Neutral Lipid Deposition in Larval and Juvenile Pacific Bluefin Tuna, *Thunnus orientalis*, under Different Rearing Temperatures

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**Abstract:** A biologically interesting lipid deposition system in tunas enables them to migrate a long distance in the ocean. On the other hand, the condition of lipid deposition in their meat is important to evaluate their meat quality as the human food. This study investigated the ontogenetic change of neutral lipid deposition in reared larval and juvenile Pacific bluefin tuna (PBT), *Thunnus orientalis*, under different rearing temperature regimes; (I) at 27°C throughout the experiment, (II) at 24°C until the flexion stage, 25°C until juvenile stage, and at 27°C thereafter. Neutral lipid deposition was examined histologically by making cryosections stained with oil red O, and haematoxylin and eosin. Under both regimes, lipid deposition firstly occurred in the brain, next visceral organs, and lastly in muscle when the fish metamorphosed from larvae to juveniles. Lipid deposition in all body parts except for the internal dark muscle occurred earlier under the regime I than II. The effect of lower temperature on the lipid deposition under the regime II continued after the temperature was raised to the same temperature of regime I. These results reveal that the effect of temperature on lipid deposition during early development continues even in later developmental stages.

**Key words:** Pacific bluefin tuna; Lipid; Larvae; Juveniles

Tunas have many characteristic physiological traits as highly migratory fishes such as the endothermy (Brill et al. 1994; Graham and Dickson 2001) and an effective cardiovascular system (Sanchez-Quintana and Hurle 1987; Brill and Bushnell 2001). Among such traits, there should be an effective lipid deposition system in their body like migratory birds, which can make them to migrate a long distance in the ocean where the food is localized and limited (McWilliams et al. 2004; Pierce et al. 2005). Such an unique lipid deposition system in tunas is biologically interesting.

On the other hand, the condition of lipid deposition in their meat is important to evaluate their meat quality as the human food. Especially, the oily meat of tunas termed as “Toro” has the highest value as the material of ‘Sashimi’ and ‘Sushi’ in Japanese market. Therefore, as in the cattle (Cameron et al. 1994), swine (Lee et al. 1973; Mersmann et al. 1975), and poultry (Adrizal et al. 2002) production, control of lipid content and composition in their meat should be the central challenge in the improvement of meat quality of farmed tunas. However, the system of lipid deposition in farmed tunas is less well understood, especially for its ontogeny.
Feeds (Bandarra et al. 2006), environmental conditions (Ibarz et al. 2005; Ibarz et al. 2007), and developmental (Cejas et al. 2004a) or mature stage (Cejas et al. 2004b) are factors which affect the lipid deposition in fishes. In the larval and juvenile rearing of the Pacific bluefin tuna (PBT), Thunnus orientalis, the present feeds, rotifers, Artemia nauplii, yolk-sac larvae of striped knifejaw, and minced fish meat of sand lance, are difficult to be replaced by other ones (Miyashita 2002) because of the difficulty to find feeds fulfilling their nutritional requirement, although the nutritional defectiveness of present feeds has been identified (Sawada et al. 2000; Miyashita 2002; Seoka et al. 2007). Therefore, to establish the control technology of lipid deposition in PBT larvae and juveniles, the modification of rearing conditions is the easier way than the change of the feeds. Among the environmental factors in the fingerling production, the rearing temperature is the highest priority to modify the lipid content, because of its convenience and importance suggested by Ibarz et al. (2007).

This study histologically investigated the ontogenetic change of neutral lipid deposition, the main energy source, in larval and juvenile PBT under different rearing temperatures.

**Materials and Methods**

**Fish and rearing**

Fertilized eggs, obtained from the 1998 year class PBT brood stock on 6 August 2005, were transported to the Ohshima Experiment Station of the Fisheries Laboratory of Kinki University. Eggs were disinfected by ozonation during somitogenesis, and thereafter 95,000 eggs were put into each four 20 m³ concrete tanks. Resulting larvae and juveniles were reared under two different temperature regimes. Under the higher temperature regime, the temperature was maintained at 27°C after raising it from the hatching temperature of 25°C at the rate of 1°C/day. For the lower temperature regime, it was maintained at 24°C until the flexion of notochord end begins, at 25°C until the metamorphosis from larvae to juveniles begins and at 27°C thereafter (Fig. 1). The rearing of each temperature regime was duplicated. The rearing finished on 40 days posthatch (dph) under the higher temperature regime and on 47 dph under the lower one when the fish grew to the same size between two temperature regimes.

DHA enriched rotifers and Artemia nauplii (Marine Gros, Nisshin Marine Tech Co. Ltd., Yokohama), live yolk-sac larvae of striped knifejaw, Oplegnathus fasciatus, and minced meat of sand lance, Ammodytes personatus, were fed to larvae and juveniles according to their growth as shown in Fig. 2.

Water temperature, dissolved oxygen concentration, and pH were measured two times daily, at 08:00 and 14:00.

**Sampling and body size measurement**

Five fish were sampled from each tank every day and were preserved in 5 – 10% formalin solution. These fish were all used for the histological examination of adipose tissue development. In addition, 10 fish were sampled from

![Fig. 1. Temperature (lower stand), dissolved oxygen (DO) concentration (middle stand), and pH (higher stand) in the rearing water of Pacific bluefin tuna larvae and juveniles in four rearing tanks. Lower 1 and Lower 2 indicate the rearing under the lower temperature regime, and Higher 1 and Higher 2 indicate the rearing under the higher temperature regime.](image-url)
each tank every day and the digital images of these fish were obtained by the digital camera (Fujix Digital Camera HC-300, Fuji Photo Film Co., Ltd., Tokyo) attached to the binocular. The body length (BL; the length from the upper jaw tip to the notochord end for larvae before the postflexion stage and from the upper jaw tip to the posterior edge of hypurals from postflexion stage) was measured every other day within these digital images by using the image analyzing software (Scion Image Beta 4.02 Win, Scion Corp, Fredrick). In addition, these fish were examined for the developmental stages according to Miyashita et al. (2001).

Making cryosections and staining

The body length was measured for every fish. Fish or fish body blocks were rinsed under running water to remove formalin for 25 h and immersed in Holt’s hypertonic gum-sucrose solution until the fish or fish body blocks sink down. Thereafter, they were embedded in 10% OCT compound (Tissue-Tec, Sakura Finetechanical Co., Ltd., Tokyo). Ten \( \mu \)m thin cross sections of the fish body were made by a cryostat (CM1850, Leica Microsystems GmbH Co. Ltd., Wetzlar). The sections were pasted in glass slides coated with meshcement (Bioden meshcement, Okenshoji Co., Ltd., Tokyo), and dried for 30 min to one h before staining.

Sections were stained by oil red O. To examine the tissue cytologically, neighboring sections were made from the tissue block pasted an adhesive film (Finetec Co., Ltd., Tokyo) at the surface, and stained by hematoxylin and eosin (HE). Sections stained by oil red O were enclosed by a water soluble sealing liquid (Mount-Quick Aqueos, Daido Sangyo Co., Ltd., Toda), and the sections stained by HE were enclosed by 30% glycerin solution.

Results

Environmental conditions

The rearing temperature changed according to the programmed two regimes (Fig. 1). The DO concentration was higher than 90% saturation level during the experimental period, and pH was higher than 7.8. These values are within the suitable ranges for PBT larval and juvenile rearing.

Growth and development

The survival of fish at the end of the rearing experiment was 0.42 – 0.63% for each rearing tank. This is the ordinary survival in the present PBT fingerling production. No outbreak of diseases was observed during the experiment.

The BL growth of the fish reared under the higher temperature regime was accelerated from 18 dph (12.15 ± 1.32 mm, mean ± SD, \( n=50 \)) and its average reached at 99.01 ± 8.37 mm on 40 dph (Fig. 2). The BL growth of the fish reared under the lower temperature regime was accelerated from 24 dph (11.76 ± 1.47 mm) and its average reached at 98.05 ± 12.01 mm on 47 dph. The difference of fish growth between the two regimes became larger from 18 dph than in the previous period.

Fish reached the postlarval stage on the same 2 dph under both two temperature regimes, and thereafter the development progressed faster under the higher temperature regime (Fig. 3). However, the fish development in relation to BL
under two temperature regimes had the same speed within the difference of one mm of BL.

Change of neutral lipid distribution from the view of age in day progress

In this study, the neutral lipid distribution was examined for five body regions, those are brain, auditory vesicle, liver, vertebra, and lateral muscles.

The first deposition of neutral lipids occurred on 2 dph at the periphery and inside of brain for the fish reared under both temperature regimes (Fig. 4A). Also inside of the auditory vesicle, it was observed on the same 14 dph for the fish reared under both temperature regimes (Fig. 4B). In all other body regions, the first deposition of neutral lipids was observed earlier for the fish reared under the higher temperature regime than those reared under the lower temperature regime (Fig. 5). This pattern was especially pronounced in the gill arch and the subcutaneous region of dorsal and ventral ordinary muscles. In these regions the neutral lipid deposition began more than eight days earlier for the fish reared under the higher temperature regime than those reared under the lower temperature regime. In addition, this pattern continued after the rearing temperature became the same 27°C on 24 dph for the both regimes.

Change of neutral lipid distribution from the view of body length growth

At the periphery and inside of brain, the neutral lipid deposition was firstly observed in the smallest size of fish among the regions examined, that is 3.42 mm body length (BL) for the fish reared under the higher temperature regime and 3.96 mm BL for those reared under the lower temperature regime (Figs. 4A and 6). Inside the auditory vesicle, it was firstly observed for the fish of 7.91 mm BL and 5.86 mm BL reared under the higher and lower temperature regime, respectively (Fig. 4B). In the liver, it was firstly observed at 7.92 mm BL and
Fig. 4. Neutral lipid deposition in the Pacific bluefin tuna larval and juvenile body parts. Red color in the figures indicates the neutral lipids stained by oil red O. Larvae and juveniles of A, B, C, E, G, I, and K were reared under the higher temperature regime, and those of D, F, H, J, and L were reared under the lower temperature regime. A: brain (b), 3.42 mm in body length (BL); B: auditory vesicle (a), 7.91 mm BL; C: liver (l), 7.92 mm BL; D: liver, 8.10 mm BL; E: subcutaneous region of the ventral ordinary muscle, 45.90 mm BL; F: subcutaneous region of the ventral ordinary muscle, 47.67 mm BL; G: subcutaneous region of the dorsal ordinary muscle, 59.23 mm BL; H: subcutaneous region of the dorsal ordinary muscle, 78.88 mm BL; I: internal dark muscle, 69.44 mm BL; J: internal dark muscle, 78.88 mm BL; K: superficial dark muscle, 47.63 mm BL; L: superficial dark muscle, 59.25 mm BL. Bars indicate 0.1 mm in A and B, and 0.5 mm in others. "e" means eye in Fig. 4A. Arrows indicate lipid deposition in brain (4A), auditory vesicle (4B), liver (4C and 4D), subcutaneous region of dorsal ordinary muscle (4G and 4H), internal dark muscle (4I and 4J), and superficial dark muscle (4K and 4L).
8.10 mm BL for the fish reared under the higher and lower temperature regime, respectively (Fig. 4C, D). The neutral lipid deposition in the subcutaneous region of ventral ordinary muscle was firstly observed at 45.90 mm BL (Fig. 4E) and 47.67 mm BL (Fig. 4F) under the higher and lower temperature regime, respectively.

In other regions, the difference of body length at which the first deposition occurred between the fish reared under two temperature regimes was more prominent. In centrum and its periphery, lipid deposition was firstly observed at 12.82 mm BL and 20.13 mm BL under the higher and lower temperature regime, respectively. In gill arch, it was firstly observed at 12.82 mm BL and 33.65 mm BL under the higher and lower temperature regime, respectively.

**Fig. 5.** Deposition of neutral lipids in body parts of Pacific bluefin tuna larvae and juvenile. The forefront of bars in the figure indicates the first day posthatch of the neutral lipid deposition. Open and close bars indicate the rearing under the higher and lower temperature regime, respectively. Histological examination was conducted for five individuals collected from each rearing tank every day.

**Fig. 6.** Deposition of neutral lipids in body parts of Pacific bluefin tuna larvae and juvenile. The forefront of bars in the figure indicates the body length of the first neutral lipid deposition. Open bars indicate the rearing under the higher temperature regime, and closed bars indicate the rearing under the lower temperature regime. Histological examination was conducted for five individuals collected from each rearing tank every day.
Lipid Deposition in Tuna Larvae and Juveniles

The neutral lipid deposition in the subcutaneous region of dorsal ordinary muscle was firstly observed at 59.23 mm BL and 78.88 mm BL under the higher and lower temperature regime, respectively (Figs. 4G, H). In the internal dark muscle,

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**Fig. 7.** Neutral lipid deposition in the subcutaneous region of ventral ordinary muscle (A and B) and internal dark muscle (C and D). Juveniles of A and C were reared under the higher temperature regime, and those of B and D reared under the lower temperature regime. A: a juvenile of 59.21 mm BL; B: a juvenile of 58.34 mm BL; C: a juvenile of 92.13 mm BL; D: a juvenile of 92.65 mm BL. Bars indicate 0.3 mm in A and B, and 1 mm in C and D. Arrows indicate the subcutaneous region of ventral ordinary muscle.

**Fig. 8.** Neutral lipid deposition in the superficial (A) and internal (B) dark muscles of a PBT of 92.13 mm BL and 38 dph. Neutral lipid deposition (C, oil red O staining) and cellularity (D, hematoxylin and eosin staining) in the ventral ordinary muscle subcutaneous region of a PBT juvenile (78.80 mm BL, 42 dph). Arrows indicate the internal dark muscles. Bars indicate 0.1 mm.
it was firstly observed at 69.44 mm BL and 78.88 mm BL under the higher and lower temperature regime, respectively (Figs. 4, J). However, in the superficial dark muscle, it occurred at the smaller size of 47.63 mm BL for the fish reared under the lower temperature regime than that for those reared under the higher temperature regime, 59.25 mm BL (Figs. 4K, L).

In addition to the body size difference of first neutral lipid deposition, the difference of degree of neutral lipid deposition recognized by the difference of staining depth and stained area between two temperature regimes was observed. At the same size of 8 mm BL, the neutral lipid deposition was observed in the liver of fish reared under both temperature regimes, however the fish reared under the higher temperature regime had more neutral lipids in the liver (Figs. 4C, D).

At the size of 58-59 mm BL, in the subcutaneous region of ventral ordinary muscle, more neutral lipids were deposited for the fish reared under the higher temperature regime (Figs. 7A, B). The fish reared under the higher temperature regime had lipids in the myoseptum of this region as the intermuscular lipid, in contrast, the same size fish reared under the lower temperature regime did not have it. In the region where neutral lipids were deposited, a mass of adipose cell was observed in the cryosections stained with hematoxylin and eosin.

At the size of 92 mm BL, in the internal dark muscle, neutral lipid was deposited in the broader area for the fish reared under the higher temperature regime than that reared under the lower one (Figs. 7C, D).

In superficial and internal dark muscle, neutral lipid was deposited inside the myocytes (Figs. 8A, B). On the other hand, in dorsal and ventral lateral muscle, neutral lipid was deposited as the subcutaneous and intermuscular lipid (Figs. 4E-H, 7A, B). In addition, at the subcutaneous region of ventral muscle, the cells were containing the neutral lipids (Figs. 8C, D). These cells were not observed at the subcutaneous region of dorsal muscle during the period examined.

Discussion

Neutral lipids have many important physiological functions such as the energy source, metabolic regulation, insulating material, buoyancy regulation in fishes (Akazaki 1987; Helfman et al 1997; Moyle and Cech 2000). In the early life of fishes, their survival is generally poor and a large reduction often occurs (Cushing 1975; Lasker 1975), because fish larvae and juveniles have less energy deposited in their body which is necessary to resist various stressors such as the starvation, attack of disease-causing microbes, and external injuries. This causal relation is also true in the fingerling production of the PBT aquaculture (Miyashita et al. 2000; Miyashita 2002; Sawada et al. 2005). Therefore, the quantity of neutral lipid as the main energy source in larvae and juveniles is an important determinant factor of their early survival. From this point of view, the investigation of lipid metabolism and the condition of lipid deposition in the early stage is important to understand well the fish population dynamics, where the population abundance depends on the early survival largely (Hjort 1914), and to improve the efficiency in fingerling production in aquaculture. However, there is very little information on the ontogeny of lipid deposition except for a few studies of Umino et al (1996). This study provides valuable information on the ontogenetic change of neutral lipid distribution in the PBT larvae and juveniles.

In PBT, neutral lipid deposition occurred in the order of brain, visceral organs, and muscle. Fishes are known to utilize the lipids in brain as the energy source (Johnston and Roots 1964; Kreps 1975; Tocher and Harvie 1988). In PBT, lipid was deposited both inside and at the periphery of brain on 2 dph. This may mean the function of lipid as the energy source and the material which maintains the brain temperature and protects the brain from extraneous impact. Since these functions are fundamental for the vital activity of fish larvae, it is rational that lipid deposition firstly occurs at the very early developmental stage inside and at the periphery of brain.
Umino et al. (1996) investigated the adipose tissue development in the juvenile red sea bream, *Pagrus major*. Generally, lipids are deposited in the fish body in the adipose tissue such as visceral lipid, subcutaneous lipid, and intermuscular lipid, as well as in other tissues such as liver, brain, and inside of muscle. Therefore, results of this study cannot be referred Umino et al. (1996) in a precise sense.

They reported that the neutral lipids were firstly deposited in liver and pancreas on 37 dph in red sea bream, and at the later developmental stage (on 42 dph) they had an adipose tissue attached to the outside of intestine. The PBT also deposited lipid in liver at the very early stage. Liver is the most important organ among the viscera concerning lipid metabolism, such as the synthesis of lipid (Mayes and Botham 2003), and the neutral lipid is abundant in fish liver as well as polar lipid (Craig et al. 2000; Cejas et al. 2004). Therefore, early lipid deposition in liver is also rational in the early development of fishes. On the other hand, in PBT the lipid deposition was not observed in pancreas and outside of intestine during the period examined. In addition, the PBT are known not to have visceral lipid except for that in liver and attached to the outside of genital gland. Therefore, it is possible that the visceral lipid deposition is different among fish species. Detailed study on such difference and the mechanism brings such difference is required. In the PBT muscle, lipid deposition occurred from juvenile stage when the feed changed from live yolk-sac larvae to minced meat of sand lance. Minced meat of sand lance used in this study contained approximately 2.4% lipid of the wet body weight, 1.3 times more lipid than live yolk-sac larvae (1.8% lipid). Therefore, the start of the lipid deposition in lateral muscle at juvenile stage means that juveniles became possible to deposit lipid into the muscle from the high lipid content feed.

Lipid is preferentially deposited in the myosepta and with increases in muscle lipid content, additional lipid is deposited along sparsely distributed thin connective tissue in yellowtail, *Seriola quinqueradiata* (Thakur et al. 2003), Atlantic salmon, *Salmo salar* (Zhou et al. 1996), amberjack, *Seriola dumerili*, and striped jack, *Pseudocaranx dentex* (Ando et al. 1993). In contrast, tiger puffer, *Takifugu rubripes*, and flounder, *Paralichthys olivaceus*, deposit lipids in the liver but a little in the muscle where the lipid is deposited inside the sarcolemma (Ando et al. 1993). The lipid deposition in the PBT ordinary muscle is categorized as the former type, although the deposition along the connective tissue was not observed. However, the neutral lipid was differently deposited in dark muscle where the lipid was deposited inside the myocytes. Dark muscle is known to be a site of lipid storage in fish (Henderson and Tocher 1987) as well as the ordinary muscle. It is interesting how the difference of deposition is related to the function of these portions of muscle.

Under the higher temperature regime, in many body parts, the neutral lipid deposition started earlier and at smaller size, and the quantity of lipid deposition was larger than under the lower temperature regime. At the higher temperature, it is supposed that the metabolism of fish larvae and juveniles is elevated and the energy demand increases resulting in the increase of feed intake. These could bring the increase of neutral lipid deposition synthesized from the surplus energy. Exceptionally, neutral lipid deposition in the superficial dark muscle delayed under the higher temperature regime. The cause of this phenomenon is unknown. Because fish dark muscles have unique physiological functions such as the use in constant swimming (Altringham and Shadwick 2001) and endothermy (Graham 1975; Carey 1981), the research on the detailed lipid metabolism of dark muscle is necessary to get the answer.

Alterations of lipid metabolism and use of energy depots by the ambient temperature have been reported for some fishes, such as gilthead sea bream, *Sparus aurata* (Ibarz et al. 2005; Ibarz et al. 2007), European sea bass, *Dicentrarchus labrax* (Skalli et al. 2006), Atlantic salmon (Ruyter et al. 2006), flounder (Kim et al. 2006). However, no information is available on the effect of ambient temperature on lipid metabolism or lipid deposition during fish early development. In addition, there is
no available information on the control of lipid deposition or adipose tissue development by the ambient temperature except for a few study such as Fauconneau et al. (1997) who reported that the increase of mean diameter of ventral adipose cells was accentuated in the low temperature environment for the rainbow trout fed on the corn-oil supplemented diet than in the cod liver oil group. In this study, lipid deposition started at the smaller size, and the deposition was faster and higher under the higher temperature regime than the lower one after that the rearing temperature became the same between two different regimes. This indicates the possibility of controlling the lipid deposition of the PBT by the rearing temperature during early life stage, although it is necessary to follow-up how long the effect continues. However, the acceleration and increase of lipid deposition should improve the survival of early stage of PBT in aquaculture and in the natural environment through the strong stress resistance.

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クロマグロ仔稚魚の異なる飼育水温下での中性脂質蓄積

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マグロ類特有の脂質蓄積機構は大回遊を可能にしている。一方でマグロ肉の脂質蓄積状態は食品としての肉質評価で重要である。本研究では、飼育クロマグロ仔稚魚で、脂質蓄積の発育変化を異なる飼育水温下で解明することを目的とした。飼育水温は、(I) 一貫して27℃、(II) 前期曲期まで24℃、後期曲期まで25℃、稚魚期以降27℃、の2系列とした。魚体の脂質分布は、凍結切片にオイルレッドO、ヘマトキシリンエオジン染色を施し、組織学的に調べた。2系列ともに、最初の脂質蓄積は脳周囲で、続いて内臓、稚魚期に入って筋肉で生じた。中性血合筋を除き、調べた全ての部位で、系列ⅠでⅡより早い発育段階で脂質蓄積が生じた。系列Ⅱにおける発育初期の低温による脂質蓄積の遅延は、飼育水温が系列Ⅰと同じ27℃にまで上昇した後も持続した。これらの結果は、低水温による脂質蓄積の遅延、発育初期の水温の脂質蓄積への影響の後の発育段階までの持続を示している。