenile stage demonstrates an extremely low optomotor reaction under twilight conditions compared with other teleosts. It is considered that, owing to the low ability of scotopic vision, juvenile PBF collided with the tank wall or net of the sea cage during the scotophase. Hence, providing night-time lighting can increase visibility and mitigate mortality.

4-2 Advantages of night-time lighting for growth

In Chapter 4-2, we validate the use of nighttime lighting to combat mortality and observe its effects on PBF growth. We hypothesized that the viability and growth of PBF juveniles could be improved

by the intake of gathered feed items under nighttime lighting when they are transferred to sea cages. This experiment was performed in land-based tanks, such that changes in environmental conditions did not affect the observations. The results showed that the survival rate was similar between the PBF juveniles fed at night (treatment) and those not fed (control). However, growth significantly improved under treatment, in which 69–78% of juveniles showed evidence of having fed during night-time. We confirmed that various wild live prey gathered under nighttime lighting conditions were ingested by PBF juveniles in addition to the rearing diet. Our results from experimental tanks and observations from sea cages suggest that the growth of juvenile PBFs can be enhanced by providing nighttime lighting conditions.

In Chapter 4, we found that the mechanism underlying collision death occurred because of the low scotopic visual ability of PBF juveniles. Thus, providing night-time lighting effectively prevents mortality by increasing visibility during the scotophase. Likewise, nighttime lighting plays an important role in the sea cage by gathering various feed items for PBF juveniles and subsequently resulting in growth promotion. Accordingly, mortality due to poor growth can be mitigated by providing nighttime lighting. Thus, it is suggested that night-time lighting possess two potential positive benefits for PBF juveniles.

Chapter 5. Technological development of night-time lighting in the sea cage

5-1 Optimal periods of night-time lighting

The period of nighttime lighting should be limited to minimize negative impacts on the surrounding environment and aid management. Therefore, we investigated the optimal period for night-time lighting by evaluating the stress parameters, growth performance, and survival of PBF juveniles in four cages with different periods of night-time lighting (i.e. unlit, 4-day, 8-day, and 12-day lighting). The results showed that almost all fish died a day after transfer to the unlit cage. In comparison, the other groups (4-day, 8-day, and 12-day lighting) had high survival rates (92.5–96.0%) without significant differences. However, in the 4-day-lighting group, an obvious stress response was recorded on day 5, and the growth performance was significantly lower. In the 8-day-lighting group, whole-body cortisol levels were slightly elevated on day nine; however, no significant elevation was recorded on day 12. These results indicate that the recommended lighting period for nighttime lighting in sea cages is approximately 10–12 days. This shortened period decreased not only the impacts on the surrounding environment, but also the cost and management of nighttime lighting.

5-2 Essential light intensity of night-time lighting and area of illumination

Suitable nighttime lighting intensity and area of illumination for sea cages have not been systematically investigated. We addressed these issues using seven different night-time light intensities ranging from 0 as a negative control (NC) to 329.4 μmol m^{-2} s^{-1} covering the same area (Exp 1) and different areas of illumination (0.12%, 1.34%, and 1.78% per cage; Exp 2). The results of Exp 1 showed that surface night-light intensity and survival rate were strongly correlated (r = 0.9643), and significant differences were found among the three treatment groups, with survival increasing in the rank order of NC–1.2 < 4.5–38.0 < 116.7–329.4 μmol m^{-2} s^{-1}. The results of Exp 2 showed no differences in survival when the illuminated areas were altered. There were no differences in growth performance between Exp 1 and 2. These results indicate that providing night-time light intensity > 116.7 μmol m^{-2} s^{-1} at the water surface can enhance PBF juvenile survival, and that light intensity rather than area of illumination is an important parameter in PBF sea cage culture.

In conclusion, we have elucidated the mechanism defining the first 3 days after transportation as a critical period for recovery from transportation stress and it is necessary to develop appropriate management techniques to alleviate high mortality due to insufficient growth caused by stress responses in Chapter 1. The definition of collision death is reviewed in Chapter 2 by investigating the blood chemistry of juvenile PBFs. Juvenile PBFs are subject to mortality due to respiratory failure and homeostatic imbalances in acid-base regulation, even in the absence of physical injury. Thus, the prevention of collision deaths in land-based tanks and sea cages is important for establishing a sustainable culture of PBF fingerlings. In addition, in Chapter 3, we discuss the development of countermeasures against these mortalities. Although controlling light intensity changes at dawn did not improve PBF viability, nighttime lighting that eliminates the scotophase, which induces the death of PBF juveniles, was found to be an effective technique in land-based tanks. Our results also suggested that an appropriate light intensity greater than 150 lx is optimal. Chapter 4 reveals that nighttime lighting plays an important role in sea cages. Our study demonstrated that nighttime lighting is a useful technique that has direct consequences on preventing collision deaths by increasing visibility and implies a subsidiary benefit of inducing nighttime feeding of live prey, which results in improved growth of PBF juveniles. Finally, we successfully determined the optimal period for nighttime lighting and the essential light intensity. These technical developments enable the minimization of impacts on the surrounding environment and contribute to production efficiency. Our

research can be devoted to stabilizing the viability of PBF juveniles and contributing to sustainable PBF aquaculture.

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