Title	Studies on the Early Development of Bluefin and Yellowfin Tuna(Dissertation_)
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Citation	Kyoto University ( )
Issue Date	2000-03-23
URL	http://dx.doi.org/10.11501/3167397
Right	
Type	Thesis or Dissertation
Textversion	author

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of Bluefin and Yellowfin Tuna

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Contents

(This thesis was submitted for the degree of Doctor of Agriculture in Kyoto University)

General Introduction

Marine teleosts generally produce huge number of small pelagic eggs during the spawning season. Consequently, larvae at just after hatching are very small and premature, and are at relatively early stage of development; larvae hatch with a large yolk mass. Most of the external and internal organs at this stage have not yet differentiated, and larvae utilize the yolk as energy source (Kamler, 1992). Yolk-sac larvae then develop to the adult-like form via two drastic developmental phases; first feeding and transformation to juvenile (Blaxter, 1988; Fukuhara, 1992). The first feeding usually occur around several days after hatching. Although the basic organs which enable the larvae to feed on exogenous nutrients have differentiated at the first feeding stage, larval body structure is still primitive form compared to that of adult fish. The second marked developmental event, transformation to juvenile, is defined as metamorphosis (Blaxter, 1988; Youson, 1988) which is characterized by completion of fins and ossification of vertebral column. Notable developmental event associated with metamorphosis is observed also in the digestive system; differentiation of gastric glands in the stomach followed by differentiation of pyloric caeca (Tanaka, 1973). Various patterns of the developmental process, however, can be seen in the early ontogeny of marine teleosts depending on species-specific life history strategies (Moser, 1981; Blaxter, 1988). Metamorphosis in the early life history of marine fish has been given much attention due to its importance as a significant developmental strategy (Policansky, 1982; Youson, 1988; Thorrison, 1994).

Bluefin tuna *Thunnus thynnus* is an oceanic pelagic scombrid species that are distributed in the Atlantic and Pacific Oceans (Collet and Nauen, 1983). This fish is one of the largest species among the osteichthyes, reaching up to 300 cm in length, and spawn enormous number of small buoyant eggs. As a results of this high fecundity, the bluefin tuna larvae at hatching are as small as other marine teleosts larvae, and undergo altricial and immature larval period. Because tunas constitute important commercial fisheries and recreational fishing throughout the major oceans of the world, many researches on their early life history have been devoted to them. At the beginning, investigations on tuna larvae were mainly focused on the taxonomy, morphology, and distribution in open oceans because of

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their primary importance as the prerequisite of assessment of recruitment and year-class strength (Ueyanagi, 1966; Yabe *et al.*, 1966; Richards and Dove, 1971; Matsumoto *et al.*, 1972; Nishikawa, 1985). These studies revealed morphological features of sea-caught tuna larvae, being characterized by a big head with large mouth and eyes, well developed preopercular spine, and posterior shift of the anus with growth (see review by Okiyama and Ueyanagi, 1978). Therefore, scombrid fishes, including tunas, have been considered having high growth potential and unique developmental process, called scombriform-type metamorphosis. Judging from these morphological characteristics, Hunter (1981) inferred that mackerel and tunas had a "large prey-fast growth" survival strategy during their early life history. Then further researches were conducted on ecological traits such as growth, food habits, and diel pattern of vertical distribution (Uotani *et al.*, 1981, 1990; Young and Davis, 1990; Davis *et al.*, 1990a, 1990b, 1991; Leis *et al.*, 1991; Scot *et al.*, 1993; Lang *et al.*, 1994). However, basic developmental biology on the early ontogeny of tuna larvae, such as physiological and biochemical aspects, have been poorly understood because of difficulty in conducting larval rearing experiments.

Bluefin tuna is the most expensive fish in Japan, and its fishery has still been at high level of economic value. However, at the meeting of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) held in 1992, there was a proposal that the bluefin tuna in the Atlantic Ocean should be listed as the endangered species. Although it was not accepted, Japanese government decided to develop seed production techniques for this species. The seed production ability of marine fish species has recently growing vigorously in Japan. Improvements in techniques for rearing marine fish larvae have increased the number of species available for aquaculture and for subsequent experiments. Bluefin tuna larval rearing beyond metamorphosis was first conducted in 1979 (Harada, 1980). Recently, the Japanese Fisheries Agency and the Japan Sea-Farming Association (JASFA) started a project to develop rearing techniques for bluefin tuna for stock enhancement, and also for yellowfin tuna, as preceded species for bluefin. However, survival rates of both tunas are still much lower than those of other marine teleosts due to high mortalities during the early life stages, in spite of a tremendous effort done by many researchers.

To establish a suitable seed production technique and facilities, it is indispensable to

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accumulate fundamental knowledge about species-specific features of the early life stages. Various kinds of technique on finfish seed production have been well established in Japan, but these are mainly based on important coastal fish species, e. g., Japanese flounder *Paralichthys olivaceus* (Takahashi, 1990) and red sea bream *Pagrus major* (Arakawa, 1999). From the external morphology of tuna larvae mentioned above, we could postulate that tuna larvae would have a specific developmental process in internal organ-system as well as external morphology, at which physiological and biochemical changes occur. Therefore, in order to establish appropriate techniques for bluefin tuna larval seed production, more fundamental research on the ontogeny from various aspects are needed, it contributing to reveal the survival strategy of scombriform-type metamorphosis.

The purpose of this study is to accumulate basic information on the early life stages of bluefin and yellowfin tuna mainly from physiological and histological aspects. The largest hurdle in studying tuna larvae is that sampling enough number is very restricted due to high mortalities. Thus in the present study, the author adopted primarily methods by which analyses could be conducted in individual larva or juvenile basis.

In chapter I, in order to describe the most basic developmental process of tunas, the author studied growth and morphological development of artificially reared bluefin tuna larvae and early juveniles. The main focus was laid on detailed observations on development of the digestive system with histological and histochemical techniques. The digestive system is closely related to the growth and food habits, thus would be essential to establish a suitable feeding schedule. Yellowfin tuna larvae were also reared and studied in a same manner, in order to compare the developmental characteristics of bluefin tuna. During these studies, it was revealed that artificially reared larvae of both tunas had uninflated swimbladder. It is well known that fish have uninflated swimbladders exhibit abnormal swimming behavior and result in skeletal deformities (Kitajima *et al.*, 1994), decreased growth and low survival rate (Chatain, 1987). Thus differentiation of normal swim bladder of bluefin tuna larvae was also studied in this chapter, with histological technique.

In chapter 2, the author studied the ontogenetic changes in physiological aspects; growth hormone cells in the pituitary and digestive physiology. These are considered to be very important for regulating larval development and growth. Although development of the

endocrine system has been documented in some teleosts (Naito *et al.*, 1993; Ayson *et al.*, 1994; Miwa and Inui, 1987b; Tanaka *et al.*, 1995; Hiroi *et al.*, 1997), there is no study on the development of this system in tuna species. Thus, the author examined development of the pituitary and its growth hormone cells during the early life stages of yellowfin tuna, using histological and immunohistochemical techniques. Developmental profiles of the trypsin activity and gastric glands in the stomach were also studied individually in this chapter, using a highly sensitive fluorescence technique (Ueberschär, 1988; Ueberschär *et al.*, 1992) and immunohistochemical technique.

Nucleic acid and protein contents of larval fish are commonly used to asses the nutritional condition and growth rate of sea-caught larvae (Bulow, 1978; Westerman and Holt 1994; Clemmesen, 1996). Moreover, developmental profiles of these biochemical contents would also reflect rates of cell proliferation, cell enlargement and metabolic activity. Thus some reports have shown a reasonable relationship between biochemical changes and morphological, physiological, and behavioral changes in the early life stages of some fish species (Fukuda *et al.*, 1986a, 1986b; Steinhart and Eckman, 1992; Takii *et al.*, 1992, 1994; Tanangonan *et al.*, 1998; Gwak, 1999). In chapter 3, RNA, DNA, and protein contents on bluefin and yellowfin tuna larvae and juveniles were examined individually in order to make clear the developmental process from the biochemical aspect.

In chapter 4, the author conducted an additional experiment on density-dependent survival after handling in yolk-sac larvae of bluefin and yellowfin tuna, using small plastic containers. The results obtained here includes a possibility for improving survival during the yolk-sac period of tunas, and presumably other marine fish species.

Finally, in the concluding remarks, the author discussed the developmental characteristics of two species of tunas in relation to the survival strategy of scombriform-type metamorphosis. Based on basic biological information obtained in the present study, some proposal comments for seed production on bluefin tuna are also noted.

Chapter 1: Growth and Morphological Development of Bluefin and Yellowfin Tuna Larvae and Juveniles

1) Growth and Development of Pacific Bluefin Tuna Larvae with Special Reference to the Digestive System

Abstract

Pacific bluefin tuna *Thunnus thynnus* larvae were experimentally reared from 2 day-old yolk-sac larvae through 30 day-old early juveniles in June and July 1994. The larvae initially fed on rotifers on Day 3, *Artemia* nauplii, fish eggs and larvae around Day 13 and thereafter were fed *Artemia* larvae and an artificial diet. The larva had transformed to the juvenile stage after 30 days. The primitive digestive system differentiated on Day 3. The gastric gland and pyloric caeca first appeared on Day 11 and 14, respectively. The pharyngeal and jaw teeth became fully functional with gastric gland differentiation. The number of gastric glands and pyloric caeca and volume of the gastric blind sac increased markedly with development to the juvenile stage. Although the external morphological development of the tuna resembles the pattern of many other marine fish larvae, the basic digestive system developed at an earlier larval stage. This precocious development of the digestive system may relate to the early appearance of piscivory and the high growth potential of tuna larvae.

Introduction

The bluefin tuna is a particularly valuable fish in Japan and natural stocks are low mainly because of overfishing. The Japanese Fisheries Agency and the Japan Sea-Farming Association (JASFA) started a project to develop rearing techniques for bluefin tuna larvae for future enhancement trials. Rearing beyond metamorphosis was first successful in 1979 (Harada, 1980). Although several subsequent trials succeeded in producing juvenile bluefin tuna, survival remained very low. High mortality occurred at particular developmental stages: just after first feeding, the early larval phase of about 10 days after hatching and around transformation to the juvenile phase. The timing of these periods of high mortality

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may correspond to particular ontogenetic phases in the development of the digestive system of larval teleosts (Tanaka, 1973).

Despite successful laboratory rearing of bluefin tuna larvae, little is known about larval morphology or development of the organ systems. A few studies on morphological development of sea-caught bluefin tuna larvae have been published (Yabe *et al.*, 1966). The main purpose of this article is to describe development of the bluefin tuna reared in the laboratory, particularly ontogeny of the digestive system because this may be important information for the establishment of an appropriate ontogenetic feeding schedule.

Materials and Methods

Experimental Animals

Fertilized eggs of bluefin tuna were obtained on 6 July 1994 from spontaneous spawning of broodstock in net pens maintained by the Uchiumi Fisheries Biotechnology Research and Development Centre, Nippon Formula Feed Co. Ltd. Newly hatched larvae were transported by air from the Centre to the JASFA Yaeyama Station on Ishigaki Island, Okinawa (24° 27' N, 124° 17' E), Japan, on 9 July. Two-day-old yolk-sac larvae were transferred to a 5000 *l* fiberglass reinforced plastics (FRP) tank (5 m length, 1 m width and 1 m depth) supplied with filtered seawater (1 μm mesh membrane) after temperature acclimatization. Initial stocking density was 6 larvae per *l* (30000 fishes in the 5000 *l* tank). Water temperature was maintained around 25 °C by a cooling system. Photoperiod was natural and surface light intensity was about 100 lux. Rotifers enriched by "Super Rotifer II" were fed from Day 3, and *Artemia* nauplii and coral trout (*Plectropomus leopardus*) eggs and larvae were fed from Day 12. *Artemia* larvae and an artificial diet were added several days before transformation to juveniles (Fig. 1).

Sampling

Twenty fishes were sampled daily from Day 2 to Day 10 after hatching, every 2 days from Day 12 to 20, and several fishes on Days 25 and 30. Sampled fish were anesthetized with MS-222 and total length (TL) and standard length (SL) were measured to the nearest 0.1 mm. Half of the fish were fixed in 4% formaldehyde to observe morphological development,

relative growth and ossification. The following body parts were measured in formaldehyde-preserved fishes: total length (TL), standard length (SL), snout length (SNL), upper jaw length (UJL), eye diameter (ED), head length (HL), body depth (BD), preanal length (PAL) and pectoral fin length (PFL). Relative growth was derived by expressing each of these measurements as a percentage of SL.
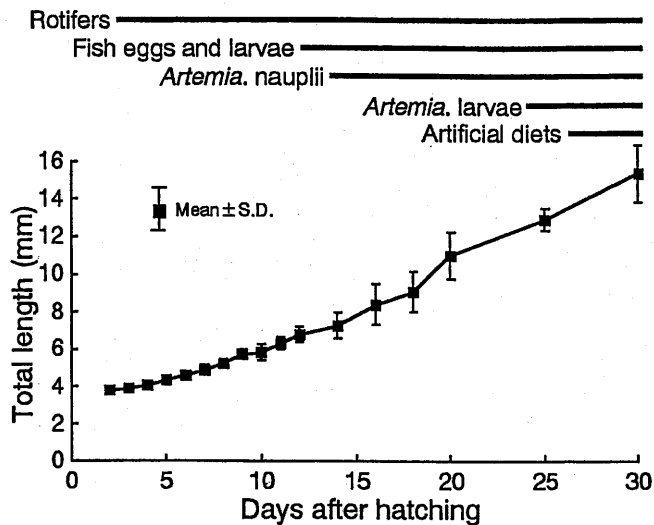

Fig. 1. Total length (mean±S.D.) of anesthetized bluefin tuna larvae reared at the JASFA Yaeyama Station in 1994. The feeding schedule is illustrated at the top of the figure.

Formaldehyde-preserved larvae were weighed on an electro-microbalance (Mettler M3) after careful removal of the surface fluid. Some of the preserved larvae were processed by the clearing and staining method described by Taylor (1967) for ossified bone and by Dingerkus and Uhler (1977) for bone and cartilage. The remaining fish were fixed in Bouin's solution and preserved in 80% ethanol for histological preparation. Whole bodies of these larvae were embedded in paraffin (parahisto, Nakaraitesque), sagittally sectioned to 5 μm thickness and stained with Mayer's hematoxylin and eosin. The developmental phase was determined by the criteria described by Kendall, Jr. *et al*. (1984). The best specimen of each developmental phase, yolk-sac, preflexion, flexion, postflexion larvae and juvenile, was selected and sketched under a camera lucida attached to a dissecting microscope.

Results

Growth and Morphological Development

The larvae grew from a mean length of 3.7 mm TL on Day 2 to 15.6 mm TL 30 days after hatching (Fig. 1). The growth process appeared to be slightly curvilinear. The average

growth rate during the first month of life was approximately 0.43 mm day^{-1} with relatively small individual size variation. Wet body weight increased exponentially with length (Fig. 2). The relationship between wet weight (W, in mg) and standard length (L, in mm) was expressed as $W= 0.000229L^{4.77}$ ($r^2=0.922$, $n=95$). The shift from preflexion to flexion phase was first noted 10 days after hatching. On Day 14 the postflexion phase was noted and on Day 25 the juvenile phase. In terms of length these transformations occurred around 5, 7 and 11 mm (Table 1).
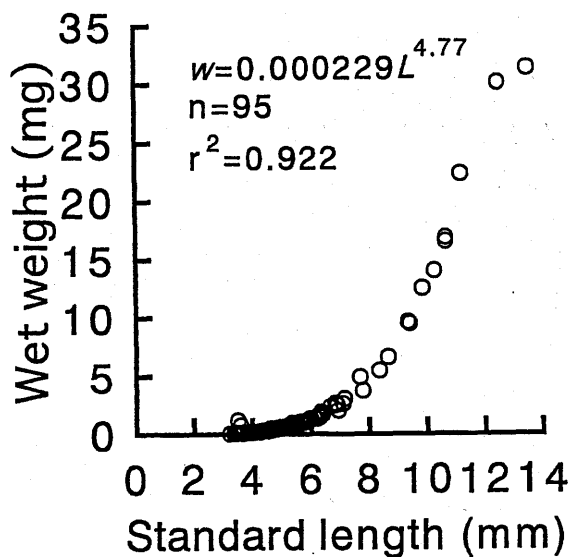

Fig. 2. Length-weight relationship of bluefin tuna larvae reared at the JASFA Yaeyama Station in 1994. The wet body weight for formaldehyde-preserved specimens was measured 4 and 5 months post-fixation and is not corrected for shrinkage. Relationship between length (L, SL in mm) and body weight (W, wet in mg) is expressed by an equation $W= 0.000229L^{4.77}$ ($r^2=0.922$, $n=95$).

Table 1. Developmental phase composition (number of individuals) of bluefin tuna larvae reared at the JASFA Yaeyama Station in 1994, showing in terms of age (upper) and length (lower). Materials used here include both formaldehyde- and ethanol-preserved fishes.

Developmental phase							Days after hatching											
	2	3	4	5	6	7	8	9	10	11	12	14	16	18	20	25	30	Total
yolk-sac larva	21																	21
preflexion larva		20	20	20	20	20	20	20	6	2								148
flexion larva									14	18	26	18	4	1	1			82
postflexion larva												2	6	12	9	9		38
juvenile																1	6	7
Total	21	20	20	20	20	20	20	20	20	20	26	20	10	13	10	10	6	296

Developmental phase				Standard length (mm)									Total
	3	4	5	6	7	8	9	10	11	12	13	14	
yolk-sac larva	6	5											11
preflexion larva	5	44	49	1									99
flexion larva			22	57	9								88
postflexion larva					10	14	5	4	3				36
juvenile									2	2	1	2	7
Total	11	49	71	58	19	14	5	4	5	2	1	2	241

According to definition by Kendall, Jr. *et al* . (1984)

2-day-old yolk-sac larva (3.5 mm TL): Mouth and anus begin to open and eyes are slightly pigmented. Patched melanophores are clearly visible on the dorsal edges of the body above the pectoral-fin base, median part of the body, near tip of notochord and dorso-posterior part of the rectum (Fig. 3A).

4-day-old preflexion larva (3.5 mm TL): Mouth completely opened and eyes are fully pigmented. Patched melanophores no longer visible. Nine or 10 melanophores are arranged on the ventral edges of the tail and 3 on the dorsal edge (Fig. 3B).

14-day-old flexion larva (6.5 mm TL): Head and mouth become larger and body depth increased. Small, sharp teeth are visible on both jaws. Several spines are distributed on preopercula and opercula. The notochord tip has begun to turn upward with the appearance of urophysial bones. First dorsal and caudal fin rays have begun to differentiate (Fig. 3C).

18-day-old postflexion larva (8.8 mm TL): Body form is intermediate between larva and juvenile. Head with large eyes and mouth is further enlarged. Notochord-tip upturn is
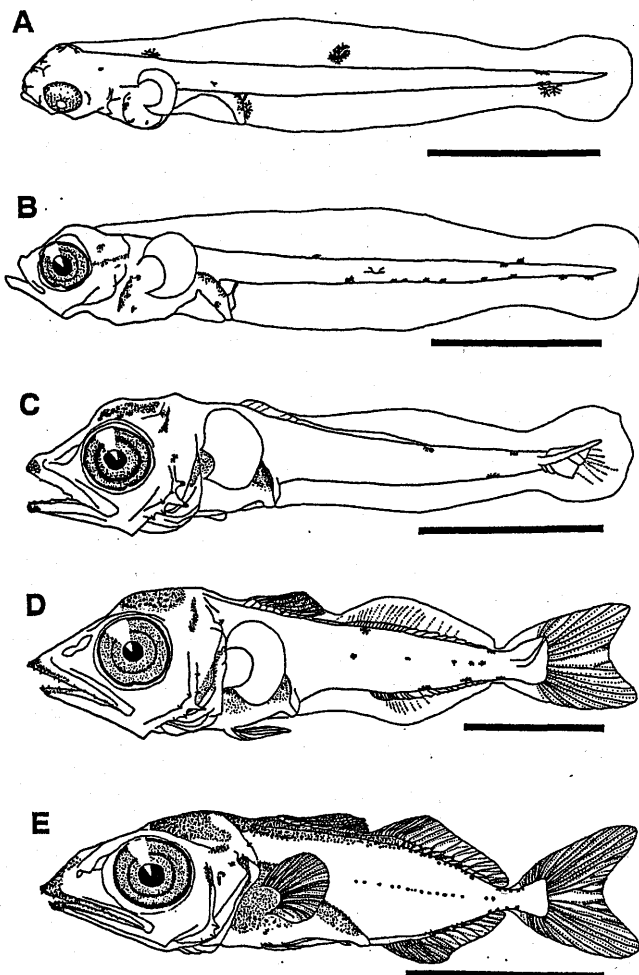

Fig. 3. Morphological development of bluefin tuna larvae reared at the JASFA Yaeyama Station in 1994. Formaldehyde-preserved specimens were illustrated with the aid of a camera lucida. A, 2-day-old yolk-sac larva; B, 4-day-old preflexion larva; C, 14-day-old flexion larva; D, 18-day-old postflexion larva; E, 30-day-old juvenile. Scales; A and B: 1 mm, C and D: 2 mm, E: 5 mm.

finished and equipped with a nearly completed caudal fin. Dorsal fin spines appeared with heavily aggregated melanophores. Second dorsal fin and anal fin bases appeared. Ventral fin is completed but pectoral fin is still membranous without fin rays (Fig. 3D).

30-day-old juvenile (15.6 mm TL): All fins are fundamentally established. The anterior part of snout, dorsal body surface and dorsal half of the abdominal cavity are heavily pigmented. Body depth increased, particularly in the tail. Anus has shifted posteriorly (Fig. 3E).

Relative Growth

Relative growth, expressed as percentage of standard length, is shown in Fig. 4. There appear to be three sizes at which relative growth rates change; 6, 8 and 10 or 11 mm SL. For example, relative growth of TL and PAL increased from 6 mm SL, whereas that of TL, HL, SNL, ED, UJL and BD was slower from 8 mm SL. Relative growth of PFL was constant from 6 mm SL. Most body parts showed a constant relative growth value after fish had reached 10 mm SL, although PAL/SL increased continuously beyond this size.
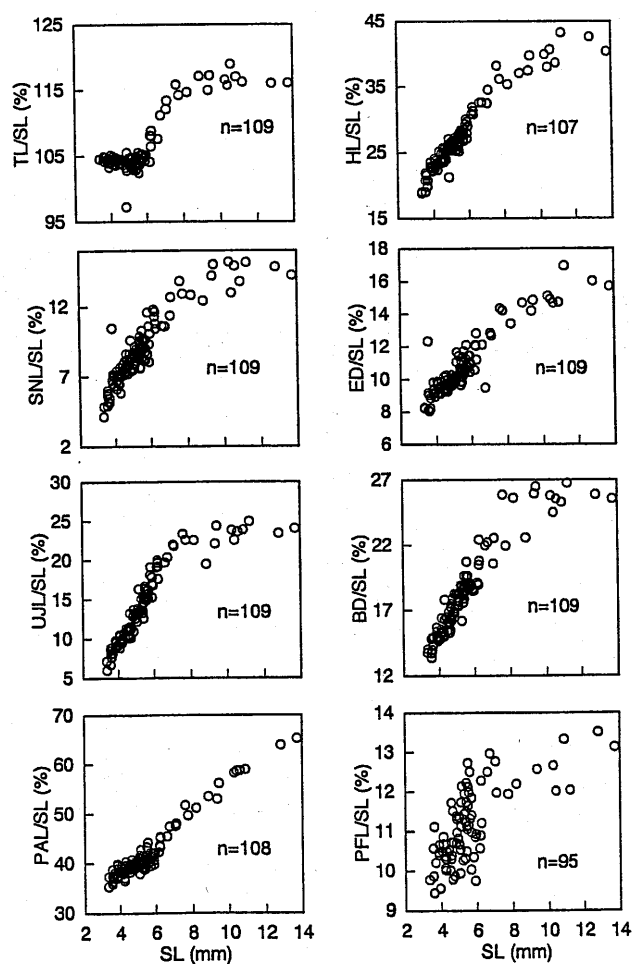

Fig. 4. Relative growth (percentage of standard length) of bluefin tuna larvae reared at the JASFA Yaeyama Station in 1994. TL, total length; SL, standard length; HL, head length; SNL, snout length; ED, eye diameter; UJL, upper jaw length; BD, body depth; PAL, preanal length; PFL, pectoral fin length.

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Ossification

No ossified elements were found in first-feeding larva (3.3 mm SL). In an early flexion larva (6.1 mm SL) upper and lower jaws, preopercula, opercula and cleithlum were ossified, but there was no sign of ossification in central skeletal elements. In a postflexion larva (7.4 mm SL) the anterior 10 or 11 centra and basal parts of the neural spines had begun to ossify, caudal and ventral fin rays were nearly ossified, but rays of dorsal and anal fins showed little ossification. All centra with neural and hemal spines and hypural bones were nearly ossified in a juvenile (12.6 mm SL).

Development of the Digestive System

2-day-old yolk-sac larva: The gut, divided into oropharyngeal cavity, oesophagus, rudimentary stomach, intestine and rectum, has opened from mouth to anus. The epithelia of the gut are still differentiating, and the liver and pancreas are differentiated from the gut.

3-day-old larva (first feeding stage, 3.3 mm SL): The primitive digestive system which enables the larva to feed on exogenous food is established (Fig. 5*B*). The stomach is short and rudimentary as the gastric gland and blind sac have not differentiated (Fig. 5*C*). The anterior part of the intestine has begun to rotate and the epithelium consists of columnar absorptive cells with a well developed striated border. The rectum has a similar type of epithelium but acidophilic granules stained with eosin are visible in the upper half of the epithelial cells (Fig. 5*D*). Pancreatic exocrine tissues produce zymogen granules to accumulate within acini. Taste buds are observed on the oral epithelium. Primordial swimbladder has differentiated just on the dorsal part of the stomach, but is not yet inflated. Pharyngeal tooth buds appear on Day 5 and become functional on Day 8.

12-day-old flexion larva (6.1 mm SL): The gastric mucosa becomes thick with an increase in functional gastric glands (the first appearance of gastric gland was noted on Day 11, 5.6 mm SL) and the stomach is elongated posteriorly to form the blind sac (Fig. 6*C*). The intestinal epithelium, particularly at the middle part, is highly vacuolated by fat absorption and accumulation (Fig. 6*D*) and acidophilic granulation have become more prominent in the rectal epithelium (Fig. 6*E*). The jaw teeth first appear simultaneously with gastric gland differentiation. Pharyngeal teeth have increased in size and number (Fig. 6*F*).

18-day-old larva at postflexion phase (7.4 mm SL): The stomach has developed
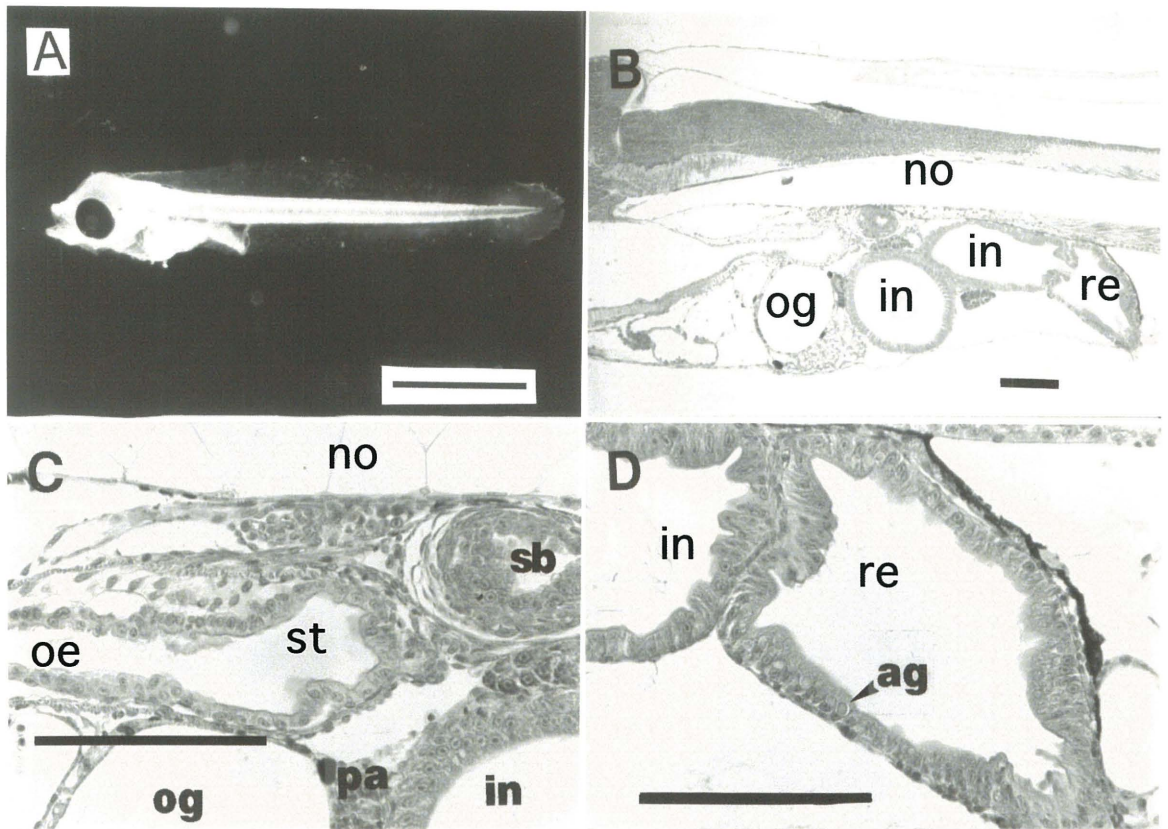
Fig. 5. Photomicrographs of a 3-day-old bluefin tuna larva at first feeding (60 hours after hatching, 3.3 mm SL).
A: whole body photograph, B: sagittal section of the body axis showing development of the digestive tract, C: sagittal section of the rudimentary stomach (st), oil globule (og), intestine (in), swimbladder (sb) and pancreas (pa), D: sagittal section of the rectum (re) showing the appearance of acidophilic granules (ag with arrowhead). no: notochord, oe: oesophagus
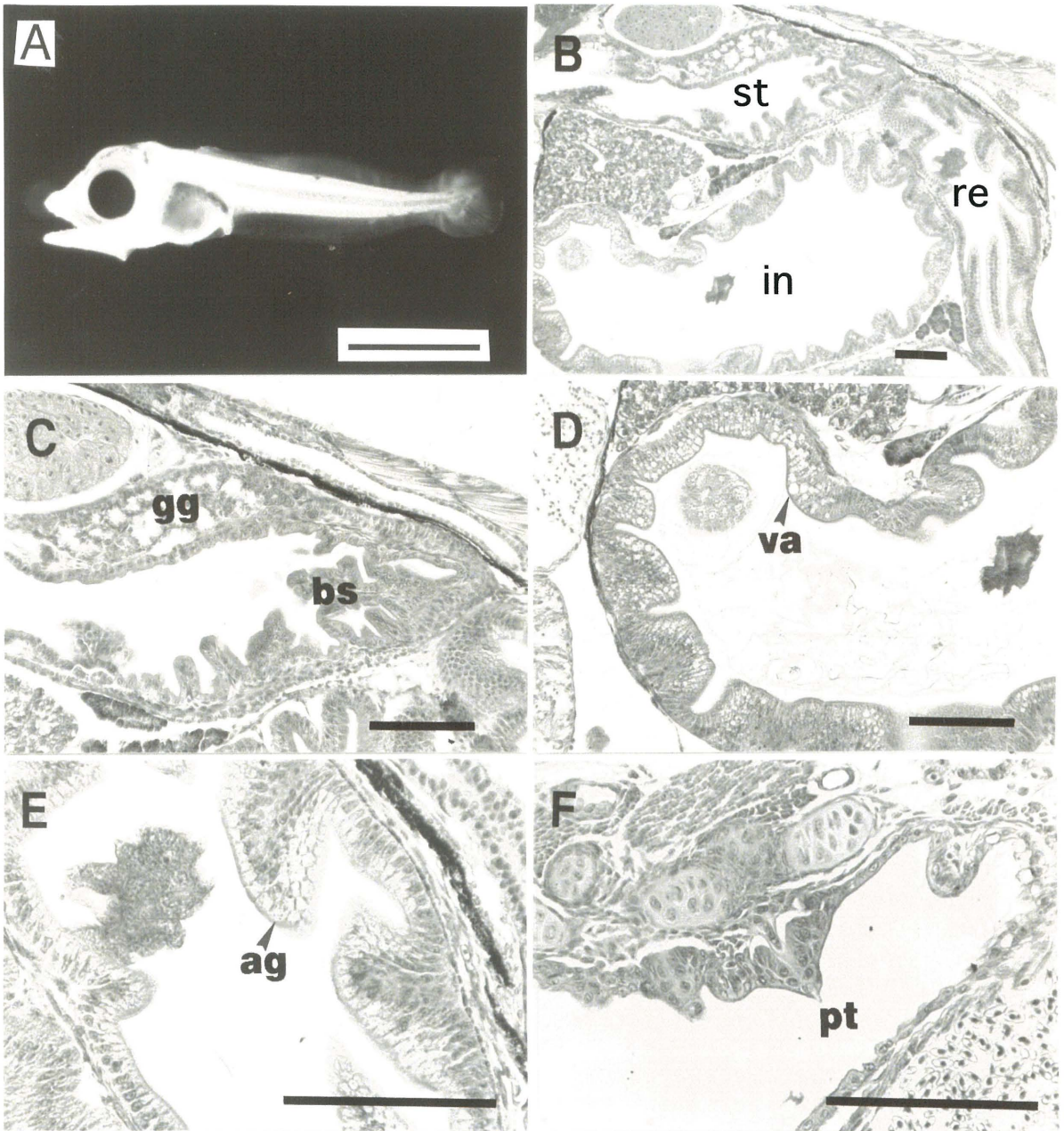Scales; A: 1 mm, B to D: 100 µm.

Fig. 6. Photomicrographs of a 12-day-old bluefin tuna larva at the flexion phase (6.1 mm SL).
A: whole body photograph, B: sagittal section of the body axis showing the stomach (st), intestine (in) and rectum (re), C: sagittal section of the stomach showing functional gastric glands (gg) and posteriorly enlarging blind sac (bs), D: Intestinal epithelium with a large number of vacuoles (va with arrowhead), E: highly granulated rectal epithelium (ag with arrowhead), F: Pharyngeal teeth (pt) on the dorsal pharynx. Scales; A: 2 mm, B to F: 100 μm.
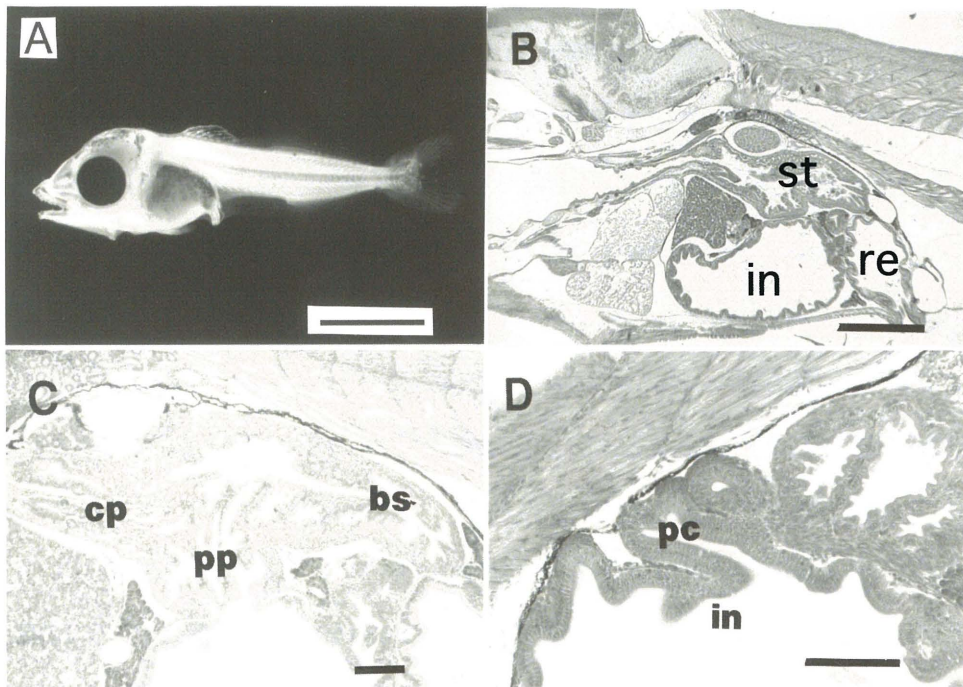
Fig. 7. Photomicrographs of a 18-day-old bluefin tuna larva at postflexion phase (7.4 mm SL).
A: whole body photograph, B: sagittal section on the body axis showing the stomach (st), intestine (in), and rectum (re), C: sagittal section of the stomach showing differentiation of 3 portions, cardiac portion (cp), pyloric portion (pp) and blind sac (bs), D: pyloric caecum (pc) differentiated from the most anterior wall of the intestine (in). Scales; A: 2 mm, B: 500 μm, C and D: 100 μm.

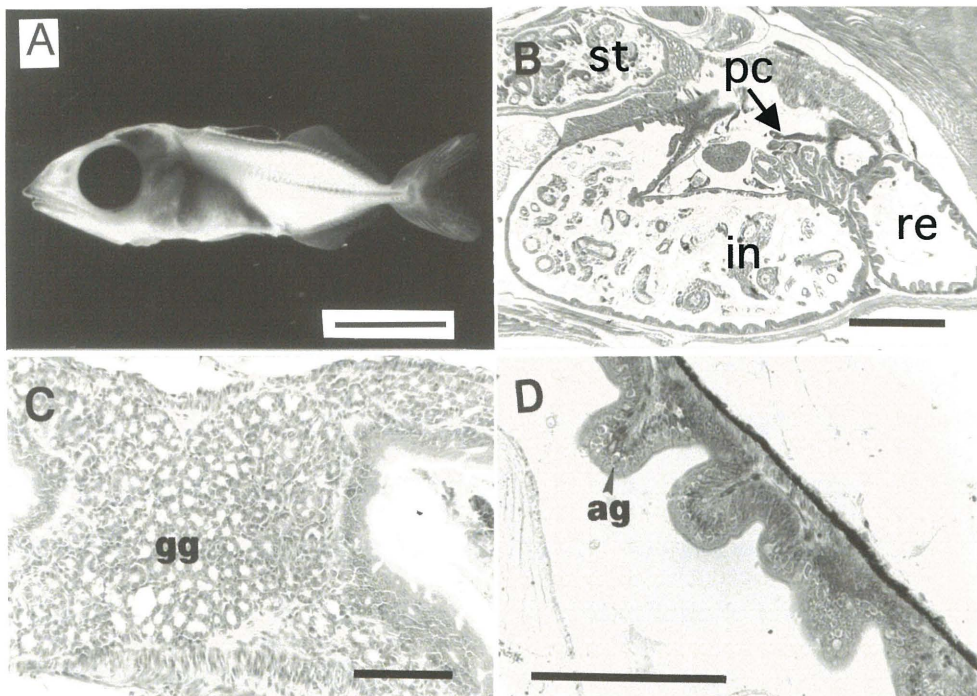**Fig. 8.** Photomicrograph of a 30-day-old bluefin tuna at the early juvenile phase (12.6 mm SL).
A: whole body photograph, B: sagittal section of the body axis showing the stomach (st), intestine (in), pyloric caeca (pc) and rectum (re), C: densely distributed gastric glands (gg), D: rectal epithelium with acidophilic granules (ag with arrowhead). Scales; A: 4 mm, B: 500 μm,
 C and D: 100 μm.

further, with increased numbers of gastric glands, an elongated blind-sac and differentiation of the pyloric portion (Fig. 7C). The pyloric caecum which appeared on Day 14 protrudes from the most anterior part of the intestine (Fig. 7D). The rectal epithelium is more heavily granulated than in flexion-phase larva. Jaw and pharyngeal teeth have increased further in number and size.

30-day-old juvenile (12.6 mm SL): The stomach is further enlarged and most of the gastric mucosa have densely stratified gastric glands (Figs. 8B, 8C). The anterior part of the intestine is surrounded by numerous pyloric caeca (Fig. 8B). Well developed goblet cells are seen in the intestinal epithelium. Acidophilic granules are still visible in the rectal epithelium (Fig. 8D); however, the size and number is reduced from the postflexion larva.

Discussion

Survival during this bluefin tuna rearing trial was very low and was estimated to be 0.19% at the final harvest, 35 days after hatching. Heavy mortalities occurred during the first week after onset of feeding and during the late flexion and postflexion phases. The causes of these heavy mortalities may be maternal in origin and/or may be related to post-hatch rearing conditions. Egg quality (Kjørsvik *et al.*, 1990) and quality of food and/or nutritional problems (Watanabe, 1993) may be of primary importance. Food quality seems to be a likely explanation for the observed high post-first feeding mortality, and is indicated by the slow growth rate exhibited by larvae in the trial. Although the bluefin tuna larval growth rate of 0.43 mm day^{-1} is of the same order as that of many species it may represent a slow rate for a species of the family Scombridae. Rearing trials for bluefin tuna larvae carried out in other hatcheries revealed twice or more higher growth rates (Y. Sawada *et al.*, personal communication; F. Endo, personal communication). This difference may be attributed to growth rates during the later part of the larval period, when *Artemia* larvae and artificial diet were mainly fed in the present experiment, whereas minced fish meat was fed in the other experiments.

The low larval survival and growth in the present rearing trial may affect the author's estimates of the timing of developmental events. Development of fish is a function of both age and length; for example the age and length at differentiation of the stomach and pyloric

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caecum could be represented by hyperbolic curves in the ayu *Plecoglossus altivelis* (Tanaka, 1973). This suggested differentiation of the digestive system occurred at a relatively small size and an older age in cohorts whose growth was slow due to low food. Age and length at differentiation of an organ obtained here may be modified to some extent by improved rearing trials, and the present results may represent development of relatively slow-growing bluefin tuna larvae.

Scombrids can grow very rapidly during the early life stages and this may be related to the precocious development of the digestive system in this group (Tanaka *et al*, 1996*a*). Growth to about 10 cm was observed during the first month of life in a Spanish mackerel *Scomberomorus niphonius* and two species of frigate mackerel *Auxis thazard* and *A. tapeinosoma* under experimental rearing conditions (Fukunaga *et al.,* 1982; Harada *et al.*, 1973*a*; 1973*b*). Spanish mackerel may represent an extreme example of precocious digestive system development as the basic structure of an adult-type digestive system are established prior to first feeding (Tanaka *et al.* 1995*a*). Development of the bluefin tuna digestive system during the yolk-sac stage seemed to be similar to the developmental stage observed in marine fishes from various families that hatch from small pelagic eggs (Tanaka, 1973). The most primitive digestive system, which enables the larvae to digest and absorb the exogenous nutrients, is established just prior to onset of feeding. In most marine fishes a digestive system, consisting of a functional stomach with well developed gastric glands and pyloric caeca, occurs associated with transformation to juvenile stage. In the bluefin tuna larvae, however, such a system was established prior to the postflexion phase. Such precocious development of the digestive system is also observed in yellowfin tuna *T. albacares* larvae which were simultaneously reared at the JASFA Yaeyama Station (Kaji *et al.*, 1999b). These findings suggest that precocious development of the digestive system is a general feature in scombrid fishes although its extent is species specific.

The most pronounced feature of digestive system development during the larval period of bluefin tuna was the rapid expansion of the gastric blind-sac and increase in number of gastric glands. Functional development of the digestive system, evaluated by enzyme activity, has been observed in several marine and freshwater fish larvae and juveniles (Tanaka *et al.*, 1972; Segner *et al.*, 1993; Walford and Lam, 1993; Tanaka *et al.* 1996*b*). These studies show that activity of pepsin-like enzyme increased and the pH in the stomach

decreased with histological and morphological development of the stomach. Nearly the same result was found for species with high-growth rates such as amberjack *Seriola lalandi* and rockfish *Sebastes schlegeli* (Kawai and Tanaka, unpublished), and the author assume that pepsin-like enzyme activity increases with stomach enlargement and increase in number of gastric glands in bluefin tuna larvae. Proteolytic capability would subsequently increase during the postflexion phase in this species, enabling a shift in food habits to piscivory.

The precocious development of piscivory is probably important to the rapid growth of scombrids. Species such as Spanish mackerel may feed on fish larvae from the first feeding stage (Tanaka *et al.*, 1996a; Shoji *et al.*, 1997). Uotani *et al.* (1990) demonstrated that bluefin tuna larvae collected off the Nansei Island fed primarily on copepod nauplii and copepodites. The gut contents of 1946 sea-caught larvae including a few early juveniles were examined; however only one fish egg was found (Uotani *et al.*, 1990). Those authors also noted that the main food shifted from copepod nauplii to copepodites in larvae during the flexion stage. Their wild 5 mm SL may be nearly the same as reared 6 mm SL, when the caudal fin began to develop. In the present rearing experiment, eggs and larvae of coral trout were fed to postflexion larvae and juveniles, but ingested fish tissues were not observed in histological sections and stomach contents were not separately analyzed. Despite the lack of evidence of piscivory, bluefin tuna larvae could potentially feed on fish larvae from the postflexion phase, when a well developed basic digestive system and a well developed jaw and pharyngeal teeth are established. Under the rearing conditions bluefin tuna larvae exhibited active cannibalistic behavior at the postflexion phase and early juvenile stage. These observations suggest that the bluefin tuna larvae, when they reach the postflexion phase, may prey on fish larvae when they are abundant.

The present findings on larval development should contribute to improvement of rearing techniques for bluefin tuna. Based on the author's finding that a morphologically functional stomach is established in the early phase of the larval period, feeding fish larvae and/or minced fish to bluefin tuna in the later part of the larval period should be beneficial. It may be important to feed other fish larvae to bluefin tuna under rearing conditions because such a practice could, by reducing cannibalism, improve survival above the increases realized by improved nutrition .

2) Growth and Development of Yellowfin Tuna Larvae with Special Reference to the Digestive System

Abstract

Yellowfin tuna *Thunnus albacares* larvae were reared from hatching beyond metamorphosis in May and June 1996. The larval size was 2.65 mm SL at just after hatching and 27.68 mm SL on Day 37. Transformation to juveniles occurred around 30 days after hatching at about 13 mm SL. The larvae initially fed on rotifers since Day 4, on fish larvae and *Artemia* nauplii since Day 16, and then on frozen fish and minced fish meat. The primitive digestive system differentiated on Day 4. The gastric gland and pyloric caeca first appeared on Day 14 and 16, respectively. The pharyngeal and jaw teeth became fully functional synchronized with gastric gland differentiation. The number of gastric gland and pyloric caeca and volume of the gastric blind sac increased markedly toward the juvenile stage. Although external morphological development of yellowfin tuna resembled to that of other marine fishes hatched from pelagic eggs, the digestive system developed precociously. The rapid development of the digestive system allows the early appearance of piscivory which can support the high growth potential as shown in other scombrid fishes.

Introduction

The yellowfin tuna *Thunnus albacares* is a commercially important scombrid fish which is distributed in tropical and subtropical waters (Collet and Nauen, 1983). In general, scombrid fish have a high growth potential and externally unique morphological features during the early life stages, characterized by a large head with large mouth and eye, well developed preopercular spine, and a posterior shift of the anus with growth. From these morphological characteristics, Hunter (1981) inferred that mackerel and tunas had a "large prey-fast growth" strategy during their early life history. Judging from these morphological peculiarities, their internal organ-system would be expected to have specialized developmental processes, however, the basic biology of the early life history of these species is poorly known.

Recently, increasing interest has been placed on tunas as a candidate for future stock

enhancement or aquaculture in Japan (Kaji *et al*., 1996). Experimental rearing of yellowfin tuna larvae was first conducted at the Shirahama Fisheries Laboratory of Kinki University in 1970, and preliminary observations on external morphological development were described (Mori *et al*., 1971; Harada *et al*., 1971). The Japan Sea-Farming Association (JASFA) has started to develop techniques of seed production for yellowfin tuna as a model for production of Pacific bluefin tuna *T. thynnus*. In the JASFA Yaeyama Station, spontaneous spawning of yellowfin tuna in a floating net pen first occurred in 1992,* and rearing trials for the larvae and juveniles have been conducted. However, survival rates have been very low due to heavy mortalities occurring at particular developmental stages: a few days after initiation of feeding and around transformation phase to juveniles. This suggests that basic biological information on ontogeny appears to be indispensable to improve the rearing technique of tunas.

The main purpose of this article is to describe development of the yellowfin tuna reared in the laboratory as a part of studies in the early development of tunas, particularly ontogeny of the digestive organ-system because of its basic importance and a practical application to establishment of a suitable feeding schedule.

Materials and Methods

Rearing

Fertilized eggs of yellowfin tuna were obtained on 21, May 1996 from spontaneous spawning of the broodstock maintained in a net pen at the JASFA Yaeyama Station, Ishigaki Island (24° 27' N, 124° 17' E), Okinawa, Japan. Detailed information on the rearing was described in chapter 2-1)

Sampling

Fourteen to 80 fishes were sampled daily or every other day from Day 0 to 20, and 22 to 53 fishes on Days 25, 30, and 37. Some of the sampled fish were anesthetized with MS-222, and total length and standard length measured.

*S. Masuma and M. Oka: Abst. Metg. Japan Soc. Fisheries Sci., April 1994, p. 78 (in Japanese).

Half of the fish were fixed in 4% neutral formaldehyde for morphological investigation. The following body parts were measured in formaldehyde-preserved specimens: total length (TL), standard length (SL), snout length (SNL), upper jaw length (UJL), eye diameter (ED), head length (HL), body depth (BD), and preanal length (PAL). Relative growth of each body part was examined by expressing it as a percentage to SL. Some of the larvae were weighed on an electro-microbalance (Mettler M3) after careful removal of the surface fluid.

A portion of each sample was fixed in Bouin's solution for 24 hours and preserved in 80% ethanol for later histological preparation. The larvae were dehydrated and embedded in paraffin (parahisto, Nakalaitesque). Serial sections (sagittal plane) were made by a microtome at a thickness of 5 μm. Sections were stained with Mayer's hematoxylin and eosin (HE, 50 fishes) or Periodic acid schiff (PAS, 44 fishes), and observed under a microscope system (Olympus). Developmental phase after Kendall, Jr. *et al*. (1984) was used in the present study.

Results

Growth, Survival, and Development

Newly hatched larvae were 2.65 ± 0.07 mm SL (mean ±S. D.), and grew to 27.68 ±3.11 mm SL on Day 37 (Fig. 1). The growth process appeared to be curvilinear with an
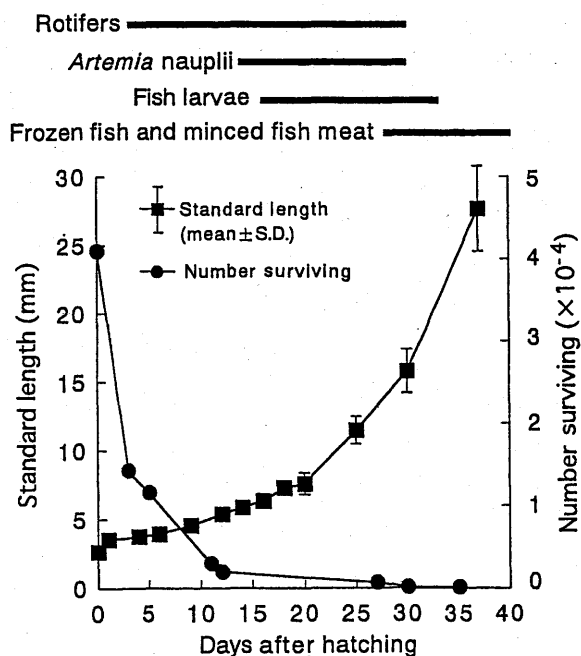

Fig. 1. Standard length (mean ±S. D.) increase of anesthetized fish and number surviving of yellowfin tuna larvae and juveniles reared at the JASFA Yaeyama Station in 1996.
Feeding schedule is illustrated at the top of the figure.

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increase in growth rate with age. Average daily growth during the experiment was approximately 0.68 mm. Wet body weight increased exponentially with increase in length (Fig. 2). The relationship between wet weight (W, in mg) and standard length (L, in mm) was expressed as $W=0.00179L^{3.846}$ (n=89, r^2=0.934).
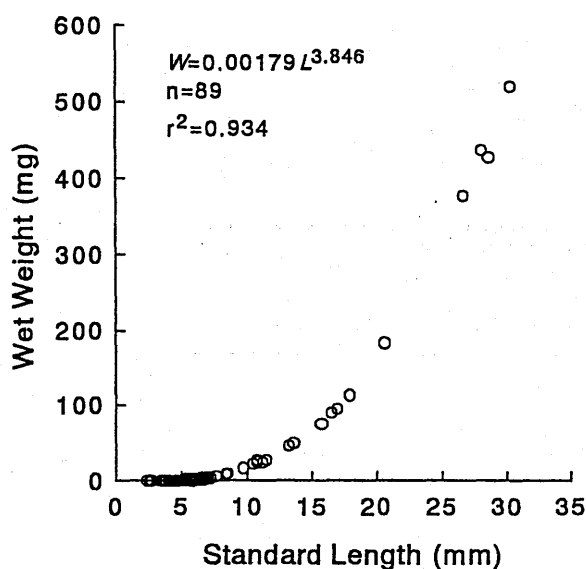

Fig. 2. Length-weight relationship of yellowfin tuna larvae and juveniles reared at the JASFA Yaeyama Station in 1996.
The wet body weight for formaldehyde-preserved specimens was measured 4 months after fixation. Relationship between length (L, SL in mm) and body weight (W, wet in mg) is expressed by $W=0.00179L^{3.846}$ (n=89, r^2=0.934).

Hatching rate of larvae was very high, almost 100%. High mortalities occurred a few days after commencement of feeding and around the transformation phase from larvae to juveniles (Fig. 1). Although routine sampling could not be made because of the restricted number of fish remaining after Day 37, rearing was continued and the fish finally reached the maximum of 122.0 mm in TL and 20.85 g in wet body weight on Day 79.

The shift from yolk-sac phase to preflexion phase occurred on Day 4. The consecutive developmental phase shifts largely varied with individuals (Table 1). Average dates are roughly estimated Day 16 (to flexion) and between 20 and 25 (to postflexion). The larvae transformed from postflexion phase to juvenile on Day 30. In terms of the standard length of fish, these shifts occurred on 4, 6, 7, and 13 mm, respectively (Table 1).

Relative Growth

There were two sizes in each body part at which the relative growth pattern changes (Fig. 3). In HL, UJL, BD, and ED, the value increased rapidly from 4-5 mm SL and became a

Table 1. Developmental phase composition (number of individuals) of yellowfin tuna larvae and juveniles reared at the JASFA Yaeyama Station in 1996, showing distribution of fish in terms of age (upper) and length (lower).

Developmental phase	0	1	2	3	4	5	6	7	9	10	12	14	16	18	20	25	30	37	Total
										Days after hatching									
Yolk-sac larva	40	80	25	28															173
Preflexion larva					40	14	40	40	40	20	36	17	4						251
Flexion larva											4	26	25	24	8				87
Postflexion larva														16	23	49	1		89
Juvenile																4	36	22	62
Total*	40	80	25	28	40	14	40	40	40	20	40	43	29	40	31	53	37	22	662

developmental phase	2	3	4	5	6	7	8	9	10	11	12	13	14	15<	Total
					Standard length (mm)										
Yolk-sac larva	19	50	46												115
Preflexion larva		15	130	56	6										207
Flexion larva				10	43	24									77
Postflexion larva					1	21	12	4	13	22	3		1		77
Juvenile											1	7	8	46	62
Total*	19	65	176	66	50	45	12	4	13	22	4	7	9	46	538

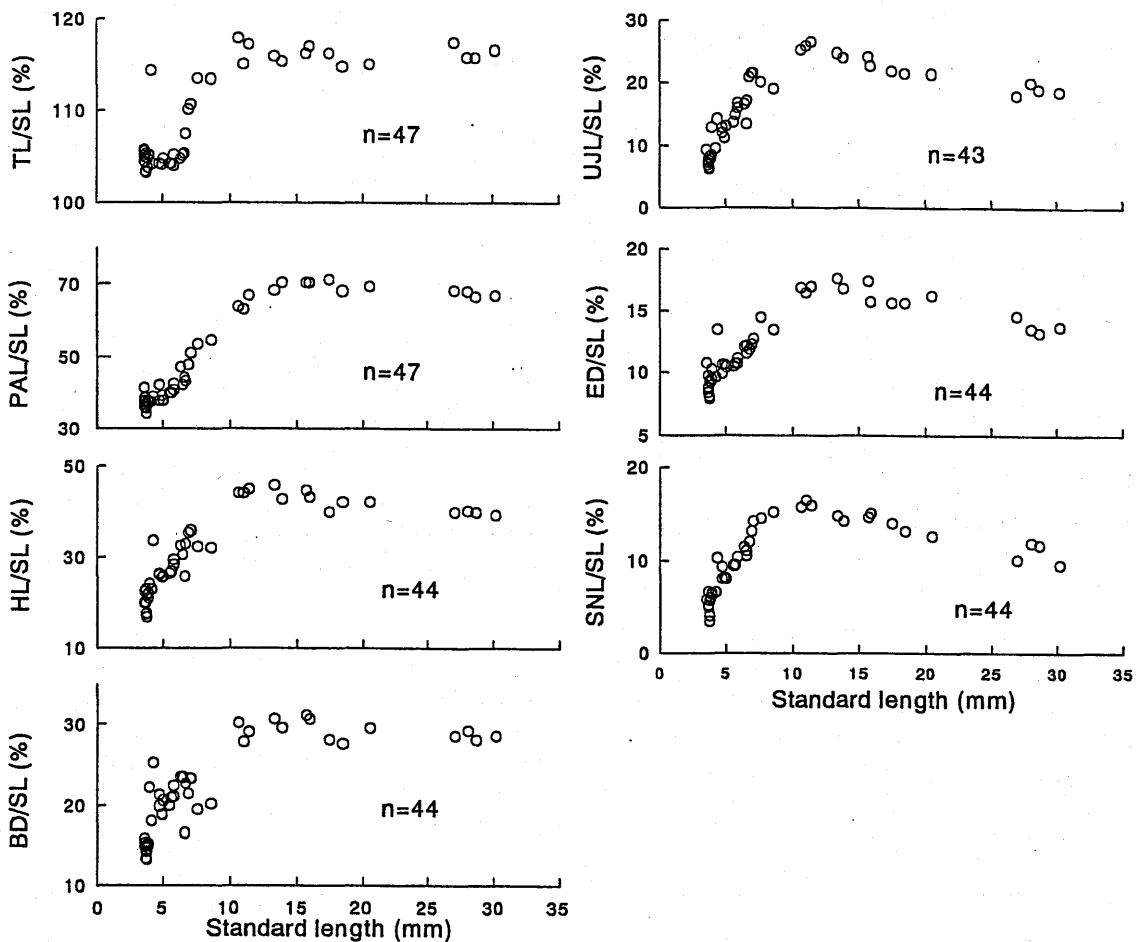* Materials used here include both formaldehyde- and ethanol-preserved fishes

Fig. 3. Relative growth (percentage of standard length) of yellowfin tuna larvae and juveniles reared at the JASFA Yaeyama Station in 1996.
TL, total length; SL, standard length; PAL, preanal length; HL, head length; BD, body depth; UJL, upper jaw length; ED, eye diameter; SNL, snout length.

constant or decreased slightly above 11-13 mm SL. The same pattern was shown at 4 and 9 mm SL for SNL. On the other hand, the value of TL increased more rapidly above 7 mm SL, and became constant around 11 mm SL. The same pattern was shown at 7 and 14 mm SL for the relative growth value of PAL.

Development of the Digestive System

Yolk-sac larvae: In 0-day old larvae just after hatching (Fig. 4A), most of the body cavity was occupied with yolk (Fig. 4B). The yolk showed a positive reaction to eosin and weak positive reaction to PAS. The gut was a simple and almost straight tube, and its posterior part was open. The mouth had not yet opened (Fig. 4B).

On Day 1, 24 hours after hatching, the lumen of the gut was slightly enlarged, especially in the oral cavity. From mid to anterior part of the gut, the striated border showed PAS positive reaction. The yolk became small and round in its shape, and showed slightly positive reaction to PAS. The liver differentiated behind the yolk mass. The rudimentary swimbladder began to differentiate from the dorsal wall of anterior gut.

In 2-days old larvae, hepatocytes were differentiated, and PAS positive granules were present among these cells. The granules were identified as glycogen by diastase-digestion test. Upper and lower jaws were differentiated, and the mouth opened completely. The pancreas with a pancreatic islet differentiated at the left side of posterior part of yolk mass.

Preflexion larvae: In 4-days old larvae (Fig. 5A), rotifers first appeared in the digestive tract. The basic structure of the digestive system which enables the larvae to ingest and digest exogenous nutrients was established (Fig. 5B). Mucous cells were not seen on the epithelium of oral cavity and oesophagus. A rudimentary stomach was apparent between the oesophagus and the intestine. Gastric glands were not observed in the stomach mucosa (Fig. 5C). The intestine coiled once and its epithelium was composed of columnar absorptive cells provided with well developed striated borders. The rectum completely separated from the intestine by a well developed rectal valve (Fig. 5D). In most of larvae hepatocytotic glycogen disappeared. The volume of pancreas increased markedly, and zymogen granules were accumulated in the acini. Some larvae had small eosinophilic granules in the rectal epithelium cells (Fig. 5D). A small amount of yolk remained in the liver (Fig. 5C). A few thyroid follicles with thyrogloburine differentiated in the lower jaw.

On Day 5, many large eosinophilic granules appeared in the rectal epithelium. Glycogen storage in the hepatocytes increased again.

On Day 7, upper pharyngeal tooth buds appeared. Yolk was still found among the hepatocytes. A few PAS positive mucous cells appeared in the oesophagus epithelium, and secreted mucous substance into the lumen.

On Day 10, mucous cells in the oesophagus epithelium increased in number, but no mucous cell was observed in the oral cavity epithelium. Upper and lower pharyngeal teeth became fully functional.

On Day 12, a few mucous cells appeared in the oral cavity epithelium. Mucosal folds of the stomach developed and its dorso-posterior part enlarged to form the blind sac. Gastric glands, however, were not seen in the mucosa. Jaw teeth first appeared.

Flexion larva: On Day 14, mucous cells in the oral cavity were still scattered. In the oesophagus epithelium, however, mucous cells increased in number, particularly in its posterior part. Gastric glands first appeared in the dorsal part of the stomach. The blind sac enlarged posteriorly.

On Day 16 (Fig. 6A), gastric blind sac was enlarged (Figs. 6B, D). Gastric glands were distributed from dorsal to lateral walls of the stomach (Fig. 6D), and included PAS positive materials in its lumen. Jaw and pharyngeal teeth (Fig. 6C) increased in number. The pyloric caeca began to differentiate.

Postflexion larva: On Day 20 (Fig. 7A), the digestive system markedly developed in number and volume. The number of jaw and pharyngeal teeth, gastric glands, and pyloric caeca increased. The stomach was separated into three parts, cardiac and pyloric portions, and blind sac (Figs. 7B, C). The blind sac extended postero-ventrally. Pyloric caeca were well formed at the anteriormost part of the intestine (Fig. 7D). Mucous cells on the oral cavity epithelium were still small in number. High number of goblet cells showing PAS positive reaction appeared among the columnar epithelium cells of the intestine and pyloric caeca.

Juvenile: On Day 30 (Fig. 8A), PAS positive mucous cells increased markedly in the oral cavity epithelium (Fig. 8C). Gastric glands increased in number, and were distributed throughout the stomach with the exception of the pyloric portion. Gastric lumen contained a large volume of food resulting in expansion of the blind sac toward posterior edge of the
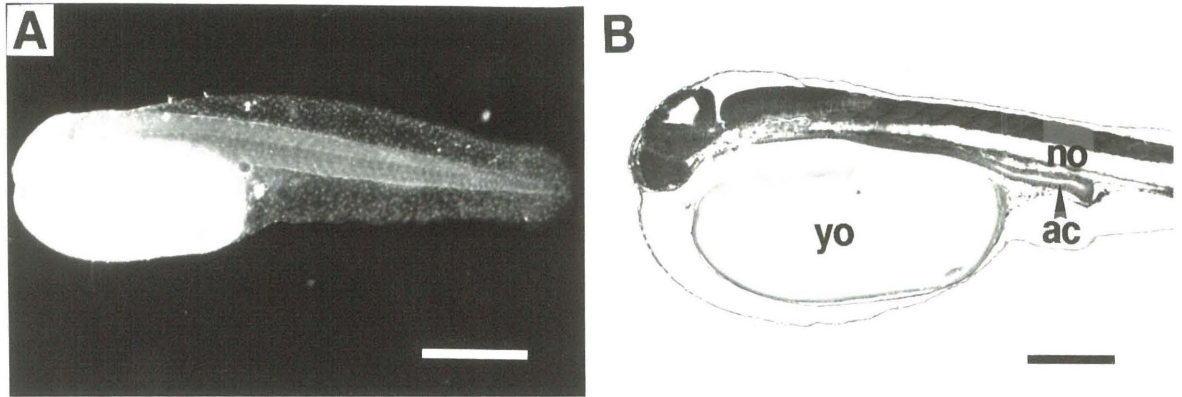
Fig. 4. Photomicrographs of a 0 day-old yellowfin tuna larva at just after hatching. (A) whole-body photograph. Scale bar=0.5 mm. (B) sagittal section of the body axis showing large yolk (yo) and undifferentiated alimentary canal (ac). Scale bar=200 μm. no; notochord.
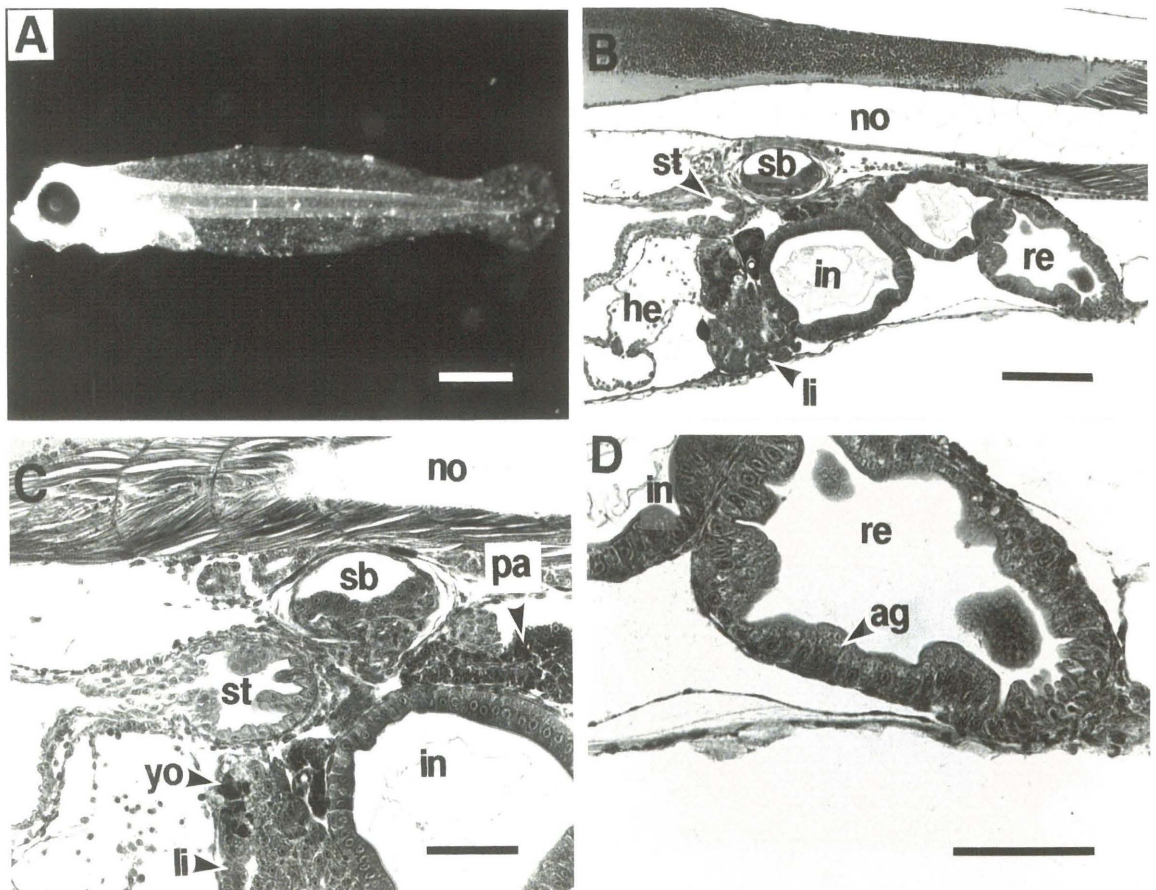

Fig. 5. Photomicrographs of a 4 day-old yellowfin tuna larva at first feeding stage. (A) whole-body photograph. Scale bar=0.5 mm. (B) sagittal section of the body axis showing the differentiated digestive tract. Scale bar=100 μm. (C) sagittal section of the rudimentary stomach (st). Scale bar=50 μm. (D) sagittal section of the rectum (re) showing the appearance of acidophilic granules (ag with arrowhead). Scale bar=50 μm. he; heart, in; intestine, li; liver, no; notochord, pa; pancreas, sb; swim bladder, yo; yolk.
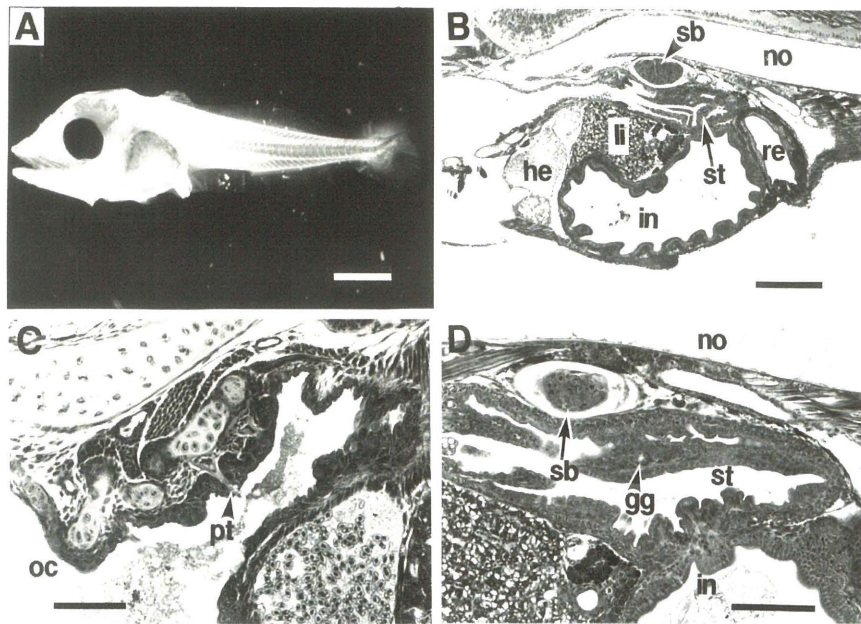
Fig. 6. Photomicrographs of a 16 day-old yellowfin tuna larva at flexion phase. (A) whole-body photograph. Scale bar=1 mm. (B) sagittal section of the body axis showing the development of stomach (st), intestine (in), and rectum (re). Scale bar=200 μm. (C) pharyngeal teeth (pt) on the dorsal pharynx. Scale bar=50 μm. (D) sagittal section of the stomach (st) showing the early appearance of the gastric gland (gg with arrowhead). Scale bar=100 μm. he; heart, li; liver, no; notochord, oc; oral cavity, sb; swim bladder.
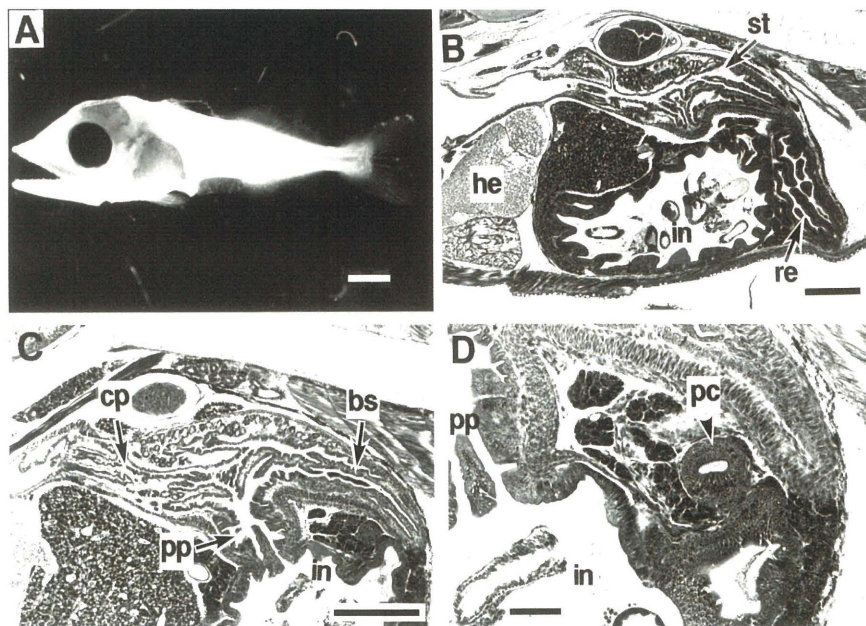

Fig. 7. Photomicrographs of a 20 day-old yellowfin tuna larva at postflexion phase. (A) whole-body photograph. Scale bar=1 mm. (B) sagittal section of the body axis showing the stomach (st), intestine (in), and rectum (re). Scale bar=200 μm. (C) sagittal section of the stomach showing differentiation of cardiac portion (cp), pyloric portion (pp), and blind sac (bs). Scale bar=200 μm. (D) pyloric caecum (pc with arrowhead) differentiated from the anterior-most wall of the intestine (in). Scale bar=50 μm. he; heart.
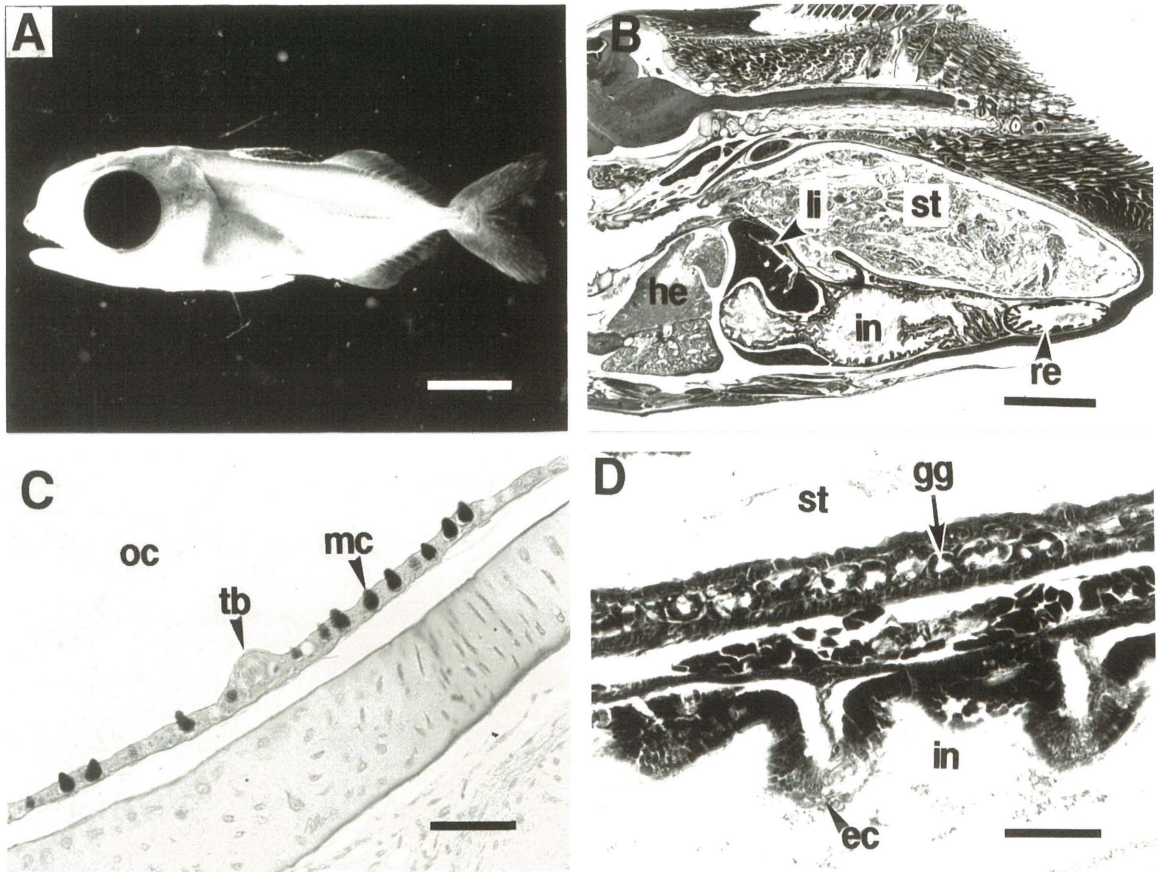
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Fig. 8. Photomicrographs of a 30 day-old yellowfin tuna at early juvenile phase. (A) whole-body photograph. Scale bar=3 mm. (B) sagittal section of the body axis showing largely expanded stomach (st), intestine (in), and rectum (re). Scale bar=1 mm. (C) sagittal section of the oral cavity (oc) epithelium showing taste bud (tb with arrowhead) and mucus cells (mc with arrowhead). PAS stain. Scale bar=50 μm. (D) sagittal section of the stomach (st) and intestine (in) showing well-prolonged gastric wall with densely distributed gastric gland (gg with arrowhead) and intestinal epithelium (ec with arrowhead) sloughing into the lumen. Scale bar=50 μm. he; heart, li; liver.

abdominal cavity (Fig. 8B). In all fish examined, intestinal epithelial cells were stained dark with hematoxylin. Some mucosal cells of intestine were sloughed into the lumen (Fig. 8D). This was also apparent in the pyloric caecum epithelium. Mucus substance showing strong PAS positive reaction was observed near the sloughed mucosal cells in the lumen.

Discussion

Compared to previous larval rearing experiments of yellowfin tuna at the Yaeyama Station, survival rates in this trial were relatively high, however, heavy mortalities occurred at specific developmental phases. The first one, occurring a few days after initiation of feeding, may be due to quality of initial food (Watanabe, 1993) and/or egg quality (Kjørsvik et al., 1990). Judging from the histological observation, this was defined as a phase when the larvae depended on both preys (rotifers) and yolk, or as a transition to complete exogenous feeding. There may be a nutritional problem during this transition of nutrient sources. Nutritional requirements of the transitional larvae, particularly in an offshore species such as yellowfin tuna, must be one of the critical issues in enhancing survival rate. Another approach to enhancement of early larval survival may be from the viewpoint of developmental endocrinology. The role of maternal hormones during early ontogeny may be important in early survival (Brown et al., 1988, 1989). Fertilized eggs of yellowfin tuna contained about 0.5 ng/g thyroxine (T4) and 1.4 ng/g 3, 5, 3'-triiodo-L-thyronine (T3) at the morula stage (Kaji et al., unpublished). The effect of thyroid hormone on enhancement of larval survival is under investigation and there may be some relationships between egg quality and thyroid hormone concentration in eggs of yellowfin tuna. The observed high mortality, which occurred around transition from the postflexion phase to juvenile, may be caused by quality of food and/or environmental factors. For example, too small a rearing tank may cause stress, increase encounters with the tank wall and also the incidence of cannibalistic behavior.

Histological evidence suggests that poor nutrition may be the primary cause of high mortalities. Changes in cellular condition in hepatocytes (Figs. 5C, 8B) and intestinal epithelium cells (Fig. 8D) were histologically observed at the critical phases. Margulies (1993) reported that histological appearance is a sensitive indicator of nutritional condition of

scombrid larvae and juveniles. The cellular changes observed in this study seemed to be similar to that of "degraded" criteria reported by Margulies (1993) and from other teleost larvae (Umeda and Ochiai, 1975; O'Connel, 1976; Watanabe, 1985; Theilacker, 1986) Thus, in the yellowfin tuna, malnourishment occurs at these critical phases, and may be the cause of high mortality.

The average growth rate of yellowfin tuna larvae and juveniles obtained in the present rearing was 0.68 mm/day, since hatching through Day 37. While that of the larval period between Day 0 and Day 30 was about 0.44 mm/day. This daily growth rate during the larval period is similar to those of wild yellowfin tuna larvae estimated by otolith daily increments (Lang et al., 1994) and of artificially reared larvae (Mori et al., 1971). Artificially reared Pacific bluefin tuna showed nearly the same growth rate in its early life stage (Kaji et al., 1996). The result obtained here could be regarded as average level of larval growth rate of *Thunnus*.

There were two body sizes at which the relative growth pattern changed (Fig. 3). The first one at 4 to 5 mm SL, observed in all body parts except TL and PAL, corresponds to the transition from yolk-sac larva to preflexion larva. The second size, about 11-13 mm SL, appears to coincide with the transition from postflexion to juvenile, or larva-juvenile transformation defined generally as metamorphosis. The change in the TL/SL ratio at about 7 mm SL corresponds to the phase of caudal fin formation. The continuous increase of relative growth value of PAL until fish reaching 14 mm SL suggests expansion of the body cavity expected to support high feeding rates related to high growth potential.

The digestive system of yellowfin tuna just after hatching was poorly developed, similar to most other marine fish hatched from small pelagic eggs (Tanaka, 1973). In the yellowfin tuna, there were two critical phases of digestive system development during the larval period. The first one, occurring around first feeding at Day 4, was characterized by establishment of a primitive digestive system (Fig. 5B) which enabled the larvae to intake exogenous nutrients. At first feeding, features of yellowfin tuna digestive system are similar to those of the altricial larvae of other marine fish species that have planktonic eggs (Tanaka, 1973). The second one, occurring around Day 16, at flexion phase, was characterized by differentiation of the functional stomach with gastric glands, a blind sac (Fig. 6D), and pyloric caecum, indicating establishment of the adult type digestive system (Tanaka, 1973).

Generally in marine fish larvae this developmental event occurs later, at the transitional phase from larvae to juveniles (Tanaka, 1973), however, precocious development has been observed in other scombrid fish such as Spanish mackerel (Tanaka *et al.*, 1996), skipjack tuna (Nishikawa, 1975), and Pacific bluefin tuna (Kaji *et al.*, 1996). The development of the digestive system has been measured by determining activity of digestive enzymes in a variety of fish species (Tanaka *et al.*, 1972; Segner *et al.*, 1993; Walford and Lam, 1993). These reports show that pepsin-like enzyme activity increased with the increase of number of gastric glands and of volume of gastric blind sac. An increase in pepsin like enzyme activity can be expected from the flexion phase in yellowfin tuna and has been observed in bluefin tuna (Miyashita *et al.*, 1998). Such precocious timing of digestive system differentiation suggests an adaptation allowing the early appearance of piscivorous food habits and high growth rate during the early life stages.

Predation capabilities and digestion in yellowfin tuna larvae appear to increase rapidly after the flexion phase. After flexion, yellowfin tuna larvae have a highly developed digestive system (Fig. 6D), and eyes and mouth become relatively large (Figs. 3, 6A). The same pattern of development was observed from artificially reared Pacific bluefin tuna (Kaji *et al.*, 1996), suggesting that postflexion tuna larvae begin to feed on fish larvae. Actually, *L. nebulosus* and *L. miniatus* larvae were supplied to the tank (Fig. 1) and the yellowfin tuna larvae fed on them. Changing the feeding schedule from invertebrate zooplankton to fish larvae associated with the functional development of the digestive system may seem to be reasonably effective in the rearing of *Thunnus* larvae.

3) Histological Observations on Development of the Swimbladder in Bluefin Tuna Larvae

Abstract

Histological study revealed that swimbladder anlage in bluefin tuna *Thunnus thynnus* differentiated in 2 day old yolk-sac larvae. The morphology of the swimbladder altered markedly during several days after the first appearance; the bladder turned to oval-shaped and enlarged, epithelial cells became thick forming the gas glands, and rete mirabile well developed connecting to the epithelial cells on the antero-ventral side of the bladder. On day 7, swimbladder inflation occurred. In the uninflated swimbladder, the epithelial cells formed folds and almost filled the entire lumen. The possibility of narrow window for swimbladder inflation were discussed.

Introduction

Inflation of the swimbladder has long been regarded as one of the most critical concerns of development in physoclistous fish larvae (Doroshov *et al.*, 1981; Blaxter, 1988). Many histological investigations have described that the developmental process of the swimbladder involves evagination of a germinal swimbladder transiently connected to the gut via a pneumatic duct followed by morphogenesis of swimbladder endothelium and dilation of its lumen. The pneumatic duct is subsequently regresses and the fish become physoclistous (Grizzle and Curd, 1978; Yamashita, 1982; Boulhic and Gabaudan, 1992; Makino *et al.*, 1995; Marty *et al.*, 1995; Goodsell *et al.*, 1996). Physoclistous species initially inflate their swimbladder when air, gulped from the surface, is transmitted via a pneumatic duct to the swimbladder (Steen, 1970). Failure of the swimbladder inflation, however, is the considerable obstacle to intensive seed production of many marine fish species, such as Australian bass *Macquaria nevemaculeata* (Battaglene and Talbot, 1990), gilthead bream *Sparus auratus* (Chatain and Ounais-Guschemann, 1990), striped bass *Morone saxatilis* (Bailey and Doroshov, 1995), and walleye *Stizostedion vitreum* (Colesante *et al.*, 1986). Fish that have uninflated swimbladders result in degeneration of the organ and larvae characterized by skeletal deformities (Kitajima *et al.*, 1994), decreased growth and low

survival rate (Chatain, 1987).

Although recent rearing trials of bluefin tuna *Thunnus thynnus*, which is one of the most important species for future stock enhancement and aquaculture, have successfully produced juveniles, survival rates have been still very low due to high mortalities during the early life stages. Preliminary histological study has revealed that most of the artificially reared larvae and juveniles of this species have uninflated swimbladders (Kaji *et al*. unpubl.). Therefore, information on the organogenesis of the swimbladder would be prerequisite for improving the rearing technique and increasing survival rate of this species. In the present study, the organogenesis of the swimbladder was examined with histological technique, as a part of studies on the early development of tunas.

Materials and Methods

Fish

Bluefin tuna larvae and juveniles were reared at the Japan Sea-Farming Association (JASFA), Amami station, located in Kakeroma island, Kagoshima, Japan in 1998. Fertilized eggs of bluefin tuna were obtained on 25 June 1998 by spontaneous spawning of broodstock maintained in a net pen. Samples examined in the present study were taken from a mass seed production rearing with a 50 m^3 tank (referred as 50t-6). Water temperatures ranged from 26.7-28.6 (mean: 27.8) ℃, and photoperiod was under natural conditions. Slight aeration was provided by airstones. Rotifers were fed from Day 3, and *Artemia* nauplii, fish larvae (striped beakperch *Oplegnathus fasciatus*, bluefin tuna, and spangled emperor *Lethrinus nebulosus*), frozen fish and minced fish meat were fed with development and growth. A microalga, *Nannochloropsis oculata*, was added to the rearing tank from Day 2 to Day 24. Information on the development and growth were described in chapter 3.

Sampling and histological procedure

Fish were sampled daily or every two days from Day 0 (hatching day) to Day 26, and on Day 31. Some of the fish were anesthetized with MS-222, and standard length was measured. Then the anesthetized fish were fixed in Bouin's solution and preserved in 70% ethanol. They were dehydrated through graded ethanol, cleared in xylen, and embedded in

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Parahisto (Nakaraitesque, Japan). Serial sections of 4 μm thickness were made at sagittal or transverse plane and mounted on slides. The sections were stained with hematoxylin and eosin. They were observed under a microscope (AX, Olympus, Japan). Developmental phases after Kendall, Jr. *et al*. (1984) were used in this study.

Results

Yolk-sac larva

In 0-day old yolk-sac larvae at just after hatching, the digestive tract was a simple, straight tube without swimbladder anlage. In 1-day old yolk-sac larvae, although the yolk-sac absorption and development of the digestive tract progressed, swimbladder anlage was not observed. In 2-day old yolk-sac larvae, swimbladder anlage which had a small lumen evaginated upward from the posterior dorsal wall of the rudimentary stomach (Fig. 1A). The epithelial cuboidal cell height of the anlage was 6-8 μm both on the dorsal and ventral side, and the cells appeared to be similar to that of the digestive tract.

Preflexion larva

Three days after hatching, larvae initiated feeding on rotifers. The swimbladder and its cavity were expanded. The bladder turned to be oval-shaped, about 48 μm in heights and 90 μm in length at body axis, and its epithelium was thin (about 2 μm) on the dorsal side and thick (about 10 μm) on the ventral side. The cytoplasm of these cells were stained slightly with eosin (Fig. 1B).

In 4-day old larvae, the swimbladder epithelial cells were densely stained with eosin, thus they appeared to be red. Capillaries including blood corpuscles were observed on the ventral side of the bladder. The pneumatic duct exited from the posterior surface of the swimbladder, extended to join the proximal posterior dorsal wall of the rudimentary stomach.

In 5-day old larvae, the plump epithelial cell height of swimbladder increased forming the folds, except for dorsal wall which was composed by very thin (1 μm) epithelium. The lumen of the bladder was more expanded than 4-day old larvae. The pneumatic duct was well established. The lumen of the swimbladder slightly contained mucus-like substance which would be secreted from the thick epithelial cells.

In 6-day old larvae, the epithelial cells of swimbladder are enlarged markedly. Consequently, the lumen of the bladder was occupied by folds composed with the plump epithelial cells in all the fish examined (Fig. 1C). The cytoplasm of these cells were stained strongly with eosin and some vacuoles were observed in the cells. The rete mirabile well developed and attached to the epithelial cells on the antero-ventral side of the bladder. The cells connected by the rete mirabile contained blood corpuscle.

In 7-day old larvae, some fish had completely inflated swimbladders (Fig. 1D). In the expanded bladder, most of the epithelial cells were squamous, except for a small crescent of low columnar epithelium that remained in the anteroventral region, forming the gas gland (about 20 μm in heights). On the other hand, some fish had uninflated swimbladder in which the lumen was occupied by plump epithelial cells.

On Day 8 to 10, larvae had the inflated swimbladder which was divided into two sections; the anterior half was occupied with plump gas glands forming complicated folds, and posterior half was expanded bladder lumen (Fig. 1E). The pneumatic duct in the inflated swimbladder fish seemed to be regressed.

The 10-day old fish with uninflated swimbladder showed heavy hypertrophy of gas glands. Gastric glands differentiated on the dorsal wall of the stomach.

Flexion larvae

On Days 12 and 14, two types of inflated swimbladder were observed. One was oval shaped bladder with fully expanded lumen. The other was a type with two separated parts, as shown in larvae of Day 8 to 10. The narrow lumen of the uninflated swimbladder contained eosinophilic mucus-like substance. The pneumatic duct was patent from its ostium on the swimbladder to mid part of the duct, but the duct of the digestive tract side was not visible in both fish with and without inflated bladder.

Postflexion larva

All the fish examined had well inflated swimbladder (Fig. 1F). The posterior edges of the bladder protruded, which seemed to be the residual tissues of the pneumatic duct. However, the other part of the duct is was not seen.
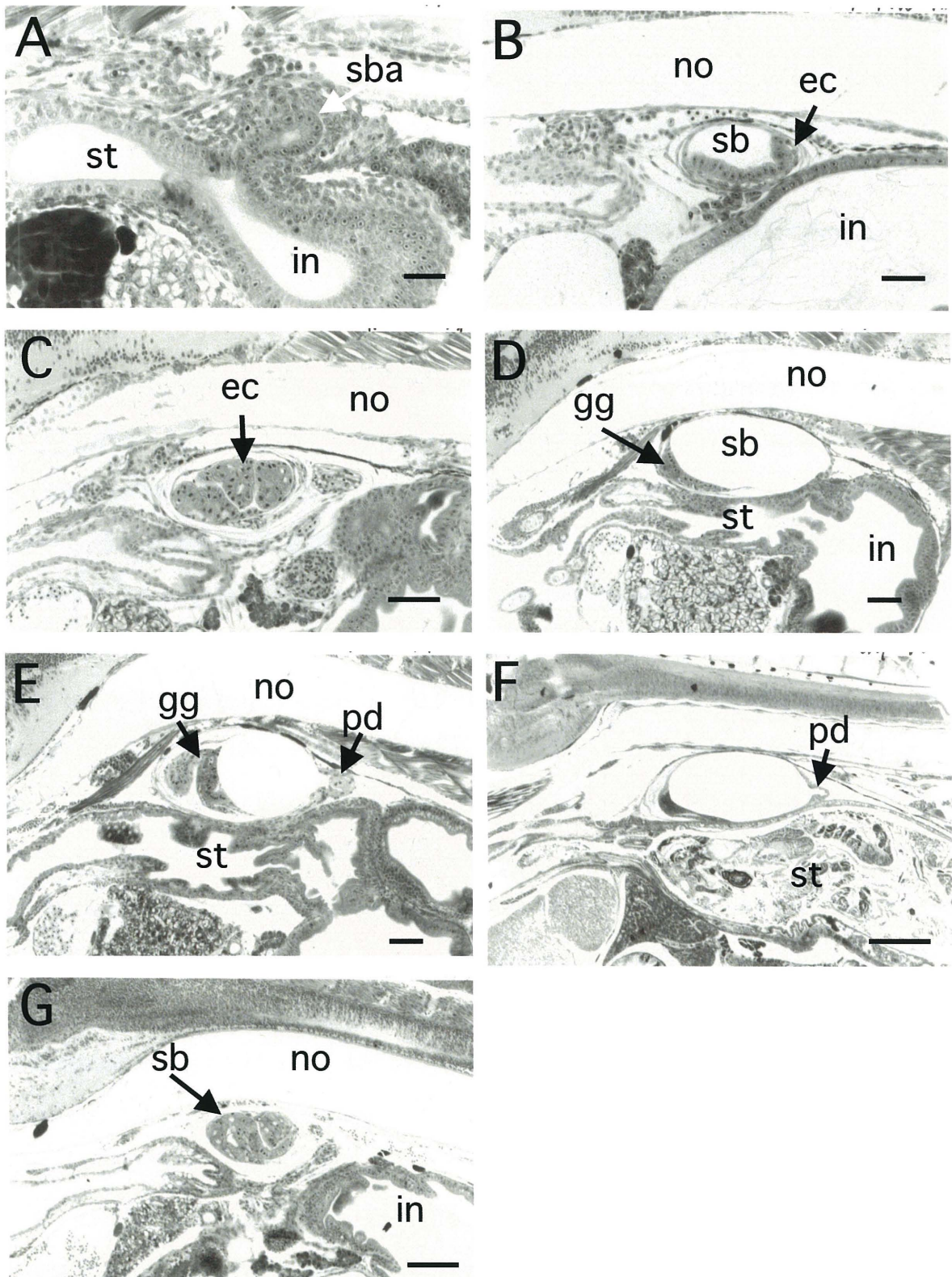
Fig. 1. Photomicrographs of longitudinal sections of the swimbladder in bluefin tuna larvae.
(A) 2 day-old yolk-sac larva. in; intestine, sba; swimbladder anlage, st; stomach. Scale bar=20 μm. (B) 3 day-old larva at first feeding stage. ec; epithelial cells, no; notochord, sb; swimbladder. Scale bar= 30 μm. (C) 6 day-old larva. Scale bar= 50 μm. (D) 7 day-old larva. gg; gas gland. Scale bar= 50 μm. (E) 9 day-old larva. pd; pneumatic duct. Scale bar= 50 μm. (F) 18 day old larva. Scale bar= 300 μm. (G) 12 day-old larva. Uninflated swimbladder with abnormally hypertrophic epithelium. Scale bar= 100 μm.

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Juvenile

The swimbladder was observed on the dorsal side of the abdominal cavity, and expanded markedly at body axis. The epithelium was very thin, and the pneumatic duct was not observed.

Discussion

Organogenesis of the swimbladder has been studied in some physoclistous fish species (Grizzle and Curd, 1978; Yamashita, 1982; Makino *et al.*, 1995; Boulhic and Gabaudan, 1992; Marty *et al.*, 1995; Goodsell *et al.*, 1996). These studies demonstrated that the primordial swimbladder differentiates during the yolk-sac period, a few days after hatching. In the present study, the swimbladder anlage of bluefin tuna also appeared at the yolk-sac period, 2 days after hatching. The organ initially evaginated from the posterior dorsal wall of the rudimentary stomach in the present species (Fig. 1A), as is in the case of Japanese seabass *Lateolabrax japonicus* (Makino *et al.*, 1995), Dover sole *Solea solea* (Boulhic and Gabaudan, 1992), and Yellowfin tuna *T. albacares* (Kaji *et al.*, unpubl.). These results, however, contradict the observations made by Yamashita (1982) and Goodsell *et al.* (1996) who mentioned that the swimbladder of red sea bream *Pagrus major* and striped trumpeter *Latris lineata* protruded from the postero-dorsal area of the oesophagus. When considered the location and appearance of the digestive tract in their photomicrographs, the author could rectify the above mentioned observations; swimbladder anlage differentiated from dorsal part of the rudimentary stomach. Thus it could be general pattern in marine fish larvae hatched from pelagic eggs.

The differentiation of gas glands and subsequent initial gas filling are the most important event in functioning of the swimbladder. In the present study, inflation of the swimbladder was first observed on Day 7 (Fig. 1D), corresponding to that of the fish reared in 1994 (Kaji *et al.*, unpubl.). The rapid developments during several days after appearance of swimbladder anlage, such as differentiation of gas gland, pneumatic duct, rete mirabile, and appearance of blood corpuscle in the rete mirabile (Figs. 1A, B, C), seemed to be similar to those of the other marine physoclistous fish larvae, which are associated with preparation for initial gas filling into the bladder. Therefore, the initial gas filling followed by

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swimbladder inflation of bluefin tuna larvae would occur around Day 7, several days after initiation of feeding.

Fish with uninflated swimbladder were observed in the present study (Fig. 1G). Several abiotic factors influenced failure of larval swimbladder inflation have been suggested in the other fish species; lack of access to the water-air interface due to turbulence, oil film, and obstruction (Doroshov and Cornacchia, 1979; Hoss and Phonlor 1984; Kitajima *et al.*, 1984), or water temperature (Bailey and Doroshov, 1995). Although various rearing conditions were tried for bluefin tuna at JASFA Amami Station, gentle aeration introduced to the present rearing. Thus the water current did not prevent the larvae from reaching the water surface. Improvements in rearing conditions such as skimmer and water quality (dissolved gasses), as well as the use of low light intensities, should be tested to solve the problem of uninflated swimbladder which generally cause future problems (e.g. lordotic deformities).

Many studies have noted a narrow window of opportunity for initial swimbladder inflation in physoclistous fish species; i. e. Australian bass, Day 6 to 11 (Battaglen and Talbot, 1990) and striped bass, Day 5 to 7 (Doroshov and Cornacchia, 1979). These duration of the window would be attributed to several factors. Degeneration of the pneumatic duct would be primary closure of the window, since the duct is passage for air bubbles. Although the exact time of degeneration of the pneumatic duct was not clarified in the present study, the duct seemed to be atrophied in the flexion larvae, and the larvae with fully inflated swimbladder were observed in postflexion phase (Fig. 1F). These observations suggest that bluefin tuna larvae would have a narrow window of opportunity for swimbladder inflation.

Another possible closure of the window would be differentiation of the gut structure. Marty *et al.* (1995) have inferred that surfactant-like secretions from the common bile duct affected fragmentation of large ingested air bubbles for transfer into the relatively small-diameter pneumatic duct in walleye larvae. After differentiation of the pyloric sphincter, however, the common bile duct in the intestine is separated from the pneumatic duct in the dorsal wall of the stomach, thus the larvae lose the chance of initial inflation of their swimbladder. Assuming that this mechanism is true also in bluefin tuna larvae, they would have very narrow window since the stomach of tuna larvae differentiates at earlier, flexion phase, than the other marine fish larvae (Kaji *et al.*, 1996; Miyashita *et al.*, 1998). The precocious development of the stomach might be a possible factor causing high proportion of

uninflated swimbladder.

It has recently been that wild-caught scombrid larvae, including bluefin tuna, display a diel rhythm of night-inflation and day-deflation of the swimbladder (Kaji *et al*., unpubl.). All the fish examined in the present study were collected in daytime. Day and night changes in swimbladder volume and/or light-intensity related inflation should be examined for further understanding the larval development and for improvement the rearing techniques.

Chapter 2: Physiological Development of Bluefin and Yellowfin Tuna Larvae and Juveniles

1) Development of Growth Hormone Cells in the Pituitary of Yellowfin Tuna Larvae and Early Juveniles

Abstract

Development of the pituitary and growth hormone (GH) cells of laboratory reared yellowfin tuna *Thunnus albacares* larvae and early juveniles were examined by histological and immunohistochemical procedures. The pituitary first appeared in the ventral edge of diencephalon of the brain on Day 2, and suspended from the brain on Day 16. The growth hormone (GH) immunoreactive cells were first detected on the day of first feeding, Day 4. Percent GH, defined as ratio of GH cell-mass volume to pituitary volume, was very high during the first 3 days after initiation of feeding. Percent GH rapidly decreased, and remained at the lowest level throughout the flexion phase. The ratio began to increase from the postflexion phase to the early juvenile stage. The %GH of yellowfin tuna was relatively higher than those of other marine fish species previously examined. These features suggest that the yellowfin tuna has a relatively high growth potential during the early life stages.

Introduction

Recently bluefin tuna *Thunnus thynnus* (Kaji *et al.*, 1996; Miyashita *et al.*, 1998) and yellowfin tuna *T. albacares* have received interest as candidates for stock enhancement and aquaculture in Japan. These species have a unique early life history which includes "scombriform-type metamorphosis" with characteristic external morphology and precocious digestive system development (Kaji *et al.*, 1996; Tanaka *et al.*, 1996; Miyashita *et al.*, 1998). Based on rearing experiments tunas appear to possess a high growth potential starting in the early life stages. Rearing of yellowfin tuna larvae preceded that of the Pacific bluefin tuna; however, survival rates of both species are low in hatcheries due to high mortality that occurs during the larval stages (Kaji *et al.*, 1996; Miyashita *et al.*, 1998). Basic biological studies of tunas should be conducted in the laboratory for establishment of seed production techniques

as well as improving our understanding of the early life history of tuna in the sea. Hormones are considered to be of primary importance in regulating development and growth of larval fish (Brown and Bern, 1989). Although development of the endocrine system has been relatively well documented in salmonid fish (Naito *et al.*, 1993; Leatherland and Barrett, 1993), tilapia *Oreochromis mossambicus* (Ayson *et al.*, 1994), and Japanese flounder *Paralichthys olivaceus* (Inui and Miwa, 1985; Miwa and Inui, 1987a, 1987b; Inui *et al.*, 1995; Tanaka *et al.*, 1995; Hiroi *et al.*, 1997), there is no study on the development of this system in tunas.

The main purpose of this article is to examine development of the pituitary and its growth hormone cells during the early ontogeny of yellowfin tuna as a part of studies in the early development of tunas.

Materials and Methods

Fish

Rearing experiment of yellowfin tuna larvae was conducted at the Japan Sea-Farming Association (JASFA) Yaeyama Station, Ishigaki Island (24° 27' N, 124° 17' E), Okinawa, Japan in 1996. Fertilized eggs of yellowfin tuna were obtained on 21, May 1996 from spontaneous spawning of the broodstock maintained in a net pen. Transparent 1000 *l* cylindrical tank was used for rearing experiment from Day 0 (hatching day) to 30, then juveniles were transferred to a cylindrical net cage (2.5 m in diameter, 1.5 m in depth) maintained in a 60 m^3 tank. Initial stocking density was 41 larvae per *l* (41000 fishes in the 1000 *l* tank). Seawater sterilized with ultraviolet light was used for the rearing. Slight aeration was provided with airstones, and daily replacement of rearing sea water was 0-250%. The natural seasonal photoperiod was maintained. Water temperature ranged from 22.9 to 28.3 (mean 26.4) ℃ and pH of rearing water ranged from 7.77 to 8.17 (mean 8.00). Fish were fed on rotifers from Day 4 and on *Artemia* nauplii, fish larvae (*Lethrinus nebulosus* and *L. miniatus*), frozen fish and minced fish meat with development and growth (Fig. 1). A microalga, *Nannochloropsis oculata*, was added to the rearing tank from Day 4 to 31. Survival of fish was estimated by random water-mass sampling using a fixed volume plastic pipe at night on Day 0, 3, and 5, and thereafter it was estimated from number of dead

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fish and the final harvest.

Fish were sampled daily or every 2 days from Day 0 to Day 20, and on Days 25, 30

and 37. Some of the fish were anesthetized with MS-222, and standard length (SL) were
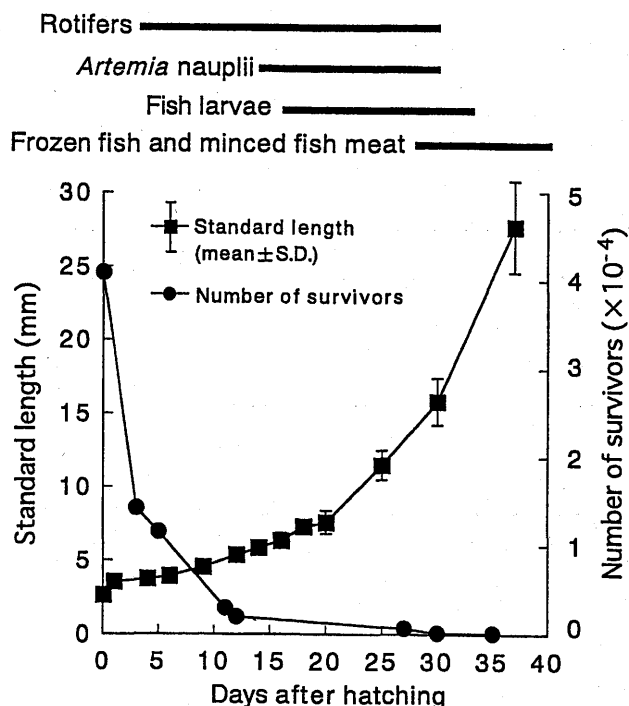
measured.

Rotifers ━━━━━━━━━━━
  Artemia nauplii ━━━━━━
    Fish larvae ━━━━━━━
Frozen fish and minced fish meat ━━━━━

Fig.1. Standard length (mean ± S. D.) increase of fish and number of survivors of yellowfin tuna larvae and juveniles reared at the JASFA Yaeyama Station in 1996.
Feeding schedule is illustrated at the top of the figure.

Histology and immunohistochemistry

Fish were fixed in Bouin's solution for 24 hours and preserved in 80% ethanol. These

were dehydrated through graded ethanol and embedded in Parahisto (Nakalaitesque, Japan).

Serial sections on sagittal plane were made at 5 μm thickness for histological observation,

and at 4 μm thickness for immunohistochemical preparates. The histological sections were

stained with hematoxylin and eosin (HE), and development of the pituitary was observed

under a light microscope.

Growth hormone (GH) in the pituitary was immunohistochemically detected using

the strepto avidin-biotin-peroxidase complex method with commercial reagents (DAKO,

Denmark). The sections were deparaffinized, rehydrated and incubated sequentially with: (1)

3% H_2O_2 for 10 min, (2) normal goat serum diluted 1:20 with 0.01 M phosphate-buffered

saline (PBS, pH 7.2) for 30 min, (3) rabbit anti-tilapia GH serum (Ayson *et al.*, 1993) diluted

1:8000 for 20 hr at 4 ℃, (4) biotinylated goat anti-rabbit immunoglobulins diluted 1:400 for

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30 min, (5) strept ABComplex/HRP for 30 min, (6) 0.08% 3, 3'-diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 for 5 min. The sections were then dehydrated, cleared and mounted with Entellan neu (Merck, Germany). Specificity of anti-tilapia GH serum was checked by the localization of immunoreactive cells at proximal pars distalis in yellowfin tuna pituitary, where no immunoreaction was detected when anti-tilapia prolactin serum was used.

The outlines of GH cell-mass and the whole pituitary was traced on paper with a camera lucida. The images were digitized with a scanner (Canon, Japan) and areas were measured on an Apple Macintosh computer using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov./nih-image/). Total volumes of GH cell-mass and of the pituitary were calculated from the areas of the each section and the thickness. The GH cell-mass volume to pituitary volume (%GH) was calculated as a relative index of GH production (Kimura and Tanaka, 1991; Tanaka *et al.*, 1995; Hiroi *et al.*, 1997).

Developmental phases proposed by Kendall, Jr. *et al.* (1984) were used in the present study.

Results

Fish growth

Newly hatched larvae were 2.65 ± 0.07 mm in SL (mean ± S. D.), and grew to 27.68 ± 3.11 mm in SL on Day 37 (Fig. 1). The shift from yolk-sac phase to preflexion phase occurred on Day 4, to flexion on Day 11, and to postflexion on day 18. The larvae transformed to juveniles on Day 30.

Pituitary development

The pituitary first appeared on Day 2, during yolk-sac phase, and was completely embedded in the bottom of the diencephalon (Figs. 2A, B). The pituitary began to protrude from the hypothalamus at the end of preflexion phase (Fig. 2C), and was suspended from the brain at the end of flexion phase (Figs. 2D, E).
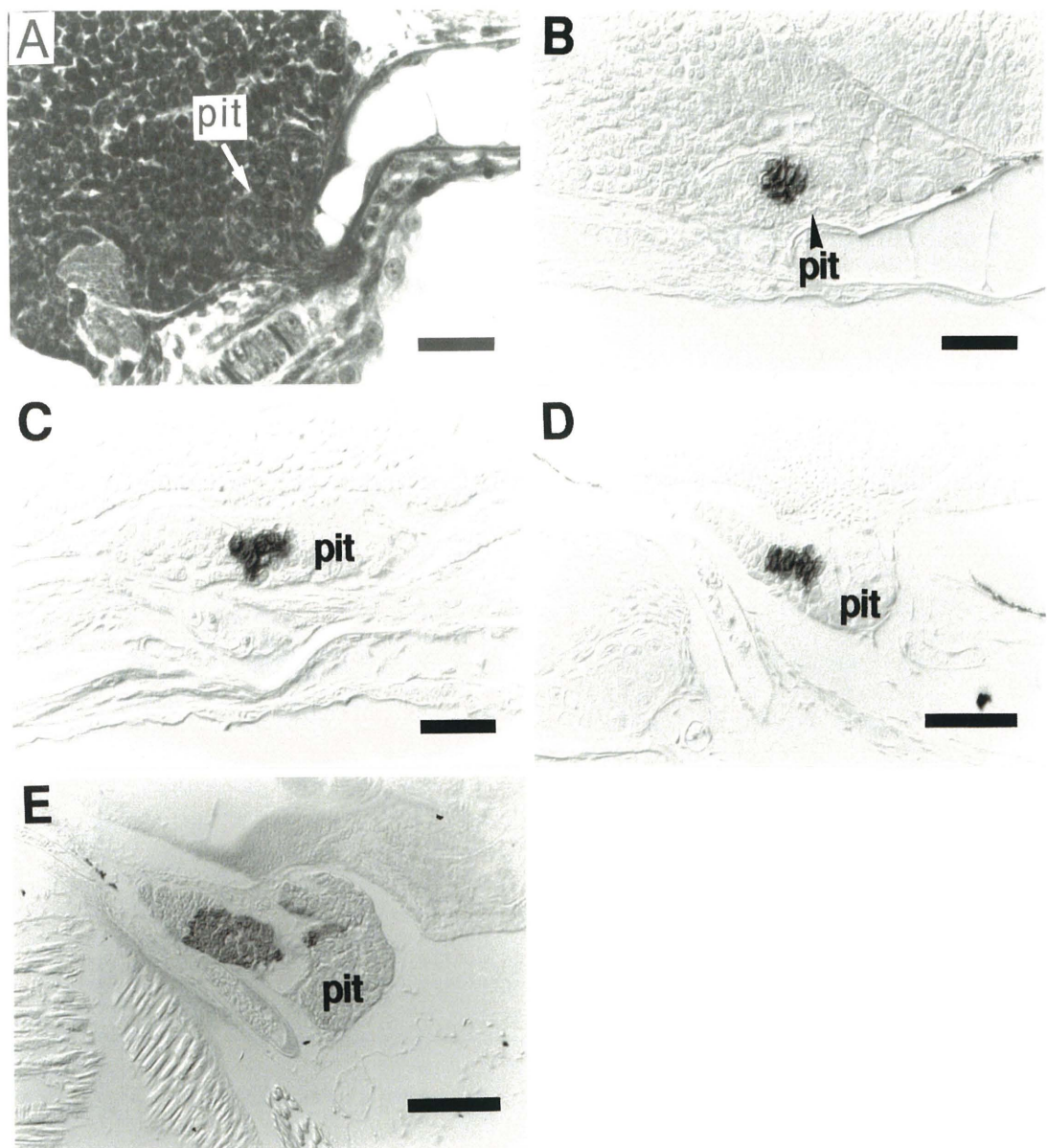
Fig. 2. Photomicrographs of sagittal sections of the pituitary (pit) of yellowfin tuna larvae and early juveniles.
(A) yolk-sac larva (2 days old). Scale bar=20 μm. (B) preflexion larva at first feeding (4 days old). Scale bar=20 μm. (C) flexion larva (14 days old). Scale bar=20 μm. (D) postflexion larva (18 days old). Scale bar=40 μm. (E) early juvenile (30 days old). Scale bar=100 μm. A was treated by HE stain and B-E by sABC for growth hormone.

GH cell development

GH-immunoreactive cells were not observed in yolk-sac larvae, and were first detected on Day 4, just at initiation of feeding (Fig. 2B). The cells immunoreactive to GH antiserum were detected in the postero-dorsal area of the pars distalis of the pituitary. Volumes of the pituitary and GH cell-mass increased exponentially with growth (Fig. 3). Figure 4 shows the developmental change in %GH with increasing age. The ratio was as high as 25% during the first 3 days after GH was first detected. There was a sudden drop on Day 7, and the ratio decreased gradually from Day 9 to Day 16, when %GH fell to the lowest level of about 14%. Percent GH increased on Day 18, and was rising 30 days after hatching when the highest value, ca. 30%, was observed. In terms of the standard length, %GH decreased from about 4 mm SL until about 6 mm SL when it reached the lowest level. The GH ratio increased markedly from 7 mm to 16 mm SL.
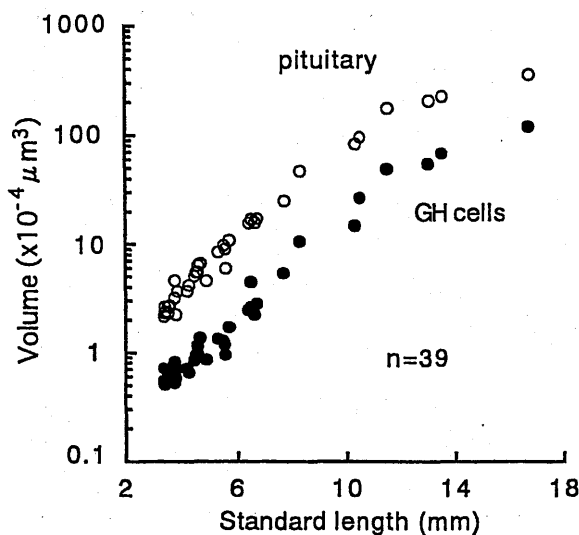
Fig. 3. Pituitary and GH cell volumes of yellowfin tuna.
Volumes were calculated from 4 μm serial immunohistochemical sections by integration of areas.
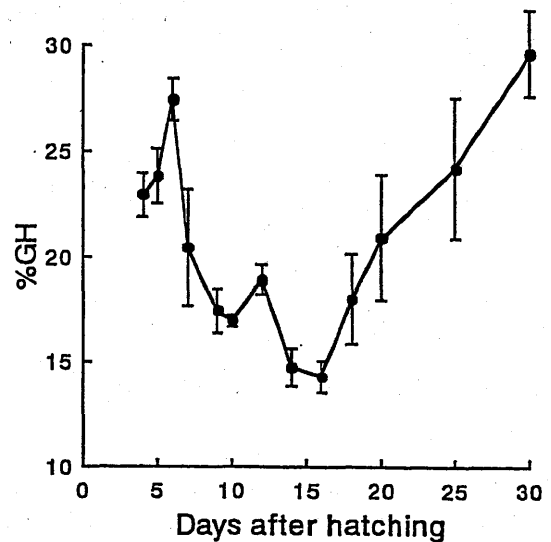
Fig. 4. Developmental changes in GH production potential expressed as percentage of GH cell volume to the pituitary volume (%GH) in terms of days after hatching of yellowfin tuna.
Each point indicates mean ± SE (three individuals). The developmental changes can be divided into 3 phases: first phase of increasing between Day 4 and 6, second phase of decreasing between Day 6 and 16, and third phase of re-increasing between Day 16 and 30.

Discussion

The developmental change in growth hormone production, as evaluated by GH ratio (%GH), of laboratory reared yellowfin tuna larvae could be divided into three phases (Fig. 4). Initially for the yolk-sac larvae there are no GH immunoreactive cells in the pituitary. The first phase occurred during a three days period after initiation of feeding with the first appearance of GH immunoreactive cells in the pituitary. During this phase high %GH, ca 25%, was observed. During the second phase, that occurred during the preflexion and flexion larval phases, a decrease of %GH was observed. During the third phase corresponding to the postflexion phase and early juvenile stage %GH increased markedly. Nearly the same pattern was obtained from the author's preliminary observation of the ontogenetic change in %GH of the Pacific bluefin tuna,* suggesting that this V-shaped ontogenetic pattern of growth hormone production is common to tuna larvae.

Tanaka *et al*. (1995) made a comparative investigation of the histological development of the major endocrine organs and immunohistochemical detection of GH in the pituitary in a variety of teleost species. Timing of the first appearances of both pituitary and GH immunoreactive cells in the yellowfin tuna larvae (Figs. 2A, B) was similar to other fishes hatched from small pelagic eggs (Tanaka *et al*., 1995). The coincidence of differentiation of pituitary and GH immunoreactive cells with eye pigmentation, as well as other pituitary hormones for example prolactin (PRL) immunoreactive cells (Tagawa and Kimura, 1991), suggests that functions of the pituitary may play an important role in the transition from yolk-sac larva to larva and/or from the endogenous to exogenous nutrient sources.

In Japanese flounder, %GH continuously increased from first feeding (ca. 9%) through post-metamorphosis (ca. 16%) (Tanaka *et al*., 1995). The lowest level of %GH observed in yellowfin tuna was nearly equal to that of the highest value of %GH of Japanese flounder. Apparently higher %GH throughout the early life stages of the present species would be a physiological background of high growth potential in their larval period, since GH has growth-promoting effects in teleosts (Donaldson *et al*., 1979), through insulin-like growth factor (IGF) I (Duan and Hirano, 1990).

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*T. Kaji *et al*.: Abst. Metg. Japan Soc. Fish. Sci., April 1996, p. 92 (in Japanese).

The high %GH during the first GH production phase suggests that yellowfin tuna larvae have a high somatic growth potential during the first feeding stage, however; larval growth at this stage was not high under laboratory rearing conditions (Fig. 1). The daily growth rate estimated by otolith daily growth increments for Atlantic yellowfin tuna collected from the Gulf of Mexico appeared to be similar to that of the present study, ca 0.47 mm/day during the first 14 days (Lang *et al*., 1994), suggesting that the early larval growth of yellowfin tuna is similar to other marine species. In contrast somatic growth during the later larval period and early juvenile stage was accelerated in association with the rapid increase in GH production as shown in Fig. 5. Although there is no information on the developmental profiles of IGF I in early life stages of fishes, it must be essentially needed in further understanding this apparent correlation.
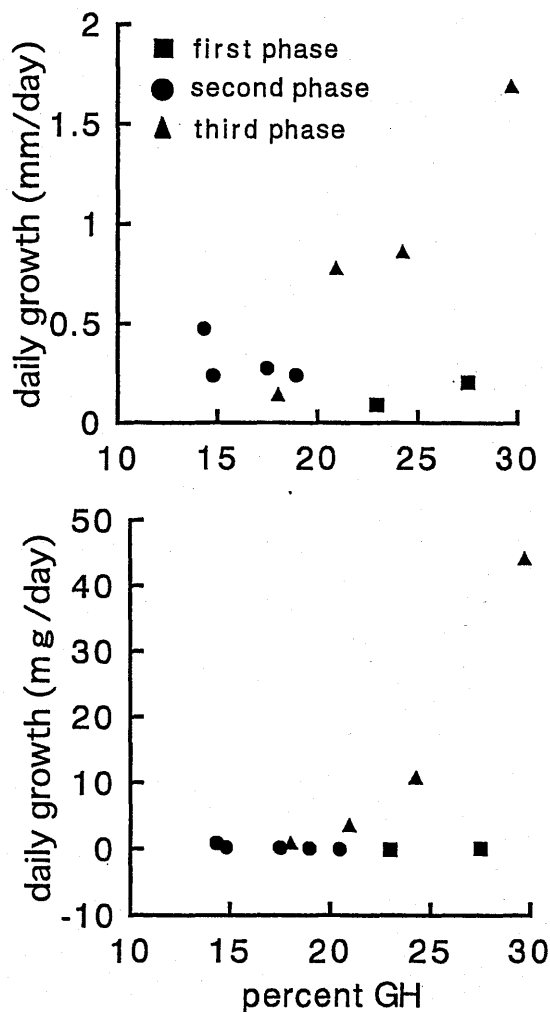

Fig. 5. Relationships between percent GH and daily growth in terms of length (upper) and weight (lower) of yellowfin tuna larvae and early juveniles.
Three phases in the upper panel are referred to Fig. 4.

Although the %GH decreased and remained relatively low level in the second phase (Fig. 4), the absolute volume of the GH cells and the pituitary continuously increased (Fig.

3). A possible explanation for this phenomena is that the other important pituitary hormone producing cells such as prolactin may increase rapidly in volume during this period, resulting in relatively low values in terms of %GH. Further studies of other hormones in the pituitary as well as the other endocrine organs are necessary to further understanding of endocrine system development in tunas.

Kaji *et al.* (1999b) demonstrated that the functional digestive system of yellowfin tuna larvae differentiated during the flexion phase while rapid elaboration of the digestive system occurred during the postflexion and early juvenile stages. The expansion of the digestive system coincides with the increase in %GH we observed and would be expected to support the shift to piscivory and rapid somatic growth observed during this period. These biological features of *Thunnus* must be essential to their "large prey-fast growth" (Hunter, 1981) survival strategy.

2) Developmental Changes in Digestive Enzyme Activities of Bluefin Tuna Larvae and Juveniles

Abstract

Activities of trypsin were measured and development of the gastric glands were observed in hatchery-reared bluefin tuna *Thunnus thynnus* from hatching beyond metamorphosis in 1998. All measurements and observations were conducted individually. There appeared to be a close relationship between digestive physiological development and the morphological and/or histological development. Activities of trypsin per ind. were low during yolk-sac stage and increased on Day 3, first feeding day. The activity remained at a low level during several days after initiation of feeding, and then increased with age. Specific activity (U/mg body weight) exhibited three peaks, Day 3, 14, and 25. These peaks coincided with first feeding, flexion phase, and transformation phase to juvenile, respectively. Pepsinogen synthesis in the stomach started on Day 10, and increased with growth. These results suggest that the digestive capability increase markedly from the flexion phase, supporting the shift to piscivorous food habit and rapid somatic growth. A phase of several days after initiation of feeding was regarded as a "critical period" of bluefin tuna larvae from present study.

Introduction

Bluefin tuna *Thunnus thynnus* has received much attentions in recent years, as one of the most important target species for aquaculture and stock enhancement, due mainly to decreasing stock as well as its considerable economic value. Fundamental information about developmental biology under laboratory-rearing conditions have been published intensively for last several years (Kaji *et al*., 1996, 1999c; Miyashita *et al*., 1998, 1999; Takii *et al*., 1997). One of the most conspicuous developmental characteristics of the tuna larvae is precocious development of the digestive system and subsequent drastic quantitative development of the system. These features would be generally observed in scombrid fish larvae (Tanaka *et al*., 1996a), and would relate closely to early appearance of piscivorous food habit and their high growth potential.

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Generally, quality and quantity of food, as well as suitable feeding schedule, have been fundamentally important for establishment of the larval rearing technique. Although some hatcheries have produced juveniles of bluefin tuna, survival rates are still very low. Therefore, more detailed information on its ontogeny, such as developmental change in digestive enzyme activities, would be necessary for establishing appropriate feeding schedule, as well as for our further understanding the scombriform-type metamorphosis.

Digestive physiology of larvae appeared to be one of the most important aspects directly influencing development and growth of fish (Govoni *et al.*, 1986). The pancreas and stomach are considered the major organs of digestive enzyme production during the larval period. The pancreatic enzymes appear to be important for digestion, particularly larval fish, because the functional stomach is not yet developed in the larval period (Tanaka, 1973). Although many researches have been devoted to the digestive physiology of fish larvae (e. g. Tanaka *et al.*, 1972; Baragi and Lovell, 1986; Govoni *et al*, 1986; Cousin *et al.*, 1987; Segner *et al.*, 1993; Walford and Lam, 1993; Tanaka *et al.*, 1996b), information on the digestive enzyme activities of bluefin tuna larvae is restricted to a paper done by Miyashita *et al.* (1998). Digestive enzyme activities of larval fish have been measured on pooled samples in this study due to the difficulty of measuring low enzyme levels. However, this method can not be applicable to some fish with difficulty in rearing because many fish are required as samples during larval period. In recent years, a highly sensitive fluorescence technique has been used to quantify tryptic enzyme activity of individual fish larvae (Ueberschär, 1988; Ueberschär *et al.*, 1992; Oozeki and Bailey, 1995).

In the present study, the author measured activities of trypsin-like protease individually from hatching beyond metamorphosis in order to examine developmental changes of digestive ability. In addition, the development of the gastric gland was observed by immunohistochemistry to trace synthesis of pepsinogen in the stomach.

Materials and Methods

Fish

The fish examined in the preset study were reared at the Japan Sea-Farming Association (JASFA) Amami Station, located in Kakeroma island, Kagoshima Japan, in

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1998. Samples were taken from two concurrent rearing trials for mass seed production, referred as 50t-5 and 50t-6 respectively. Details on the rearing conditions and on larval growth and development were described in chapter 3.

Sampling

Samples for trypsin-like enzyme assay were taken every day or every other day from hatching (Day 0) to Day 20, thereafter taken on Day 24 and 31, from the 50t-6. In 50t-5 rearing, fish were taken every day or every other day from Day 1 to 14, then taken on Day 17, 19 and 25. Sampling was performed in daytime. Fish were individually sampled, rinsed, pipetted into Eppendorf micro tubes. Then the fish were immediately frozen at -80 °C and stored at -30 °C until later analysis.

Samples for immunohistochemistry were taken from 50t-6 rearing. Sampled fish were anesthetized with MS-222, and fixed in 10% formalin in 10 mM Tris-buffered saline (TBS; pH 7.5) for 24 h. Fixed samples were washed and preserved in 70% ethanol. Then samples were dehydrated through a graded ethanol series, embedded in parahisto (Nakaraitesque, Japan) and cut into serial sections at 5 μm thickness.

Measurement of tryptic activity

The tryptic enzyme activity measurements were performed according to the fluorescence technique described by Ueberschär (1988) with some modifications. Individual larvae were homogenized in 250 μl 1/15 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.0. 250 μl of the substrate, N α -benzoyl-L-arginin-methyl-coumarinylamide (Sigma), 0.20 mM, were added to 50 μl of the homogenate in a cuvette and mixed well. The substrate was dissolved in a TRIS-HCl buffer, 0.1 M, pH 8.00.

The relative fluorescence enhancement (excitation 380 nm, emission 440 nm) was recorded every 2 min over a maximum period of 10 min, using a Shimadzu RF-5300 PC. The trypsin activities were expressed as U (units), an increase of emission min^{-1}. Specific activity of trypsin (U/body weight in mg) was calculated from the means of U at each day and mean body weight measured in specimens which were taken from the same day and preserved in 10% formaldehyde from Day 0 until 20, while the fish that were older than Day 20 were weighed individually before the homogenization, and specific activities were calculated

individually.

Immunohistochemistry

The pepsinogen in the gastric glands were immunohistochemically detected using anti-Japanese flounder pepsinogen antibody (Kurokawa *et al.*, unpubl.), as described by Kurokawa and Suzuki (1995). In brief, after paraffin was removed with xylene, sections were incubated with the antibody (1:2000 dilution) for 1 h at room temperature, and developed using a Histofine SAB-PO kit (Nichrei, Japan). Reactions to the antibody developed as a brown-colored precipitate in the sections.

Results

The growth processes of bluefin tuna larvae and juveniles were described in chapter 3.

Trypsin activity (U/ind.) was detected in the youngest larvae of both rearing trials, and increased with age (Fig. 1). Average activity was less than 5.0 U/ind. during the yolk-sac stage and then increased to about 10 U/ind. on Day 3. Although the patterns were different between the two rearings, the activities remained around constant value during several days after initiation of feeding (Fig. 1). The activity increased markedly from 12, then reached around 44000 on Day 25 of 50t-5, and around 22000 on Day 31 of 50t-6, respectively.

Specific activity of trypsin (U/body weight in mg) exhibited nearly the same developmental pattern, characterized by three peaks, in both rearing trials (Fig. 2). The activity increased during the yolk-sac stage and reached the first peak (about 40) on Day 3 when the first feeding was observed in both rearing trials. Then it decreased or remained constant value from Day 4 to Day 10, corresponded to preflexion phase. The specific activity increased on Day 10, and attained to the second peak on Day 14 in both rearing trials, at which about 54 U/mg in 50t-5 and about 43 in 50t-6 were observed. Thenafter the activity dropped to low level, below 20 U/mg during the postflexion phase. The value increased then markedly from Day 20, corresponding to the transformation to juveniles, and reached the highest value, about 80 U/mg, around Day 25.
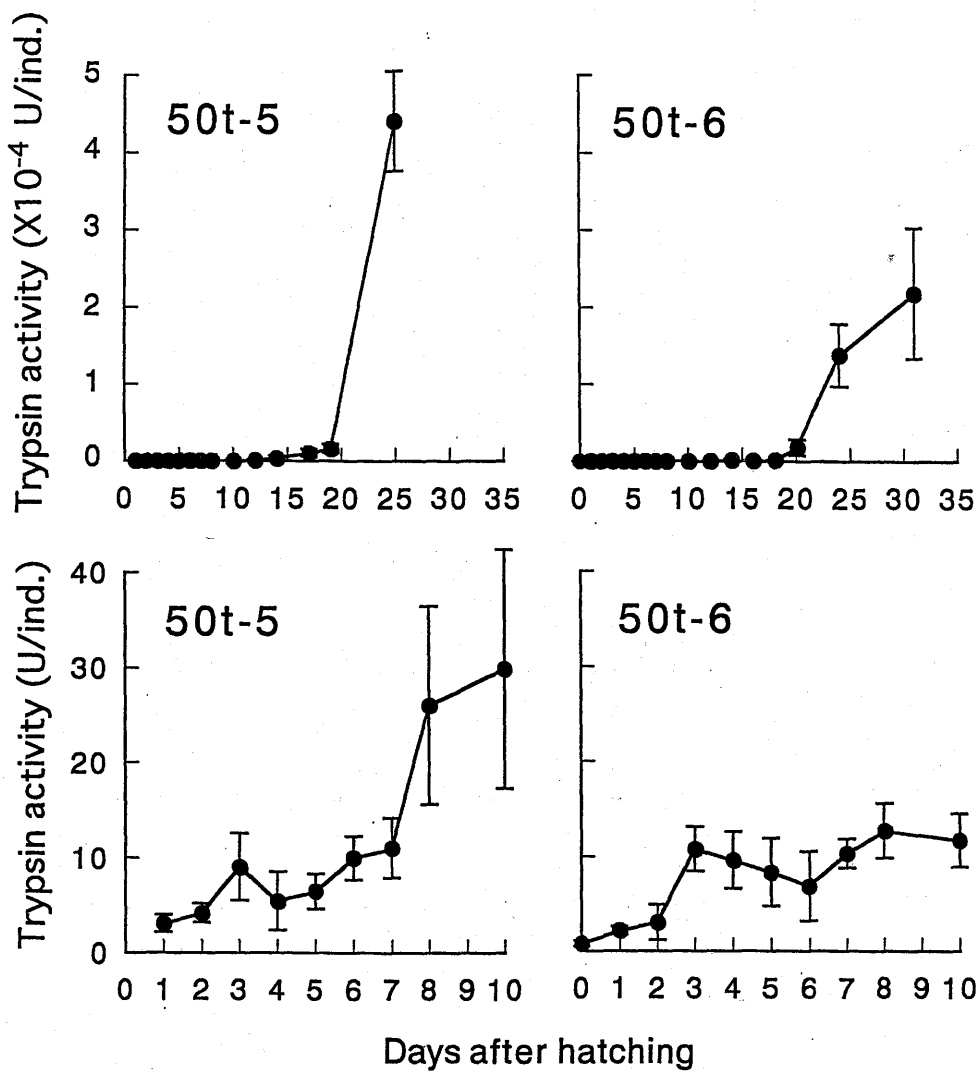
Fig. 1. Developemental changes of trypsin activity in bluefin tuna larvae and juveniles reared at the JASFA Amami station in 1998 (tank no. 50-5 and 50t-6), showing throughout rearing (top) and first 10 days after hatching (bottom). Data points with error bars (standard deviation) are means of 5 individually measured values.
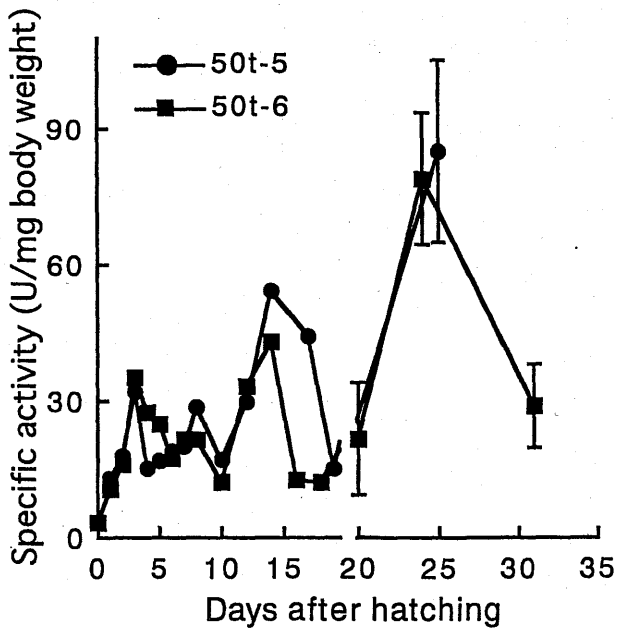

Fig. 2. Relationships between specific trypsin activity and age of bluefin tuna larave and juveniles. Values are calculated from means of trypsin activity (U/ind.) measured individually and means of wet body weight (mg) collected in same age younger than Day 20. The values older than Day 20 were calculated individually and shown by means ± S.D. of five specimens.
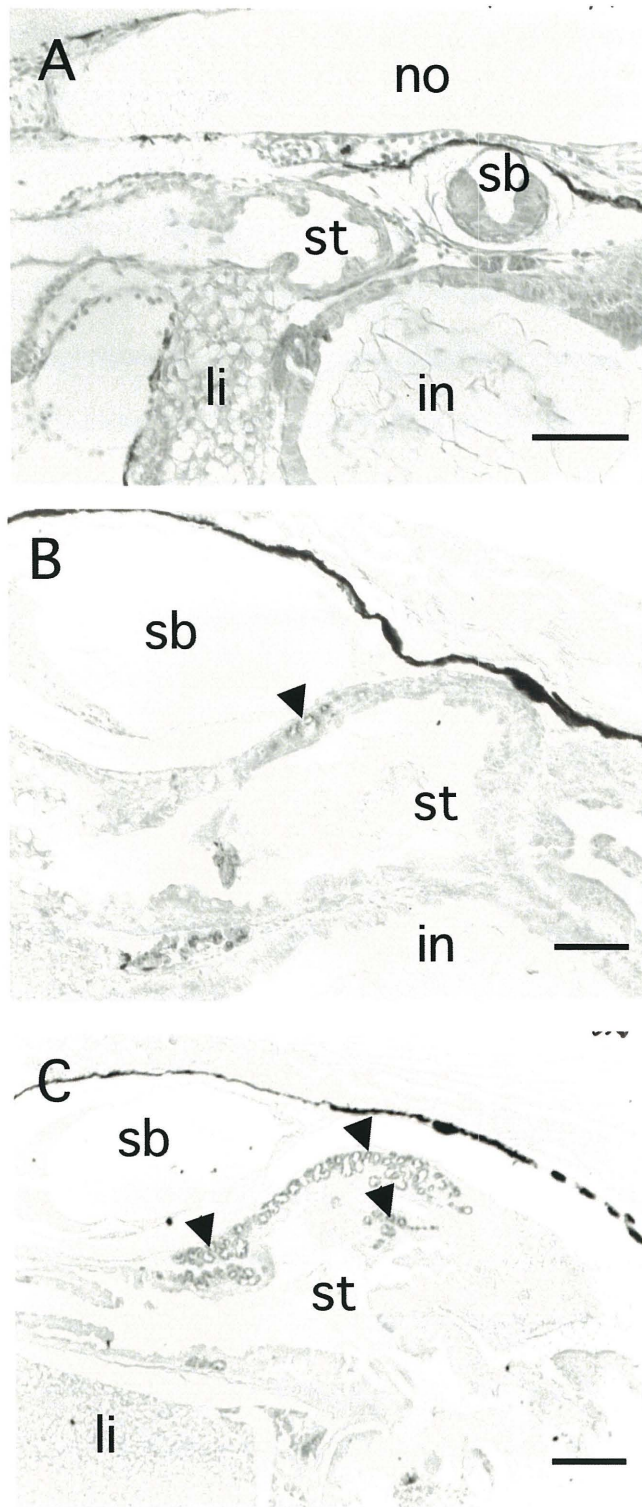
Fig. 3. Photomicrographs of sagittal sections of the stomach (st) of bluefin tuna larvae.

 Sections were stained with anti-Japanese flounder pepsinogen and counterstained with hematoxylin (arrowhead; positive reaction).
(A) preflexion larva at first feeding (3 days old). Scale bar=100 μm. (B) flexion larva (12days after hatching). Scale bar=50 μm. (C) flexion larva (14 days old). Scale bar=25 μm.
in; intestine, li; liver, no; notochord, sb; swimbladder.

Immunohistochemical observation on the gastric region revealed that, in 3 days-old preflexion larvae, no positive staining with the anti-Japanese flounder pepsinogen antibody was detected in mucosa of the rudimentary stomach, indicating that the fish have not yet developed peptic activity (Fig. 3A). One of three larvae at Day 10, weak staining with the antibody was first detected in the gastric gland-like cell masses on the dorsal wall of the stomach, indicating first appearance of pepsinogen synthesis. On Day 14, the stomach developed with 3 compartments of an elongated blind sac, cardiac and pyloric portions (Fig. 3C). The gastric glands increased in number, mainly on the dorsal and lateral wall of the stomach. These glands exhibited strong positive signals with the antibody, indicating that pepsinogen synthesis is a activated from Day 10 to 14 and gastric digestion by pepsin starts. Then the number of gastric glands increased markedly with larval development and growth during the postflexion phase to juvenile.

Discussion

The method adopted in the present study for trypsin activity assay was so highly sensitive that tryptic enzyme activities were able to be detected in a single larva from hatching (Fig. 1), as already demonstrated in other species (Ueberschär, 1988; Ueberschär et al., 1992; Oozeki and Bailey, 1995; Kawai et al., unpubl.). The method appears to be useful particularly for a difficult species like bluefin tuna, because heavy mortalities frequently occur and consequently sampling is usually restricted in number. On the other hand, the latent activity of trypsinogen, inactive precursor of trypsin, cannot be measured by this method. Ueberschär et al. (1992) mentioned that the values of trypsin activity measured by this method might include activities of trypsin-like enzymes existing in body parts aside from the digestive organs. Oozeki and Bailey (1995) reported, however, that values of eviscerated walleye pollock *Theragra chalcogrramma* larvae were low relative to whole body and did not affect the developmental trend. Although eviscerated samples were not measured in the present study, results obtained here would represent the developmental pattern of tryptic enzyme activity in bluefin tuna.

Unit/individual increased with age, and the increasing process well corresponded to the larval growth (Fig. 1). However, the value exhibited some fluctuations during the first 10

54

days after hatching. During the yolk-sac stage, the activity increased and reached a small peak on Day 3, when larvae initiated feeding on rotifers. Thus the increase would play an important role for first feeding.

Nearly the same ontogenetic trend of specific activity of trypsin (U/body weight in mg), synchronized three peaks during the experiments, was observed in both two rearing trials (Fig. 2). This suggests that ontogenetic changes in trypsin activity appeared to be an intrinsic developmental pattern of bluefin tuna ontogeny. The relationship between the marked changes of enzyme activity and development of the digestive system (Kaji *et al*., 1996; Miyashita *et al*., 1998) was clearly demonstrated. The first peak of the specific activity, observed on Day 3, corresponds to the day of first feeding. Bluefin tuna larvae at this stage have the most primitive digestive system which enables the larvae to feed on exogenous nutrients. Pancreatic exocrine tissues produce zymogen granules within acini; however, the stomach is still rudimentary form and gastric gland is not observed (Kaji *et al*., 1996; Miyashita *et al*., 1998), as shown in other fish larvae hatched from small pelagic eggs (Tanaka, 1973). Immunohistochemical observation also indicates that the stomach is not functional at this stage in terms of chemical digestion as well as physical (mechanical) digestion (Fig. 3A). The marked increase of specific trypsin activity observed at this stage appears to play an important role for their initiation of feeding. From Day 4 to Day 10, U/ind. and specific activity decreased or remained at a constant level in both rearing trials (Figs. 1, 2). A short period of a few days after first feeding is defined as transitional phase from endogenous to complete exogenous energy by histological observation (Kaji *et al*., 1996). A similar relationship between enzyme activities and ontogenetic development has been reported in other species, such as striped bass *Morone saxatilis* (Baragi and Lovell, 1986), herring *Clupea harengus* (Pedersen *et al*., 1987), and walleye pollock (Oozeki and Bailey, 1995). In bluefin tuna larval rearing, as well as in yellowfin tuna *T. albacares* (Kaji *et al*, 1999b), heavy mortalities frequently occur during a few days after initiation of feeding. Although a lot of rotifers were observed in guts of bluefin tuna larvae at this phase, growth rates and survival rates are low. The decrease in trypsin activity followed by a low level would relate to this critical period in the present species under rearing conditions, and the role of enzyme activity as a bottleneck to growth and survival should be examined in more detail.

The specific activity of trypsin increased on Day 10 (Fig. 2), corresponding to the end of preflexion phase in both rearing trials. The activity reached the second peak on Day 14, at flexion phase. The advanced digestive system, characterized by the gastric glands and pyloric caecum, differentiated during the flexion phase at which the external morphology with large mouth and eyes became prominent (Kaji *et al.*, 1996; Miyashita *et al.*, 1998). The main food shifted from copepod nauplii to copepodites at this phase in the ocean-caught bluefin tuna larvae (Uotani *et al.*, 1990). The marked increase of tryptic enzyme activity and concurrent development of gastric glands (Fig. 3C) suggest that proteolytic capability would increase at this phase, and suggest that the flexion phase is a turning point not only for external and internal morphology but also for digestive physiology

Day 20, when the most remarkable increase of the specific activity was observed (Fig. 2), coincide with the transition to juveniles. At this phase, the fins and vertebral column developed completely (Kaji *et al.*, 1996) and fish swim actively (Miyashita *et al.*, 1999). The digestive system develops further; the gastric blind sac is expanded and pyloric caecum increases in number. The drastic increase of the trypsin activity coincides with such a quantitative development of the digestive system, resulting in voracious feeding habits and rapid somatic growth of this species.

Kawai *et al.* (unpubl.) examined developmental changes in digestive enzyme activities from hatching beyond metamorphosis in various teleosts and found that the specific activities of trypsin exhibit clear peaks during the larval period. For example, larvae of *Seriola lalandi* and *S. dumerili*, which have high growth potential and piscivorous food habits like bluefin tuna, have the peak of trypsin activity during 15 to 20 days after hatching. The authors noted that this flexion point would be important for larval ontogeny and thus for larval rearing. In the present study, although marked increases of trypsin activity were observed on Day 20 and the highest value around Day 25 (Fig. 2), the trend of activity beyond this age was remained uncertain. Miyashita *et al.* (1998) reported existence of such a peak on Day 14 at flexion phase in pooled samples, as similar in the second peak in the present study, and following decrease in the activity. Further study should be required for better understanding of bluefin tuna larval ontogeny of digestive physiology.

It is well known that the trypsin activity of fish larvae is affected by various kinds of biotic and abiotic factors; e.g. nutritional condition (Ueberschär, 1995), quality of food

(Abi-Ayad and Kestemont, 1994; Zamboino-Infante and Cahu, 1994), stocking density and social hierarchy (Alvarez *et al*., unpubl.), and diel rhythm (Kawai *et al*., unpubl.). It is suspected that bluefin tuna, a typical active swimmer and voracious feeder, may have more drastic and sensitive response of the digestive physiology to the factors above than common marine fish. Further studies on effects of biotic and abiotic factors on digestive enzyme activities would help us to understand the diagnosis of the early life history of tunas, and they could contribute to improving the seed production technique.

Chapter 3: Developmental Changes in RNA, DNA, and Protein Contents of Laboratory Reared Bluefin and Yellowfin Tuna Larvae and Juveniles

Abstract

Changes in nucleic acid and protein contents of bluefin and yellowfin tuna, *Thunnus thynnus* and *T. albacares*, larvae and juveniles were measured individually. Although whole-body DNA content increased consistently during the early development, RNA content remained constant during the yolk-sac stage followed by increase, parallel to DNA content increase, from first feeding to the end of the larval period. The changes in protein content well coincided with those found in RNA content. At transformation to juveniles in both species, a marked increase of RNA content was observed. Subsequently, RNA/DNA ratio remained at constant low level during the larval period after a temporal decrease around first feeding. The ratios increased steeply at metamorphosis in both species. These results suggest that growth at cellular level in tunas is characterized by active cell division or hyperplasia during the larval period followed by a marked increase in cell size or hypertrophy from transformation to juvenile. The developmental process assessed by biochemical components in tuna larvae and juveniles was closely parallel to daily growth rate and related to morphological and physiological developmental events.

Introduction

Bluefin tuna *Thunnus thynnus* is one of the most important species for future stock enhancement and marine aquaculture in Japan. Biological information on the early life history of tunas under rearing conditions have been accumulated intensively in recent several years, with improvement of the rearing technique and subsequent increase of the number of produced juveniles (Kaji *et al.*, 1996, 1999a, 1999b, 1999c; Miyashita *et al.*, 1998, 1999; Takii *et al.*, 1997). However, survival rates during early life stages are still much lower than those of other marine finfish seed production, and are frequently influenced by unexpected and unknown conditions.

The author's previous works demonstrated that the functional digestive system of tuna larvae differentiated earlier than other marine fish larvae previously examined; during the

flexion phase, followed by rapid development of the digestive system during the postflexion and early juvenile stages (Kaji *et al.*, 1996; 1999b). The rapid development of the digestive system in a quantitative way coincides with a marked increase in growth hormone production in the pituitary (Kaji *et al.*, 1999a). These morpho-physiological developmental characteristics of *Thunnus* could allow the larvae to shift to piscivory and to bring rapid somatic growth. Since bluefin tuna larvae posses such a diagnostic developmental process, it is postulated that ontogenetic changes in biochemical components also exhibit a species-specific pattern. However, biochemical developmental characteristics on tuna larvae, such as nucleic acid and protein contents during the early development and growth, have not yet been studied.

Nucleic acid and protein contents of fish larvae are primarily used as tool to asses the nutritional condition and growth rate of sea-caught larvae for prediction of recruitment and yea-class strength (Bulow, 1987; Westerman and Holt 1994; Clemmesen, 1996). Rates of cell proliferation, cell enlargement and metabolic activity could generally reflect to developmental profiles of these biochemical contents. Thus some reports have shown reasonable relationships between biochemical changes and morphological, physiological, and behavioral changes in the early life stages of some fish species (Fukuda *et al.*, 1986a, 1986b; Steinhart and Eckman, 1992; Takii *et al.*, 1992, 1994; Tanangonan *et al.*, 1998; Gwak, 1999).

The purpose of this section is to analyze developmental changes in DNA, RNA, and protein contents of hatchery-reared bluefin tuna and partly yellowfin tuna *T. albacares* in order to clarify the early ontogenetic pattern, as a part of studies on developmental characteristics of tuna larvae.

Materials and Methods

Fish

Bluefin tuna larvae and juveniles were reared at the Japan Sea-Farming Association (JASFA), Amami Station, located in Kakeroma island, Kagoshima Japan in 1998. Samples examined in the present study were taken from two concurrent rearing trials designed for mass seed production under almost the same rearing condition with 50 m^3 tanks (referred as 50t-5 and as 50t-6). Fertilized eggs of bluefin tuna were obtained by spontaneous spawning

of the broodstock maintained in a net pen at the JASFA Amami Station. Initial stocking density were 52 eggs per *l* in 50t-5 and 25 larvae per *l* in 50t-6. Seawater sterilized with ultraviolet light was used for the rearing. Slight aeration was provided with airstones. Photoperiod was maintained under natural conditions. Water temperature ranged from 26.7 to 28.7 (mean 27.9) ℃. Rotifers were fed from Day 3, and *Artemia* nauplii, fish larvae (striped beakperch *Oplegnathus fasciatus*, bluefin tuna, and spangled emperor *Lethrinus nebulosus*), frozen fish and minced fish meat were fed according to development and growth.

Yellowfin tuna larval rearing from hatching beyond metamorphosis was carried out at the JASFA Yaeyama Station in 1996. Details on the rearing experiment were described in Kaji *et al.* (1999a).

Sampling and Preservations

Sampling was performed in daytime. Fish were individually sampled, rinsed, pipetted into Eppendorf micro tubes. Then fish were immediately frozen and stored at -80 ℃ until later analysis.

Fish were sampled also for evaluating the growth process. Sampled fish were anesthetized and measured standard length (SL) under a profile projector. The daily growth rate (mm/day) calculated from the following formula:

Growth rate=$(l_{n+t}-l_n)/t$

where l_n is the mean standard length at time n and t is the interval in days.

Some of the fish were then fixed and preserved in 10% formaldehyde for later developmental phase determination and measurements of wet body weight. Developmental phases after Kendall, Jr. *et al.* (1984) were used in this study.

Determination of RNA, DNA, and Protein Quantity

Measurements of RNA and DNA contents were carried out on an individual fish basis in both species younger than 20 days after hatching, while a part of muscle from dorsal part of bluefin tuna juvenile older than 20 days were blotted, weighed (mg) and utilized to analyze. Quantity of RNA and DNA was determined by a fluorescence technique using Ethidium bromide (Nacalaitesque Co. Ltd., Japan) described by Clemmesen (1993) with a slight modification by Sato *et al.* (1995). Salmon sperm DNA (Wako pure Chem., Japan) and

yeast RNA (Kanto Chem., Japan) were used as standards. Both RNA and DNA contents are expressed as μg or μg/mg muscle.

For bluefin tuna younger than Day 20, total protein (dissolved in NaOH) was determined by Bio-Rad protein kit using bovine serum albumin as a standard. Results are expressed as mg of protein per individual fish.

Results

Development and Growth of Tuna Larvae and Juveniles

Changes in standard length (SL) during the early life stages of bluefin and yellowfin tuna are shown in Fig. 1. Newly hatched larvae of bluefin tuna were 3.03 ± 0.08 mm SL (mean ± S.D.), and grew to 37.3 ± 4.43 mm SL on Day 25 for 50t-5 and to 40.27 ± 3.54 mm SL on Day 31 for 50t-6. The growth process appeared to be curvilinear with an increase in growth rate with age (Fig. 6). Wet body weight (Fig. 2) increased exponentially with age.
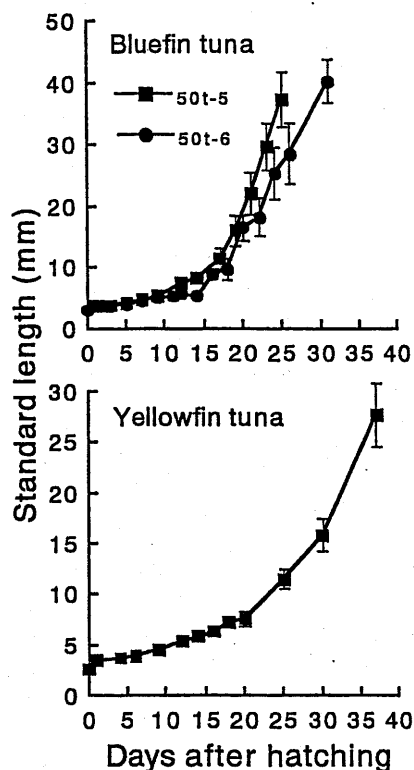

Fig. 1. Standard length of anesthetized bluefin and yellowfin tuna larvae and juveniles. Bluefin were reared at the JASFA Amami Station in 1998 and yellowfin were reared at the JASFA Yaeyama Station in 1996. Values are given as mean ± S.D.
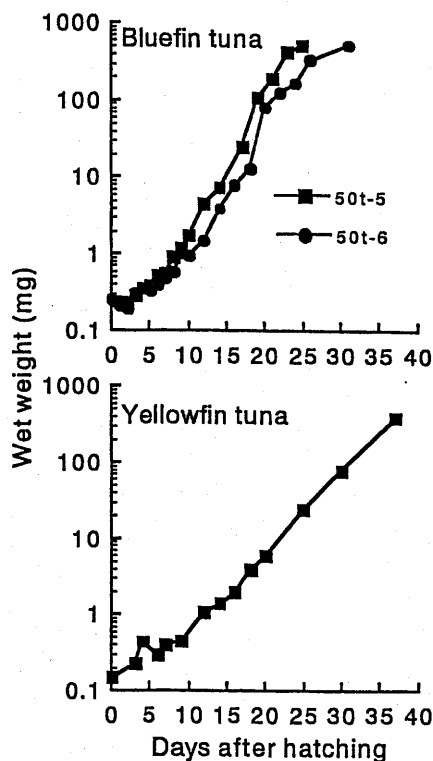
Fig. 2. Wet body weight (mg) of formaldehyde-preserved bluefin and yellowfin tuna larvae and juveniles. Bluefin were reared at the JASFA Amami Station in 1998 and yellowfin were reared at the JASFA Yaeyama Station in 1996. Values are given as mean ± S.D.

The shift from yolk-sac phase to preflexion phase occurred on Day 3 in both rearings (Table 1). Age-developmental phase relationship varied among individuals. Average phase-shift dates, however, were roughly estimated Day 12 (to flexion) and Day 18 (to postflexion). The larvae transformed from postflexion phase to juvenile on Day 20, in both rearings.

The morphological development and growth processes of yellowfin tuna larvae and juveniles used in the present study were described in Kaji *et al.* (1999b).

Table 1. Developmental phase composition (number of individuals) of bluefin tuna larvae and juveniles reared at the JASFA Amami Station in 1998, showing distribution of fish in terms of age. Results of two concurrent rearing trials were shown, reffered as 50t-5 (upper) and 50t-6 (lower).

Developmental phase	Days after hatching																	Total
	1	2	3	4	5	6	7	8	9	10	12	14	17	19	21	23	25	
yolk-sac larva	15	18																33
preflexion larva			21	17	11	14	22	25	20	18								148
flexion larva										1	13	6	1					21
postflexion larva												4	9	2				15
juvenile														9	10	10	13	42
Total*	15	18	21	17	11	14	22	25	20	19	13	10	10	11	10	10	13	259

Developmental phase	Days after hatching																	Total
	0	1	2	3	4	5	6	7	8	9	10	12	14	16	18	20	22-31	
yolk-sac larva	26	28	30															84
preflexion larva				17	17	22	17	17	12	10	9	10	1					132
flexion larva											1	4	9	10	7			31
postflexion larva															8	3		11
juvenile																7	70	77
Total*	26	28	30	17	17	22	17	17	12	10	10	14	10	10	15	10	70	335

* Materials used here were formaldehyde-preserved fishes.

Changes in Nucleic Acid and Protein Contents

DNA and RNA contents per individual of bluefin and yellowfin tuna increased rapidly with age during the larval period (Fig. 3), though RNA content showed a slight increase during the first 3 days after hatching in bluefin tuna. The changing patterns in protein content of bluefin tuna during the larval period corresponded fundamentally with those of RNA content, indicating that RNA content is an indicator for protein synthesis.

Developmental changes in RNA/DNA ratios of bluefin and yellowfin tuna are shown in Fig. 4. The lines for bluefin tuna discontinue around Day 20 due to different treatments;
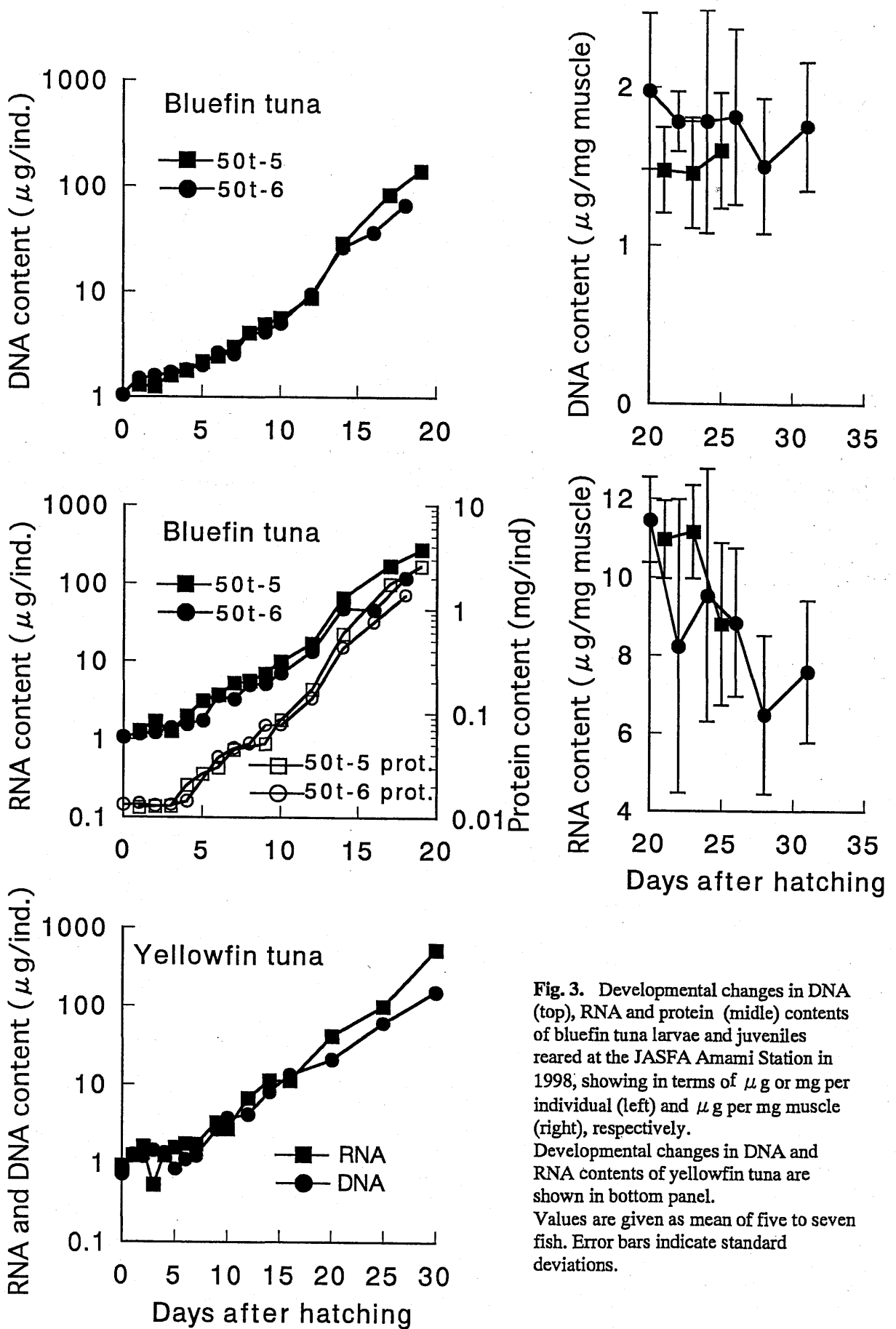
Fig. 3. Developmental changes in DNA (top), RNA and protein (midle) contents of bluefin tuna larvae and juveniles reared at the JASFA Amami Station in 1998, showing in terms of μg or mg per individual (left) and μg per mg muscle (right), respectively.
Developmental changes in DNA and RNA contents of yellowfin tuna are shown in bottom panel.
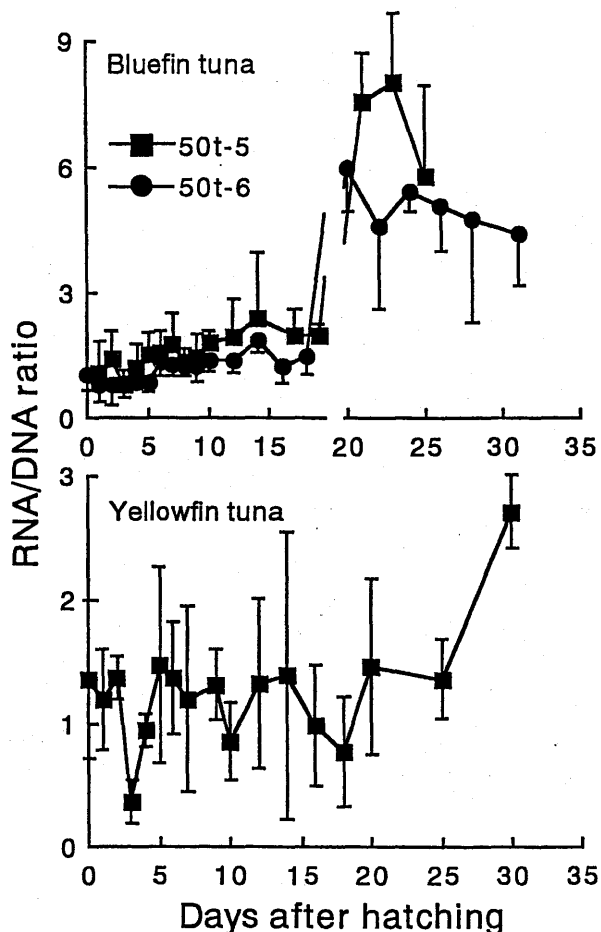Values are given as mean of five to seven fish. Error bars indicate standard deviations.

Fig. 4. Developmetal changes in RNA/DNA ratios of bluefin and yellowfin tuna larvae and juveniles. Values are given as mean ± S.D. of six to seven fish. Discontinuity of the lines in the upper panel indicates different assays using whole body and blotted muscle assays.

whole larvae and blotted muscle assays before and after Day 20, respectively. Overall developmental trends exhibited similar patterns in all the rearing trials of two tunas; the RNA/DNA ratios remained steady with some fluctuations from hatching to Day 20 for bluefin and to Day 25 for yellowfin tuna, thereafter the ratios increased steeply. These steep increases of the RNA/DNA ratios corresponded to transformation to juveniles in all the rearings (Table 1; Kaji *et al.*, 1999b).

Changes in RNA/DNA ratios and protein/DNA ratios during the first 20 days after hatching of bluefin tuna are shown in Fig. 5. The lowest levels of both ratios were detected around day 3, first feeding day. Protein/DNA ratio increased gradually with age, but temporarily decreased during the yolk-sac stage, reached the lowest level on Day 3 in both the rearings, and increased after first feeding day.

The RNA/DNA ratios closely paralleled with mean growth rates (mm/day) in all the
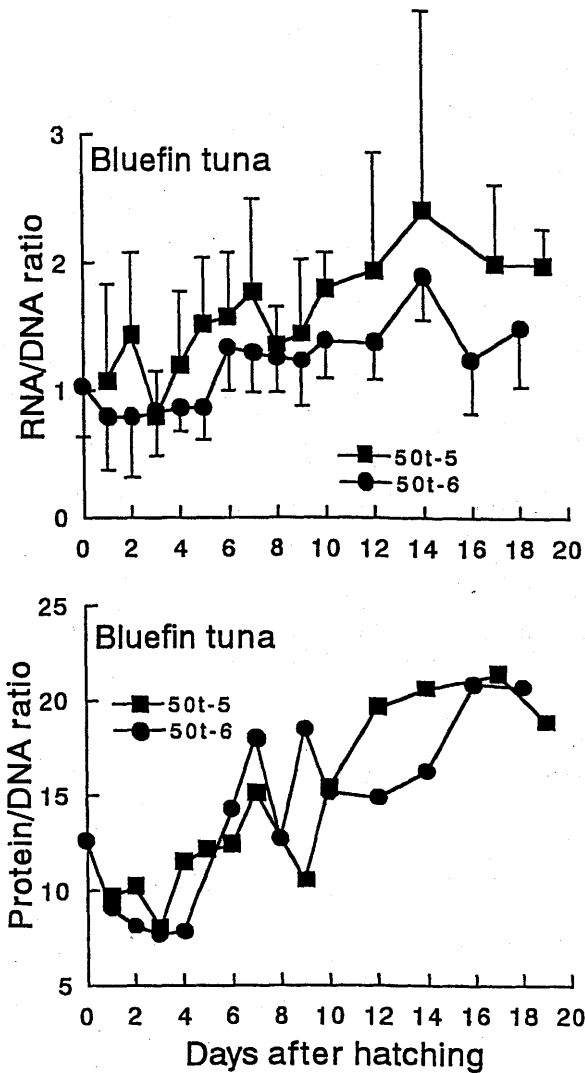
rearings (Fig. 6).

Fig. 5. Developmetal changes in RNA/DNA (top) and protein/DNA (μ g/μ g, bottom) ratios of bluefin tuna larvae reared at the JASFA Amami Station in 1998. Values are given as the mean of six to seven fish, and error bars indicate standard diviation.
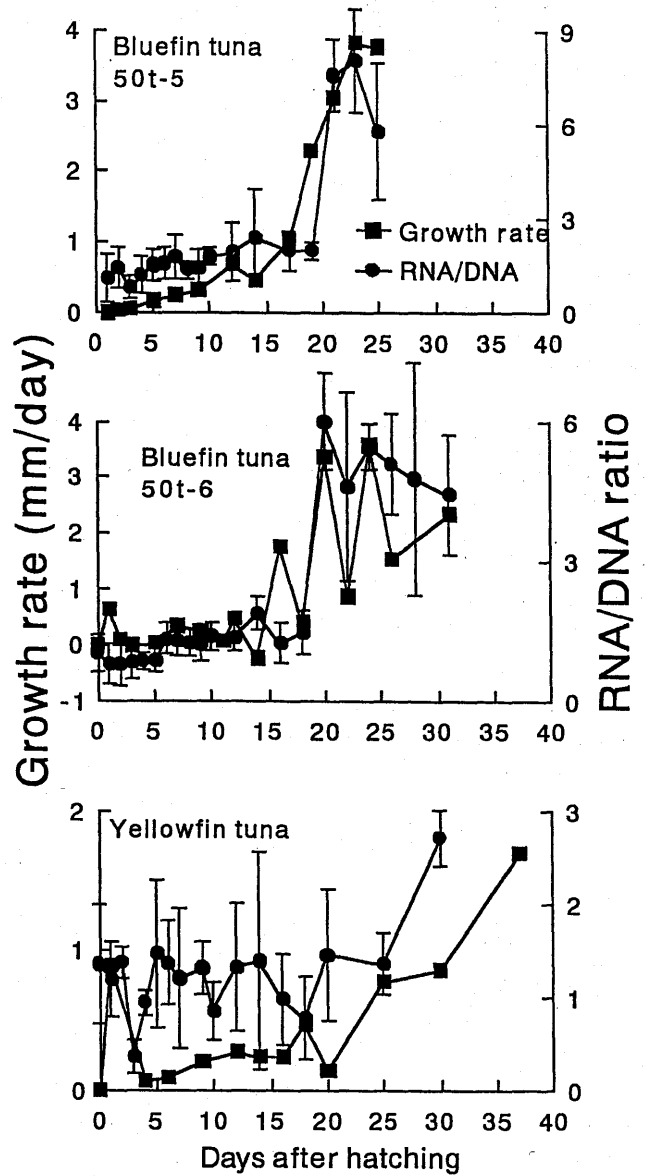
Fig. 6. Daily mean RNA/DNA ratios and growth rates in bluefin and yellowfin tuna larvae and juveniles.

Discussion

Nucleic acids play a major role in growth and development. The amount of DNA is

constant in somatic tissues and well reflects to cell numbers, while that of RNA in the cell is

directly proportional to the amount of protein synthesis occurring. The relationship between

RNA and DNA is an index of the cell's metabolic intensity (Bullow, 1987; Clemmesen, 1996).

In the present study, a piece of muscle collected from dorsal part of the body were blotted and utilized to determine the nucleic acid content in bluefin tuna older than Day 20, while whole body in bluefin tuna younger than Day 20 and all of the yellowfin tuna larvae and juveniles was used for assays. Thus the RNA/DNA ratio could not be compared directly between these two analysis methods, however, supplemental analyses demonstrated that RNA/DNA ratio obtained from a part of muscle appeared to be only about 1 higher than that from whole body analysis on a same individual juvenile of bluefin tuna. Therefore, the developmental changes in RNA/DNA ratio in the present study could be regarded as ontogenetic pattern of reared tuna larvae on the whole, in spite of some correction would be needed in the ratio of bluefin tuna older than Day 20.

The present analysis has demonstrated that overall developmental changes in RNA/DNA ratio appeared to be similar pattern in all the rearings and both species (Fig. 4). The pattern of constant low level of the ratio during the larval period followed by a steep increase around the transformations to juvenile (metamorphosis) have been observed repeatedly, suggesting that this is an intrinsic characteristic pattern for tuna development under rearing conditions. Moreover, the lowest level of the RNA/DNA ratios was observed around Day 3, the first feeding day, in all the rearing trials. Therefore, onset of feeding around Day 3 and around metamorphosis would be two turning points in development of *Thunnus* based on nucleic acid and protein content changes.

During the yolk-sac stage, tuna larvae did not feed and utilized endogenous nutrient mainly from the yolk (Kaji *et al.*, 1996, 1999b; Miyashita *et al.*, 1998). Basic organs for start feeding, such as eyes, jaws, and digestive system with rudimentary stomach, developed rapidly during a few days of yolk-sac stage. Increase of DNA contents and constant RNA content during the yolk-sac stage (Fig. 3) resulted in the lowest level of RNA/DNA ratios at around first feeding day (Fig. 5). Similar decreases of RNA/DNA ratios around first feeding have been reported from winter flounder *Pseudopleuronectes americanus* (Buckley, 1982), red sea bream *Pagrus major* (Takii *et al.*, 1992), red drum *Sciaenops ocellatus* (Westerman and Holt, 1994), striped jack *Caranx delicatissimus* (Takii *et al.*, 1994), and Japanese flounder *Paralichthys olivaceus* (Tanangonan *et al.*, 1998). Moreover, the protein/DNA also

decreased during the yolk-sac stage and exhibited the lowest level around the first feeding in bluefin tuna (Fig. 5). These results suggest that development and growth of tuna larvae during yolk-sac stage would be mainly realized by rapid cell division in relation to organogenesis of the basic organ-system for first feeding, and that larvae undergo in a state of critical condition or potential for protein synthesis around first feeding. These biochemical aspects of development of bluefin and yellowfin tuna seem to relate to the low daily growth rates (Fig. 6, Kaji *et al.*, 1999a) and heavy mortalities frequently occurring around the first feeding stage (Kaji *et al.*, 1996, 1999a, 1999b).

The author's previous morphological, histological, and physiological studies demonstrated that the period from feeding initiation to the end of postflexion phase could be regarded as a period of relatively slow growth with numerous developmental events in external and internal morphology; swim bladder developed and inflated during several days after first feeding, and functional digestive system established at flexion phase followed by rapid elaboration of the digestive system in a quantitative way during the postflexion phase to early juveniles. Simultaneous increase of both RNA and DNA contents per larva (Fig. 3) resulted in constant RNA/DNA ratio from the first feeding to the end of larval period in all the rearings (Fig. 4). On the other hand, protein/DNA ratio increased gradually during this period (Fig. 5). These patterns suggest that development and growth at cellular level of the bluefin and yellowfin tuna from first feeding to the end of larval period involve marked increase of cell number due to rapid cell division or hyperplasia, combined with increasing cell size, as reported from Pacific herring *Clupea pallasi* (Fukuda *et al.*, 1986a), cresthead flounder *Limanda schrenki* (Fukuda *et al.*, 1986b), and striped jack (Takii *et al.*, 1994).

At the transition to juvenile, marked increase in RNA/DNA ratio of yellowfin tuna was observed (Fig. 4), as a results of drastic increase in RNA content and consistent increase of DNA content (Fig. 3). Similar sharp increase of the ratio, corresponding to the metamorphosis, was also shown in bluefin tuna (Fig. 4). During increase in RNA/DNA ratio, it could be speculated that body growth of fish chiefly occurs by cell enlargement (hypertrophy) resulting from protein synthesis (Fukuda *et al.*, 1986a, 1986b). Fin formation complete and vertebral column is ossified during the transformation to the juvenile in bluefin and yellowfin tuna, and fish begin to exhibit active swimming and foraging behavior. The structure of the digestive system developed markedly in a quantitative way in both species

(Kaji *et al.*, 1996, 1999b; Miyashita *et al.*, 1998), and specific trypsin activity increased steeply at this phase in bluefin tuna (chapter 2-2). Development of growth hormone cells in the pituitary synchronized with increase of growth rates (Kaji *et al.*, 1999a). The steep increase of the RNA/DNA ratios of bluefin and yellowfin tuna during transformation to juveniles well correspond to these developmental events, and consequently daily growth rates were accelerated from the transition to juveniles (Fig. 6).

Recent findings using otolith microstructure (Jenkins and Davis, 1990; Scott *et al.*, 1993; Lang *et al.*, 1994) have demonstrated that daily growth rates of sea-caught tunas during the larval period are not so high, similar to many other marine species and to that of the present rearing (see review by Tanaka *et al.*, 1996a), while the age of wild juvenile bluefin tuna (about 20-40 mm) were estimated about 22 days after hatching*, suggesting that growth of tunas must be accelerated extremely during the later larval period and/or early juvenile stage. Results obtained here well support the possibility from biochemical developmental characteristics.

Present data suggests that growth at cellular level in tuna larvae is characterized by rapid cell divisions from hatching to first feeding followed by continuous active cell divisions associated with organogenesis. Increase in cell size concurrently occur from first feeding to the end of larval period. A drastic increase of protein synthesis capacity, well demonstrated by RNA/DNA ratio, was observed at metamorphosis. These developmental characteristics appeared in biochemical components are closely related to morphological and physiological characteristics of the early life history of *Thunnus*. Further studies on effect of biotic and abiotic factors, such as temperature, food availability, and starvation, on biochemical components should be conducted in order to understand the development and growth of tunas in more details, and to improve the rearing technique.

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*Ito, T. and Tsuji, S. (1998). Abst. Meet. Jap. Soc. Sci. Fish. Sci., April, p. 38. (in Japanese)

Chapter 4: Laboratory Study of Density-dependent Survival after Handling in Yolk-sac Larvae of Bluefin and Yellowfin Tuna

Abstract

Yolk-sac larvae of bluefin tuna *Thunnus thynnus*, yellowfin tuna *T. albacares*, and additionally coral trout *Plectropomus leopardus* were transferred to small plastic plates at various densities, and were reared without diet. Dead larvae were counted and removed every 12 hours. High mortality, which would be related to handling stress, was recorded at the first observation after the transfer in all the species. The survival at the first observation was high, near 100%, at high densities (10-30 larvae/ml), and the lowest survival rate was observed at the lowest densities, in all the species. Since the similar pattern was reported also in Japanese flounder *Paralichthys olivaceus*, this phenomenon would be general in marine fish larvae, and may provide better understanding of the larval response to stress and physical injuries.

Introduction

In seed production of marine fish, high mortality frequently occurs during larval period. Mortality at yolk-sac stage, prior to first feeding, is expected to be due to egg quality (Kjørsvik *et al.*, 1990) and/or environmental factors. Early mortality is a serious problem in the development of seed production of economically important species, such as yellowfin tuna *Thunnus albacares* (Kaji *et al.*, 1999b), bluefin tuna *T. thynnus* (Kaji *et al.*, 1996), and grouper, such as the coral trout *Plectropomus leopardus* (Masuma *et al.*, 1993). Recently, presence of density-dependent survival after handling was reported in yolk-sac larvae of Japanese flounder *Paralichthys olivaceus* * using small plastic plates as fish containers (Brown and Nunez, 1994). In this study, survival after handling stress was examined in relation to larval density in the above mentioned species with more interest for the establishment of seed production procedures than flounder.

Materials and Methods

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*M. Tagawa *et al*.: Abst. Metg. Japan Soc. Fisheries Sci., September, 1997, p. 49 (in Japanese).

Fertilized eggs were obtained by spontaneous spawning at the Japan Sea-Farming Association (JASFA) Yaeyama Station (yellowfin tuna at 27.0 ℃ and coral trout at 28.0 ℃) and at the JASFA Amami Station (bluefin tuna at 28.2 ℃) in 1997. Yolk-sac larvae were transferred from a small tank using a Pasteur's pipet to 6-well and 24-well tissue culture plates, and 9 cm dishes, filled with seawater obtained from a single tank to assure the same quality and temperature, to establish the densities shown in Table 1. After the transfer, all containers were kept in the dark at constant temperature (25 ℃ for bluefin and yellowfin tuna, 27 ℃ for coral trout) without aeration. Dead larvae were counted and removed every 12 hours. Water was not renewed, and food organisms were not supplied during the experiments.

Table 1. Experimental design

group[*1]	container	area cm^2	depth cm	volume ml	number of larvae	density ind./ml	replicates		
							yellowfin tuna	bluefin tuna	coral trout
d-10	9 cm dish	64	0.5	32	10	0.31	10	10	10
d-30					30	0.94	5	5	3
d-100					100	3.1	3	3	3
6wp-5	6 well plate	9.4	0.5	4.7	5	1.1	6	-	-
6wp-15					15	3.2	-	-	6
6wp-50					50	10.6	3	-	3
24wp-10	24 well plate	2	0.5	1	10	10	10	-	10
24wp-30					30	30	5	-	3

[*1] ex. d-10 means 10 larvae in a 9 cm dish, and 6wp-5 means 5 larvae in a 6 well tissue culture plate.

Results and Discussion

Results of these experiments indicated that the initial mortality was strongly density-dependent. Figure 1 shows the time course of larval survival for each species and density group. Although the overall pattern varied among species, high mortality was recorded at the first observation time after transfer in all the species. The timing of death was before the completion of yolk absorption, as indicated by solid arrows in the figure. When the relationship between survival at the first observation and the larval density was examined (Fig. 2), survival was high, near 100%, at high densities for all the three species. In contrast lowest survival rate was observed at the lowest density, in all the species. At the lowest density survival rate varied among species, and the relatively low survival rate (below 10%),

as well as the faster decrease in survival before completion of yolk absorption, observed in coral trout may reflect high vulnerability to handling due to the small size of this species (Masuma *et al.*, 1993).
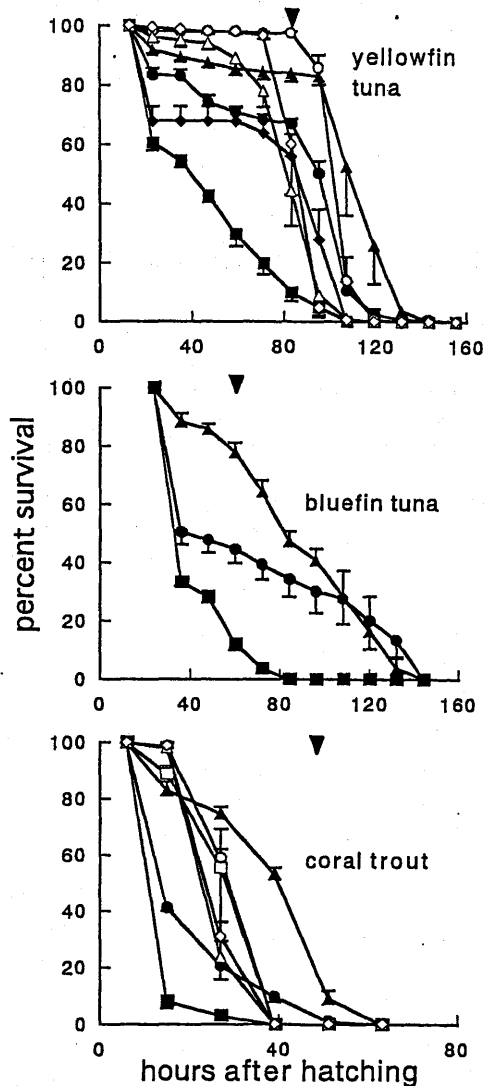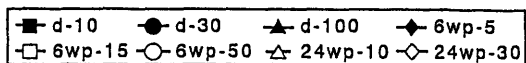

Fig. 1. Survival of unfed larvae of each species under laboratory condition. Each point represents the mean of each group. Bars are standard errors of means. Arrows indicate the timing of completion of yolk absorption.
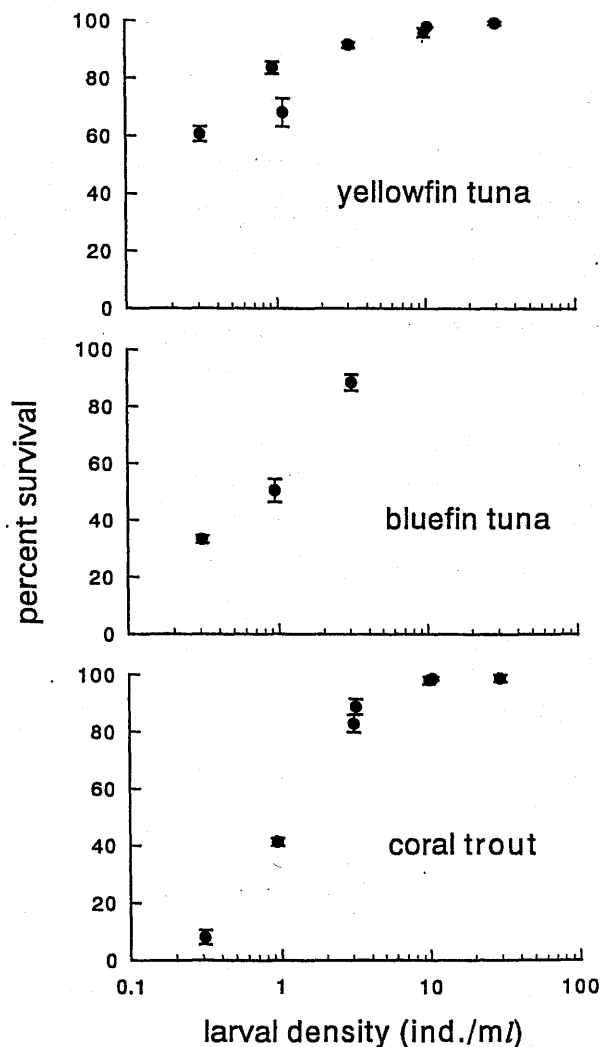
Fig. 2. Relationship between the survival at the first observation time and the rearing density. Each point represents the mean of each group. Bars are standard errors of means.

The author believes the initial mortality of larvae after transfer (Fig. 1) is related to handling stress. The narrow tip of the Pasteur's pipet could damage the larvae during the transfer from the stock tank to the plastic containers. Since larvae transferred to containers of

higher densities showed better survival (Fig. 2), it appears that survival after acute stress or damage is improved by rearing fish at extremely high density. Tagawa *et al.* * reported the similar pattern of survival in Japanese flounder larvae.

The second timing of severe mortalities in Yellowfin tuna would be related to the starvation, because the timing of the mortality was later than the completion of yolk absorption. The information on "the point of no return" of each species is not available so far, which is necessary for the further discussion on the reason of second timing of severe mortalities.

The mechanism(s) responsible for the observed density-dependent survival are not understood. Concentrations of substance(s) secreted from larvae, as well as changes in larval behavior, in high densities are possible factors to be considered in future. Those investigations may improve our understanding of the larval response to stress and thus our seed production abilities. The present finding is based on a small experimental system with stress by pipetting and larval density which is unrealistically high. The larval densities at which the highest survival was observed is 1000-3000 times higher than a practical level for seed production of Japanese flounder (Takahashi, 1990). Although these results can not be applied directly to the technique of seed production, they do suggest that further experiments of this type may help us to understand the mechanisms of larval death and/or recovery after stress.

Concluding Remarks

In the present study, the author clarified the early development of bluefin and yellowfin tuna mainly from the histological and physiological aspects. The author summarizes the present study to conclude with two special remarks on "Developmental characteristics of tunas" and "Proposal comments for seed production of bluefin tuna".

Developmental Characteristics of Tunas

Altricial Yolk-sac and Preflexion Larval Phases

Morphological, histological, physiological, and biochemical data suggest that tunas undergo altricial and immature early larval period from hatching until the end of preflexion phase. Overall developmental features during this period, e.g. primitive body structure with normal-size mouth and larval-type digestive system structure and function, appeared to be similar to those of common coastal marine teleosts hatched from small pelagic eggs.

In particular, existence of a "critical period" around first feeding, represented by slow growth and high mortalities under rearing conditions, was confirmed consistently by histological, physiological, and biochemical data. Tunas, because of their high fecundity and batch spawning mode at offshore, could be a representative group with a life history pattern characterized by very high annual production of larvae and subsequent high rates of mortality in the sea. Vulnerability of tuna larvae revealed in the laboratory-reared fish could be common to in open ocean. This mode of spawning also may force larvae to undergo altricial larval period at offshore in which densities of available food organisms are relatively low. Primitive body structure of tunas at early larval phase would be adaptive to utilize small but abundant invertebrate zooplankton, such as copepod nauplii.

Flexion Phase as a Turning Point of Development

Flexion phase should be noted as a "turning point" in tuna larvae. External morphology common to most fish species transformed to a specialized form of scombrid larvae characterized by enlarged head with large mouth and eyes. Although the other body

structures are still at less-advanced larval type, only the digestive system attained to adult type; differentiation of gastric glands in the stomach followed by differentiation of pyloric caeca. Compared to the other marine fish larvae, apparently precocious development of the digestive system coupled with enlarged head and mouth could allow the larvae to shift the survival strategy from "altricial larval life with planktivory" to "large prey-fast growth with potential piscivorous food habit".

Large Prey-Fast Growth During Postflexion Phase to Juvenile

Histological, immunohistochemical and physiological data, as well as morphological features as posterior shift of the anus position, demonstrated that structure and function of the digestive system developed extremely in a quantitative way during the period from postflexion to early juvenile phase. At the transition to juvenile, fins completed and vertebral column ossified, which are generally observed in metamorphosis of many marine teleosts, indicating marked increase in swimming ability which enable the tuna juvenile to actively exploit prey fish larvae at relatively low densities in their offshore habitat. These developmental characteristics coincide with marked increases in growth hormone production in the pituitary and in RNA/DNA ratio. Therefore, developmental feature of tunas at postflexion and early juvenile phases can be described as appearance of "large prey-fast growth".

In summary, the author could divide early development of tunas into two contrasting phases; altricial larval phase and rapidly developing postflexion to juvenile phase. The flexion phase intervenes between these two phases as a turning point.

Further studies on ecological traits of wild tuna larvae are inevitably needed to understand the early survival strategy of tunas. A comparative study on the early life stages among scombrid species is also needed.

Proposal comments for Seed Production of Bluefin Tuna

Bluefin tuna has been one of the final targets for future stock enhancement and aquaculture in Japan. Based on basic biological information obtained in the present study, the

author tries to make some proposal comments for seed production of bluefin tuna.

During yolk-sac stage, density-dependent survival after handling in tuna larvae was noted as a possible factor to improve survival rates of bluefin tuna. Since this phenomenon was confirmed in a small experimental scale and with stress induced by pipetting, it can not be adopted directly to practical rearing at this moment. However, application of the phenomenon to practical level could be useful for a certain species such as bluefin tuna of which larval transportation will be needed in future to effective seed production. In addition, the author confirmed so high survival rates as nearly 100% during 80 hours after hatching of yellowfin tuna using this method. Such a high survival rate beyond yolk absorption has never been realized in practical rearing trials. Further investigations on expansion of tank-size toward practical level would contribute improving the survival rates of tuna larvae during several days after hatching.

High proportion of fish with uninflated swimbladder appeared to be one possible obstacle for successful larval rearing in bluefin tuna. Histological study demonstrated that initial swimbladder inflation occurred around Day 7, and duration in which larvae could inflate their swimbladder was relatively short. Thus improvements in rearing conditions should be tested to enhance % inflation, particularly for first 10 days after hatching. Relationship between light-intensity and initial swimbladder inflation, as well as day and night (or light and dark) changes in swimbladder volume (Kaji *et al*., unpubl.), seem to be of primary importance in improving the rearing techniques.

The precocious development of the digestive system is closely related to piscivorous food habits and high growth potential in scombrid species (Tanaka *et al*., 1996a). Prey-capture and digestive capabilities in tuna larvae appear to be accelerated rapidly after the flexion phase at which tuna larvae have a highly developed digestive system. These suggest that postflexion tuna larvae begin to feed on fish larvae when they are abundant in ambient waters. Changing the feeding schedule from invertebrate zooplankton to fish larvae can be started associated with differentiation of the functional digestive system.

At the transition to juveniles, drastic increase in somatic growth potential was expected based on accelerated advances in the digestive system, digestive enzyme activities, growth hormone production, and RNA/DNA ratio. Preliminary observations demonstrate that body depth at anus position of bluefin tuna juvenile examined in the present study is lower than that of sea-caught juvenile at a same size range. In addition, condition factor of hatchery-reared bluefin tuna juvenile is also lower than that of sea-caught fish (Kaji *et al.*, unpubl.). Although comparative studies on larval morphology between reared and wild fish have not yet been conducted, these results suggest that quality and/or quantity of food adopted in the present hatchery-rearing may not be appropriate for nutritional requirement of bluefin tuna juvenile with such a high growth potential. Another possible explanation for these differences between hatchery-reared and sea-caught juvenile is that rearing abiotic conditions may be unsuitable for behavior and swimming ability of juvenile bluefin tuna, causing highly stressful condition.

Moreover, in hatchery-reared juvenile bluefin tuna, melanophores were more dense and caudal fin formation was delayed than those of sea-caught fish. These differences in external morphology suggest that physiological and/or biochemical aspects would also differ between the 2 groups, bringing ecological and behavioral differences. Due mainly to their unique developmental characteristics, bluefin tuna show various kinds of abnormalities under rearing conditions. Comparative studies between hatchery-reared and sea-caught fish from various aspects is indispensable to improve the rearing techniques, and could certainly contribute to understanding the early survival strategy of *Thunnus*.

Summary in Japanese
（和文要旨）

　　マグロ類は水産業上の重要魚類の一つであり，天然海域における初期発育にはこれまで多くの関心が払われてきた．主として外部形態の観察結果から，マグロ類を含めたサバ型魚類は，サバ型変態と呼ばれる独特の形態発育過程を経ることが明らかにされている．その特徴は，1）発育初期に眼や口，頭部が非常に大きくなる，2）前鰓蓋骨に棘が発達する，3）成長に伴い肛門が後退する，などである．このような外部形態から，マグロ類を含めたサバ型魚類仔稚魚は，"large prey-fast growth"という生残戦略を備えると推測されてきた．当初，分類学的，形態学的研究から始まったマグロ類の初期生活史に関する研究は，近年では食性や成長解析などの生態学的側面にまで発展しつつある．しかし，マグロ類の飼育実験には大きな困難を伴うことから，発育・成長に伴う詳細な形態・生理・生化学的研究はみられなかった．

　　クロマグロ *Thunnus thynnus* は将来の栽培漁業および養殖における最重要魚種の一つであり，近年さかんに種苗生産が試みられている．しかし，多大な努力が払われているにもかかわらず，生残率は不安定であり，概して非常に低い現状にある．本種は先に述べたように特徴的な外部形態発育を経ることから，その内部形態や生理・生化学的側面も特異な発育過程を示すものと推測される．現在までに確立されている仔稚魚の飼育技術は，主に沿岸性の重要魚種で得られた知見に基づいたものである．したがって，クロマグロ種苗生産技術確立のためには，本種の初期発育に関する多面的な知見を集積し，それに基づいた飼育技術を確立することがとりわけ重要である．本種の初期発育を多面的に理解することは，種苗生産技術の確立だけでなく，サバ型変態の初期生残戦略を理解する上でも不可欠である．本研究は，クロマグロと，クロマグロの先行種として飼育が行われたキハダ *T. albacares* をモデル魚種として，マグロ類仔稚魚の初期発育を多面的に明らかにすることを目的とした．マグロ類仔稚魚の研究において，最大の障害は生残率の低さによるサンプル数の制限である．本研究では，個体ごとに適用できる分析手法を主に採用し，限られたサンプルから最大限の情報を得ることに努めた．

　　第1章では，クロマグロとキハダの仔稚魚の成長と外部および内部形態発育，

特に消化系の発達過程を調べた．また，これらの研究から，両種の仔魚で鰾が正常に開かない未開鰾魚が高い割合で出現することが明らかとなったため，比較的開鰾魚の多かった飼育例のクロマグロ仔稚魚を用い，鰾の発達過程を組織学的に調べた．第2章では発育・成長に関わりの深い下垂体中の成長ホルモン産生細胞と消化酵素を取り上げ，これらの成長に伴う動態を調べた．第3章では生化学的側面として核酸量の変化を調べた．第4章では，クロマグロ仔魚の生残率向上に関わる実験的研究として，クロマグロとキハダの卵黄仔魚のハンドリング直後における生残率の逆密度依存性について調べた．

第1章：クロマグロとキハダ仔稚魚の成長と発育

1）クロマグロ仔稚魚の成長と外部および内部形態発育

　　クロマグロ仔稚魚の初期発育を把握する第一歩として，外部および内部形態，特に消化系の発達過程を調べた．本種仔魚はふ化後3日には発眼，開口し，摂餌を開始した．ふ化後11日(flexion phase)には胃腺が，ふ化後14日には幽門垂が分化した．ふ化後30日（体長12 mm）には背鰭および臀鰭の鰭条は定数に達し，外部形態的に稚魚へ移行するとともに脊椎骨の骨化が完了した．魚体各部位の測定の結果，いずれも体長8 -10 mmに屈曲点がみられた．本種の仔魚期における消化系の発達過程は，分離浮性卵よりふ化する一般的な海産魚よりかなり早く，仔魚期の半ばには成魚の基本型に達することが明らかとなった．

2）キハダ仔稚魚の成長と外部および内部形態発育

　　キハダ仔稚魚を飼育し，外部および消化器官の発達を観察した．ふ化直後に体長2.65 mmであった仔魚は，ふ化後37日には27.68 mmに成長した．摂餌開始はふ化後4日，外部形態から判断した稚魚への移行はふ化後30日にみられた．ふ化後14日には胃腺が，16日には幽門垂が分化し，成魚様の消化系が確立した．その後胃腺数等の量的形質には著しい発達がみられた．本種においても外部形態の変化に先行する形で消化系が発達し，これがその後の魚食性への転換と速い成長を支える内的要因の一つと考えられた．

3）組織学的観察によるクロマグロの鰾の発達過程

　　1），2）における組織学的観察から，人工飼育されたマグロ類仔魚の大半

は，鰾が正常に開かない未開鰾魚であることが判明した．そこで，比較的開鰾魚が多くみられた1998年の飼育魚を用いて，クロマグロ仔稚魚の鰾の発達過程を組織学的に調べた．鰾原基はふ化後2日の卵黄仔魚において，胃原基後方背面から膨出して形成され始めた．その後ふ化後6日までに，鰾は拡大して楕円型となり，ガス腺，気道，奇網血管が確立するなどの分化がみられた．これらの開鰾に必要な基本構造が確立した直後の，ふ化後7日に初めて開鰾魚が認められた．したがって，摂餌開始から数日後が本種の開鰾時期であると推定された．しかし，本飼育例でも未開鰾魚がみられ，その鰾内腔は肥大した上皮細胞で占められていた．気道の消失時期と消化系の構造における早期発達から，クロマグロ仔魚における開鰾可能期間が短いことが推測され，このことが本種での未開鰾魚の高い出現率の一因と考えられた．

第2章：クロマグロとキハダ仔稚魚の成長と発育における生理学的側面

1）キハダ仔稚魚の成長ホルモン産生細胞の発達

　キハダ仔稚魚の脳下垂体および成長ホルモン（GH）産生細胞の発達を組織学的・免疫組織化学的手法を用いて調べた．脳下垂体はふ化後2日目に組織学的に識別され，ふ化後16日にはほぼ下垂状態となった．GH産生細胞はふ化後4日の摂餌開始時から免疫組織化学的に検出された．GH産生細胞群の脳下垂体に対する体積比（％GH）は摂餌開始後3日間は非常に高かった．その後％GHは急減し，低い値で推移した後，postflexion期から稚魚期にかけて著しく上昇した．同様の傾向はクロマグロ仔魚においてもみられた．マグロ属仔稚魚は他の海産魚に比べて高い％GHを示し，仔魚期の後半から高い成長ポテンシャルを持つことが推測された．

2）クロマグロ仔稚魚の消化生理の発達

　　　消化生理は，仔魚の成長に深くかかわる重要な生理的側面である．近年，個体レベルでの分析が可能である高感度のトリプシン酵素活性の測定法が開発され，いくつかの魚種で知見が得られている．本節ではこの手法をクロマグロ仔稚魚に応用し，発育に伴うトリプシン酵素活性の動態を調べるとともに，免疫組織化学的手法を用いて胃腺の観察を行った．1個体当たりのトリプシン酵素活性は，摂餌開始時にかけて上昇し，その後は成長に伴って増大した．体重当たりの活性には3つのピークがみられ，これは発育の段階的進行と良く対応した．1つ目はふ化後3日の摂餌開始時にみられ，胃が機能化していない仔魚の摂餌開始にとって重要な意味を

持つものと思われた．2つ目のピークはふ化後14日のflexionフェーズに見られ，この時期には胃腺の機能化や巨顎化とともに仔魚の捕食，消化能力が飛躍的に上昇することが示唆された．3つ目のピークは最も大きく，稚魚への移行期にみられ，組織学に観察された消化系の著しい量的発達と良く対応し，この時期の活発な遊泳，摂餌と体成長を支える大きな要因と推定された．

第3章：クロマグロとキハダ仔稚魚の成長・発育に伴う核酸・タンパク量の変化

クロマグロとキハダ仔稚魚の発育・成長に伴うDNAおよびRNA量の変化を個体レベルで調べた．クロマグロ仔魚ではタンパク量の分析も行った．1個体当たりのDNA量は成長に伴い一貫して増加傾向にあった．一方，RNA量はふ化から摂餌開始までは一定の値で推移し，その後仔魚期の終わりまでDNA量と同調して増加した．タンパク量の増加傾向はRNA量の変化と良く一致した．稚魚への移行とともにRNA量の増加は加速化された．RNA/DNAは摂餌開始時に低下した後，仔魚期を通してほぼ一定した低い値で推移し，その後稚魚への移行期から急激に上昇した．これらの変化パターンは両種で良く類似しており，マグロ属仔稚魚に共通した傾向であると推測された．マグロ類仔稚魚の発育過程をこれらの核酸比の変化から概観すると，活発な細胞分裂によって組織や器官の形成に重点が置かれる時期（ふ化から仔魚期の終わりまで）と細胞肥大による体成長に重点が置かれる時期（稚魚への移行期からそれ以降）に区分できると考えられた．

第4章：クロマグロおよびキハダ卵黄仔魚のハンドリング直後における死亡の逆密度依存性

クロマグロとキハダの卵黄仔魚について，ハンドリング直後の死亡と仔魚密度との関係を実験的に究明した．

キハダとクロマグロ，ならびに比較対照としてスジアラ *Plectropomus leopardus* 卵黄仔魚を，パスツールピペットを用いて様々な密度になるように細胞培養用プラスチックシャーレに移槽した．その後，無給餌状態で12時間ごとにへい死を計数した．経時的な死亡のパターンは魚種により異なったが，3種とも移槽後12時間以内にハンドリングの影響と思われる死亡の増大が認められた．このとき，予想に反して仔魚密度が高いほど生残率が高くなる傾向がすべての魚種で認められた．特に，キハ

ダとスジアラのきわめて高密度の実験区（10-30尾/ml）では，ハンドリングによる移槽直後の死亡はほとんどみられなかった．同様の現象はヒラメでも確認されており，分離浮性卵からふ化する海産魚では一般的な現象である可能性が示された．

Acknowledgments

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