

# Development of tissues involved in eye migration and role of thyroid hormone in metamorphosing Japanese flounder (*Paralichthys olivaceus*)\*<sup>1</sup>

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## 1.1 Asymmetrical development of bones and soft tissues during eye migration of metamorphosing Japanese flounder

The symmetrical body of flatfish larvae dramatically changes into an asymmetrical one after the metamorphosis. Eye migration results in the most significant asymmetrical development seen in any vertebrate. To understand the mechanisms involved in eye migration, bone and cartilage formation was observed during metamorphosis in laboratory-reared Japanese flounder using whole body samples and histological sections. Most of the hard tissues of the cranium (parasphenoid, trabecular cartilage, supraorbital canal, and supraorbital bar) exist symmetrically in the larval period before metamorphosis, and develop twisting in the same direction as the eye migrates. Increase of skin thickness beneath the eye was observed only on the blind side at the beginning of eye migration; this is the first definitive difference between the right and left sides of the body. The pseudomesial bar (Pb), a peculiar bone present only in flatfishes, develops from this thick skin, and grows dorsad. Novel sac-like structures were found, and named retrorbital vesicles (Rvs). The Rv of the blind side grew larger and faster than that of the ocular side when the right eye moved most drastically, whereas no difference was observed between the volume of right and left connective tissue in the head. Asymmetrical presence and growth of the Pb together with inflation of the Rv on the blind side may be responsible for right eye migration during metamorphosis of Japanese flounder.

## 1.2 Fine structure of soft and hard tissues involved in eye migration in metamorphosing Japanese flounder

The body of a Japanese flounder changes from a symmetrical to an asymmetrical form during metamorphosis. To obtain detailed information on the mechanisms of the migration of the right eye to the left side, soft and hard tissues in the head of larval flounders were examined using transmission electron microscopy (TEM). Rvs are pairs of sac-like structures under the eyes. It has been suggested that the asymmetrical development of Rvs, with the right (blind) one being bigger than the left, is the driving force behind eye migration. The present study revealed that the ultrastructure of the Rv sheath is quite similar to that of a lymphatic capillary. Thus, it is possible that the Rv is a part of the lymph system, and is probably related to the secondary vascular system in teleosts. If I assume that the Rv sheath has a high permeability to liquid, similar to lymphatic capillaries, it is not plausible that the active expansion of the Rv pushes the eyeball. On the other hand, the Pb is a bone that is unique to flounders and is present only on the right (blind) side. At the beginning of eye migration, an aggregation of fibroblast-like cells is observed in the dermis under the right eye, where the Pb will subsequently be formed. These cells have a well-developed rough endoplasmic reticulum (rER) and mitochondria, and are probably responsible for the thick layers of collagen fibrils around them. Since it is unlikely that the active expansion of the Rv causes eye migration, the role played by the Pb and its rudiment in right eye migration in the Japanese flounder becomes more significant.

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(京都大学審査学位論文を基本としその一部を改変)

\*<sup>2</sup>変態期ヒラメの眼の移動における周辺組織の発達とそれに及ぼす甲状腺ホルモンの働き

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## **2.1 Histological study of deformity in eye location in Japanese flounder**

The internal structure of malformed Japanese flounder juveniles in eye location was histologically examined and deformed bones were identified as the tissue important for eye relocation. A deformed Pb was the common feature for individuals with abnormal eye location (AEL individuals). Individuals with mild AEL had undeveloped Pb and those with serious AEL had no Pb or two Pb on both sides. When the Pb was absent, left-right asymmetry of the other hard tissues in the head region was disordered. For these reasons, Pb was considered important for eye relocation, and the possibility to define the left-right asymmetry of other tissues was suggested. The skin beneath the right eye and just outside the Pb is thickened in all normal individuals, while this skin was not thickened in most Pb-absent individuals, suggesting the possibility of the presence of upper control of Pb formation by the skin.

## **2.2 Insufficient ability to synthesize thyroid hormone in abnormal juveniles during seed production of the Japanese flounder**

In flatfish hatcheries, various types of deformities occur during metamorphosis, at a relatively high ratio in some species. Since thyroid hormones (THs) are known to be essential for metamorphosis, the thyroid system was examined in a type of abnormal fish accounting for 4% of juveniles of the Japanese flounder observed in this study. From comparisons of external and internal structures between normal and abnormal fish, stasis during the metamorphosis was suggested as a fundamental reason for the abnormality, and malformed thyroid glands were found in the abnormal fish. In addition, serum thyroxine (T4) concentrations in the abnormal fish were reduced to less than 1/10 of that of normal. In order to examine the responsiveness of the abnormal fish to TH, T4 (0.1 ppm) and thiourea (TU, antithyroidal agent, 30 ppm) were administered. After 14 days of T4 treatment, all the abnormal characteristics disappeared, and fish recovered to normal, suggesting normal responsiveness to THs in peripheral tissues. In combination with the observation of thyroid follicles in T4 or TU treated fish, an insufficient ability of TH synthesis was suggested, and retardation of metamorphosis was induced by lowered TH levels in the abnormal fish in this specific case.

## **3.1 Bone development during metamorphosis of the Japanese flounder: differential responses to thyroid hormone**

The larvae of flatfish change their body structure during metamorphosis, including dramatic translocation of one of the eyes from one side of the body to the other. Such metamorphic processes are in general promoted by THs. This study focuses on the response of individual tissues to hormones, and morphological characteristics were examined in hormone-deficient larvae of the Japanese flounder. Treatment of flounder larvae with an inhibitor of TH synthesis, TU (30 ppm) inhibited translocation of the right eye, shortening of dorsal fin rays, and body pigmentation, as previously reported. Treatment also inhibited elongation of the Pb, which is important for eye translocation, and formation of bones related to larval or juvenile characteristics, the actinost and the distal radial of the pectoral fin, and the pterygiophore of the anal fin. Bones other than these, which are similarly present in both larvae and juveniles, were unaffected. These results suggest the differential responsiveness of bones to TH deficiency during early developmental stages, which seems to play a significant role in the morphological changes from larvae to juveniles. To examine the period of hormone responsiveness, T4 treatment (0.1 ppm for 2 weeks) was started at two different daily ages for the fish receiving TU treatment. When T4 was given 2 weeks later than TU, the fish completely metamorphosed through the expected bone formation that had been suppressed by TU. However, when T4 treatment was started 4 weeks later than TU, body pigmentation did not occur, and the translocation of the right eye was not complete due to failure of Pb formation. It is suggested that individual tissues that change during metamorphosis have their own timing in response to THs.

## **3.2 Effect of stage-specific thyroidal stasis on the eye migration of Japanese flounder**

Since TH is the main stimulator of eye migration in flounder metamorphosis, the timing of TH responsiveness was determined in the tissue responsible for eye migration in Japanese flounder. TU (30 ppm, an inhibitor of TH synthesis) was administered starting at different stages of metamorphosis, and the inhibitory effects on the tissue related to eye migration were histologically examined. When TU treatment was started before the E stage, eye migration was significantly inhibited due to lack of formation of the Pb, a bone important for eye migration. When treatment started after the F stage, the process was not significantly inhibited

due to the minor inhibitory effect on Pb formation. This result indicates a specific requirement by stage F for TH in the Pb. These results suggest that the thyroidal status during the early phase of metamorphosis is extremely crucial for the Pb formation and therefore important for the completion of eye relocation in Japanese flounder, although the process of eye migration is gradual and continuous.

Key words : Japanese flounder, metamorphosis, bone development, asymmetry, pseudomesial bar, deformity, abnormal fish, and thyroid hormone

## General Introduction

The Japanese flounder is the most commercially important Pleuronectid flatfish in Japan. In this species, the early life history in nature<sup>1,2)</sup> and the developmental process in rearing tanks have been elucidated<sup>3,4)</sup>, and protocols for rearing have been established. In addition, physiological information during metamorphosis has been accumulated<sup>5)</sup>.

The asymmetry of flatfishes is the most significant among vertebrates. In adult or juvenile flatfish, both eyes locate on the same side (the ocular side) of the body, whereas no eyes locate on the other side (the blind side). Pigment cells for dark colors are present only on ocular side, and mostly absent on blind side. In addition, the scales of the ocular side are ctenoid, while those of the blind side are cycloid in a large number of genera in flatfish<sup>6)</sup>. These characteristics differentiate during the metamorphosis from bilaterally symmetric larvae to asymmetric juveniles.

Eye migration also occurs during the metamorphosis, as one of the events of asymmetrical development. Several attempts have been made to elucidate the way in which the eye moves from observations on metamorphosing larvae<sup>7-18)</sup>. Based on literatures and his own observation, Brewster<sup>19)</sup> summarized the process of asymmetrical cranium formation as the relocation of the anterior blind side "frontal" (the Pb, in this study) to the ocular side, enforced by the enlargement of the blind side "lateral ethmoid". However, the direct cause for eye relocation was still unclear, and therefore the developmental process of the tissues of cranium were needed to be studied. In Chapter 1, taking advantage of laboratory-reared samples of Japanese flounder, I have started my study from the detailed description on the developmental process of bones and cartilage in relation to eye migration.

In large-scale hatcheries for seed production of flatfishes, significant occurrence of abnormal juveniles has been observed<sup>20,21)</sup>, at a ratio much higher than those in sea-caught adults<sup>6,22-26)</sup>. There are two major types of abnormality in flatfish, abnormality in body coloration and abnormality in

eye location. A large volume of information has been accumulated on abnormal and normal process of body coloration. Technical progresses, based on the understanding of the mechanism of coloration, have enabled fish farmers to reduce the occurrence of this type of abnormality<sup>27,28)</sup>. In contrast, information on abnormality in eye location is very limited. Presence of two types of abnormality in eye location was reported in brown sole (*Pleuronectes herzensteini*)<sup>29)</sup>. Both eyes move dorsad in one type, while both eyes do not migrate in the other type. The presence of abnormality in eye location has been reported in sea-caught individuals of many flatfish species<sup>30)</sup>, and the relationship with the abnormality in body coloration has been reported. Higher occurrence of abnormality in eye location was reported in albino fish of hatchery-reared mud dab (*Pleuronectes yokohamae*)<sup>31)</sup> and frog flounder (*Pleuronichthys cornutus*)<sup>32)</sup>, while the direct relation of the two was questioned in Atlantic halibut (*Hippoglossus hippoglossus*)<sup>33)</sup>. However, effective methods for reducing the occurrence of this abnormality have not been established, probably due to the lack of information on the morphological characteristics of the abnormal individuals. The first detailed examination on individuals with abnormal eye location was carried out at individual tissue level in Chapter 2.1.

The significance of THs in flatfish metamorphosis has been extensively demonstrated at the whole body level<sup>34,35)</sup> and at the individual tissue level; erythrocytes<sup>36)</sup>, skeletal muscle<sup>37)</sup>, gastric glands<sup>38-40)</sup> and chloride cells in the gill<sup>41)</sup>. Therefore, eye migration is also expected to be under the stimulatory control of THs. In anuran metamorphosis 3,3',5-triiodo-L-thyronine (T3) induced ossification in stage- and dosage-dependant manner<sup>42)</sup>, and chondrogenesis in a dosage-dependent<sup>43)</sup>. Stage-specific and tissues-specific sensitivity to T4 was also shown for ossification and chondrogenesis in urodele metamorphosis<sup>44,45)</sup>. At present however, no research has been carried out on flatfish metamorphosis focusing on bones in head and their control. It is crucial to understand the controlling mechanisms of bone formation, especially in head

region during metamorphosis, to establish the effective methods for reducing the occurrence of abnormality in eye location. The effect of TH deficiency on bone formation, as well as the tissue responsiveness to the hormone, was examined in normal (Chapter 3) and accidentally occurred abnormal (Chapter 2.2) individuals of Japanese flounder.

## Chapter 1.1

### Asymmetrical development of bones and soft tissues during eye migration of metamorphosing Japanese flounder

#### Introduction

The asymmetry seen in flatfishes is the most significant among vertebrates. The eyes become located only on one side (the ocular side) of the body, whereas the other side has no eye (the blind side). The colors and scales of the skin are also different between the two sides<sup>46)</sup>. These characteristics differentiate during the metamorphosis from bilaterally symmetric larvae to asymmetric juveniles<sup>47)</sup>. Migration of the eye occurs at the beginning of asymmetrical development.

Several attempts have been made to elucidate the way in which the eye moves based on observations on metamorphosing larvae mostly after whole-body processing with clearing and staining techniques for bone and cartilage<sup>7-18)</sup>. Brewster<sup>19)</sup> reviewed the literature and re-evaluated several hypotheses. To understand the way in which the eye migrates, detailed observations of the morphological changes in bones, cartilage, and soft tissues are indispensable. However such observations are difficult to conduct with the available methods.

The Japanese flounder is the most commercially important Pleuronectid flatfish in Japan. Its early life history in nature<sup>1,2)</sup> and developmental process in rearing tanks have been elucidated<sup>3,4)</sup> and protocols for the rearing of the species have been established. Moreover, physiological information during metamorphosis has been accumulated on the Japanese flounder<sup>5)</sup>, suggesting that the species would be a good model for studying the ontogeny of the asymmetry of flatfishes at the molecular level.

In this study, I have taken advantage of laboratory-reared samples to describe the detailed developmental process of bones and cartilage by using two methods; (1) double staining of whole-body samples and (2) microscopic observation after thin sectioning in histo-resin. In the latter,

plastic sections can provide greater insights because they typically introduce fewer artifacts and can provide a better understanding of anatomical structure and relationships than the method of double staining of whole-body samples. The contributions of hard and soft tissues in the head are discussed in relation to the process of eye migration. The "retroorbital vesicle (RV)", a novel structure, and the "pseudomesial bar (Pb)", a characteristic bone present only in flatfishes, is considered to be especially important components for right eye migration.

#### Materials and Methods

##### Experimental Animals

Fertilized eggs of Japanese flounder were obtained from the Miyazu Station of the Japan Sea Farming Association, Miyazu, and transported to the Fisheries Research Station of Kyoto University, Maizuru. They were stocked in a 500 L plastic tank at a density of 80,000 eggs per tank at 18°C. More than 40% of eggs hatched 3 days later, and the larvae were fed on the rotifer (*Brachionus rotundiformis*) twice a day for the first 20 days. Newly-hatched *Artemia* nauplii of Utah strain were also provided from 10 days after hatching (DAH).

The classification of developmental stage follows that of Minami<sup>16)</sup>, in which right eye migration starts at stage E and finishes at stage I.

##### Double staining of bones and cartilages of whole body samples

Five fish of each developmental stage (F-I) were fixed in 70% ethanol, cleared and double-stained with alcian blue and alizarin red 'S', following the standard method described by Dingerkus and Uhler<sup>48)</sup>. Using a drawing device attached to a stereomicroscope, cranial bones and cartilages were carefully identified and sketched using red for bones and blue for cartilages. Names of cartilages and bones are based on Amaoka<sup>49)</sup> and Matsuoka<sup>50)</sup>.

##### Microscopic detection of bones and cartilages

Three to five fish of each stage (E to I, covering the entire period of eye migration) were fixed in 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3-6 hours at room temperature, dehydrated through graded ethanols, and embedded in JB4 resin (Polysciences, Inc. PA U.S.A.). The head part around the eyes was transversely

sectioned at 2  $\mu\text{m}$  thick and stained with silver nitrate-toluidine blue. After the staining, cartilage undergoes metachromasy, becoming blue violet, whereas calcified bone becomes dark brown from silver-nitrate reaction. The adjacent sections were checked in order to make sure that the difference between the left and the right was not due to tilted sectioning.

### Volume measurement of soft tissues

Five fish at each F to I stages were used to examine the volume of soft tissues in head. From anterior (cranial) to posterior (caudal) edge of the eyeball, transverse sections of 2  $\mu\text{m}$  thick were selected at intervals of 100  $\mu\text{m}$  and stained with silver-nitrate toluidine blue. Outlines of the areas were traced, on right and left sides separately, with a drawing device attached to a light microscope. The area of connective tissue was distinguished from artificial cracks by the presence of materials positive to toluidine blue. The images were digitized with a flat bed scanner (Cano Scan 600, Canon, Tokyo Japan), and areas were measured on an Apple Macintosh computer using "NIH Image" (a public domain program, available at <http://rsb.info.nih.gov/nih-image/>). Volumes were calculated from the areas of each section and the thickness. Differences in the volume between right and left were tested with ANOVA.

## Results

Whole body samples of stage F to I were cleared and double stained with alcian blue and alizarin red. Developmental changes in cranial bones (red) and cartilages (blue) are illustrated from ocular and blind side in Fig. 1. In the figure, only the hard tissues related to eye migration are described.

Each stage was examined in detail using plastic-embedded sections stained with silver nitrate-toluidine blue, and photographs were taken from anterior (cranial) side of the section (Fig. 2). Therefore, the right side of the image corresponds to the ocular (left) side of the body. The right eye, situated in the left side of the image, migrates gradually to the right side with progress in the developmental stage. Some of the bones and cartilages are not visible in Fig. 1, but visible in Figs. 2, because of 3 dimensional locations and/or less sensitivity of the method.

Figure 3 indicates one of a pair of hitherto unreported sacs (the higher magnification of red square in Fig. 2H). This sac

appeared to be surrounded by a thin layer of cells and, in most cases, contained material(s) weakly stained with toluidine blue. These structures were named "retroorbital vesicles" (Rv).

The tissues appeared in the black square of Fig. 2, and related to eye migration were schematically summarized in Fig. 4.

### Development of cartilage and bone during eye migration Stage E (Figs. 2E, 4E)

The larva of stage E was almost symmetrical, although the right eye had just started to migrate, and therefore was situated slightly more dorsad than the left. The cartilage and the bone located at the bottom of the neurocranium were identified as the trabecular cartilage (Tc) and the parasphenoid (Ps), respectively. At the beginning of eye migration, they were located symmetrically, but grew larger twisting slightly toward the ocular side (clockwise on the figures) during eye migration thereafter. The flat bone plate on the top of head was the frontal (Fr). The Fr and Ps were only the cranial element already ossified prior to eye migration. A pair of cartilages located on the dorsal edge of eyes was the supraorbital bars (Sob). Loose connective tissue (C) filled the space around the eyes, bones, and brain.

### Stage F (Figs. 1F, 2F, 4F)

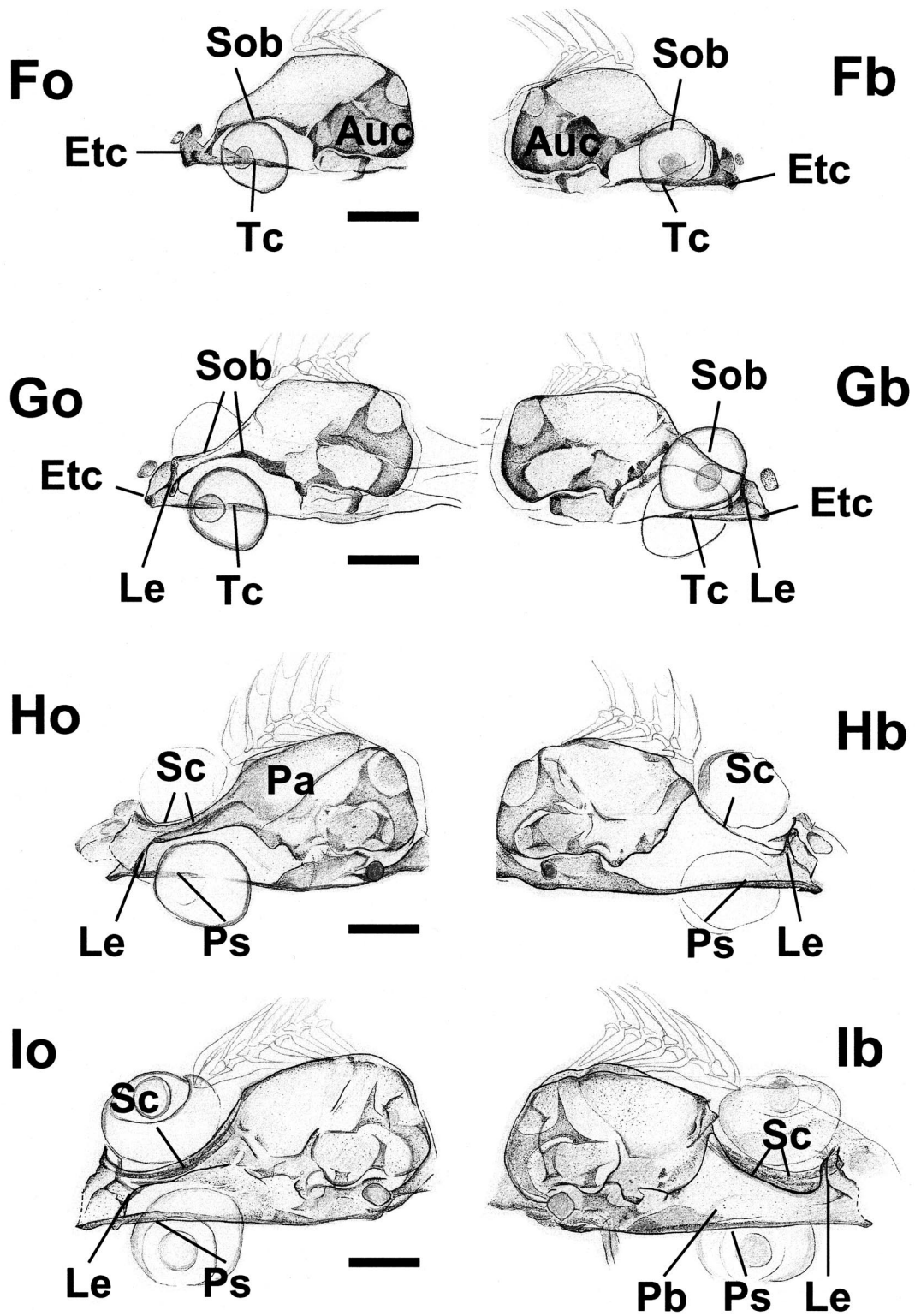
In stage F, the right eye moved towards the dorsal margin, but still could not be seen from the left (ocular) side. The two cartilages located anterior and posterior to eyes were the ethmoid cartilage (Etc) and the auditory capsule cartilage (Auc), respectively. A pair of hollow bones, supraorbital canal (Sc), appeared between the eyes forming the dorsal wall of the neurocranium. Only beneath the right eye (blind side, left side of the figure), an increase of skin thickness was observed. A pair of Rv first appeared beneath the eyes.

### Stage G (Figs. 1G, 2G, 4G)

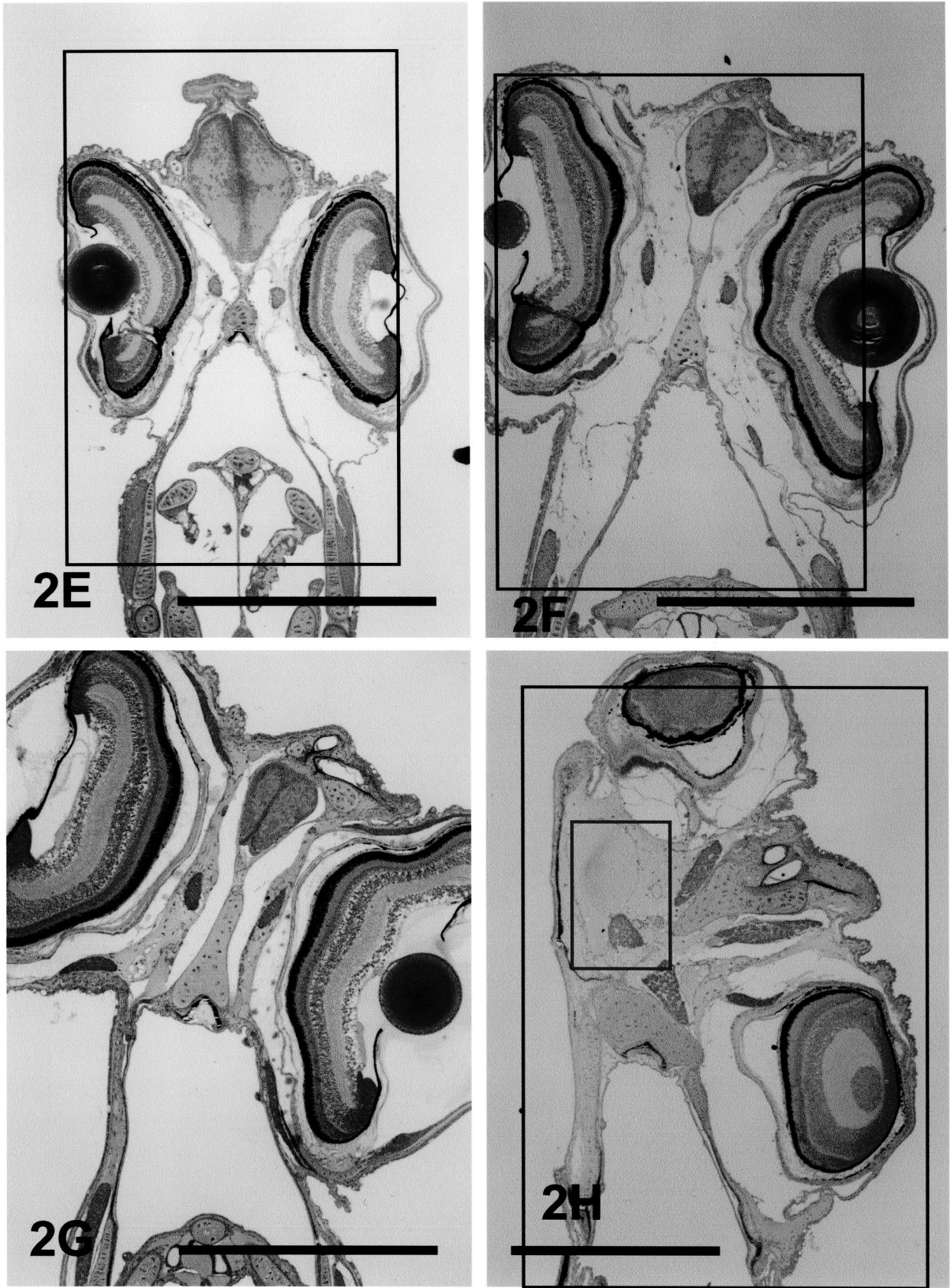
The right eye became visible from the ocular side in stage G, but had not reached the dorsal midline. In front of the eyes, a pair of bones covering right and left eyeballs from blind side was the lateral ethmoid (Le). In the sections anterior to Fig. 2G, it was observed that the right Le elongated dorsad, the left toward the ocular side, and the calcification of them just started at this stage.

### Stage H (Figs. 1H, 2H, 4H)

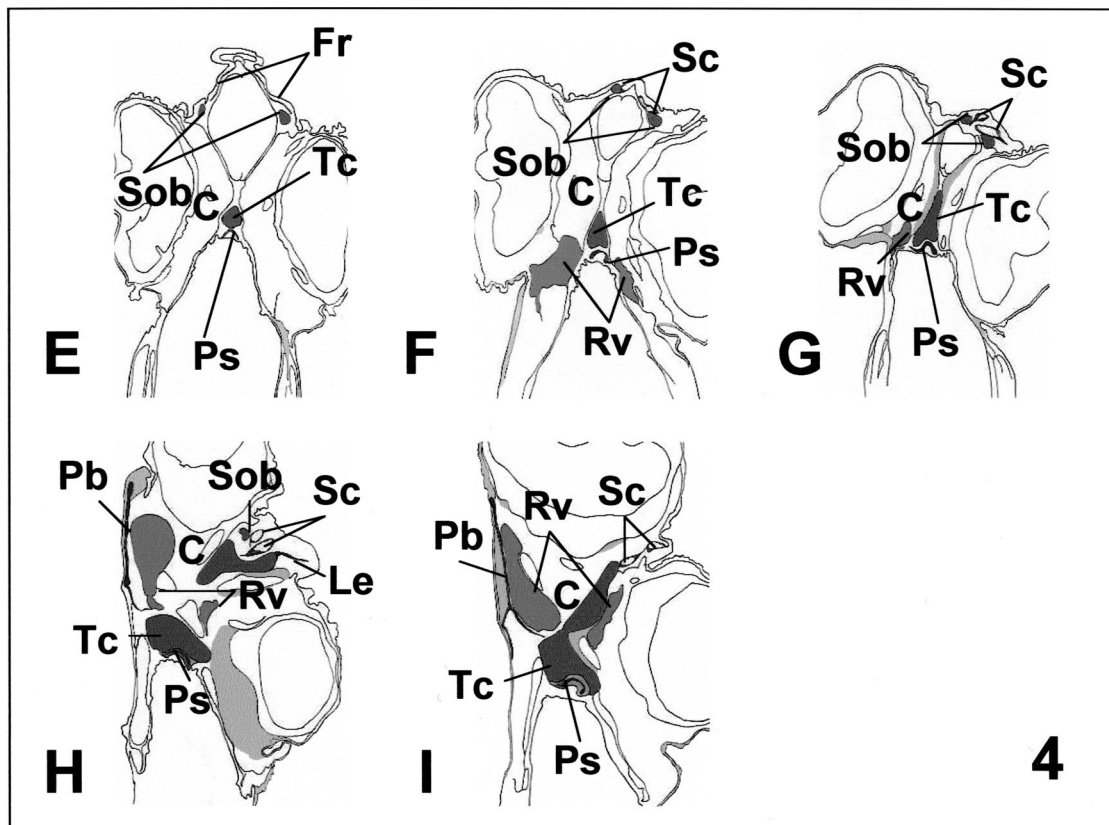
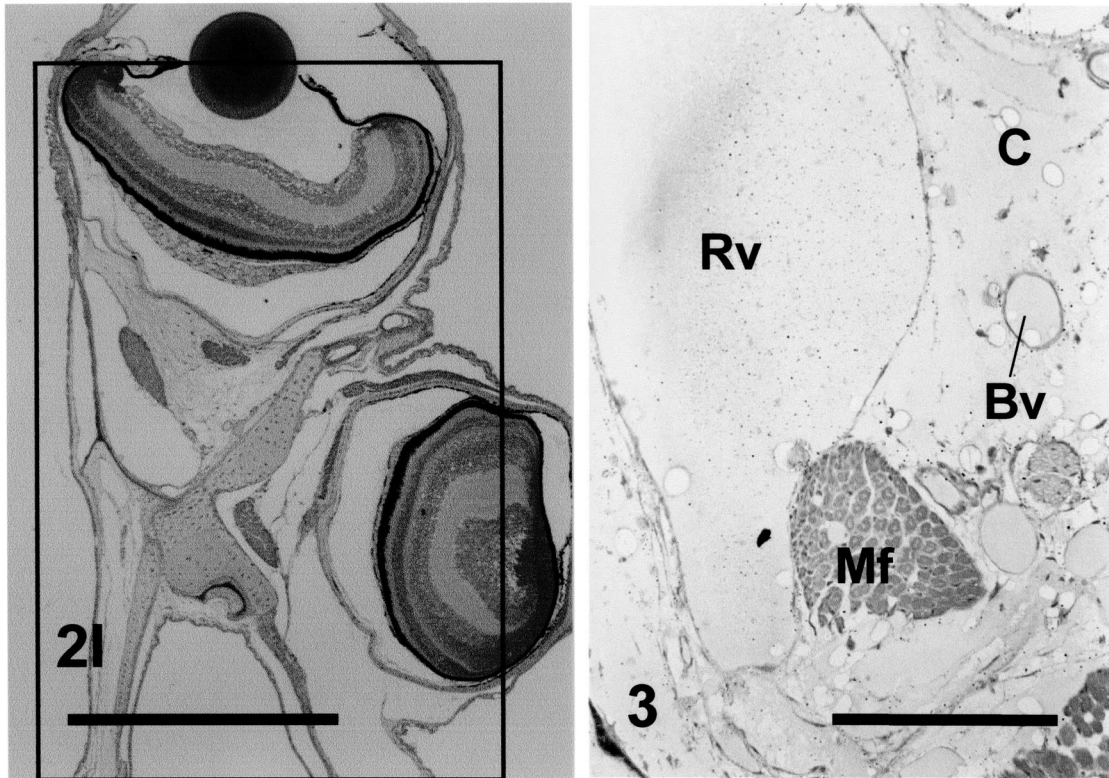
When the larvae reached stage H, the right eye was just on the dorsal margin. The Sob and the Sc twisted toward the ocular side. The characteristic bone present only on the blind



**Fig. 1** Development of neurocranium during eye migration in the Japanese flounder. Large characters (F, G, H, I) indicate developmental stages; o and b refer to the ocular left and the blind right side, respectively. Standard lengths of specimen are 7.4 mm (F), 8.4 mm (G), 9.0 mm (H), and 10.5 mm (I). Ossified bones are indicated in red and cartilages in blue, the colors seen after staining. [Auc auditory capsule (cartilage), Etc ethmoid cartilage, Le lateral ethmoid (bone), Sc supraorbital canal (bone), Pa parietal (bone), Pb pseudomesial bar (bone), Ps parasphenoid (bone), Sob supraorbital bar (cartilage) Tc trabecular cartilage]. Bars 0.2 mm



**Fig. 2** Transverse sections of the middle eye ball region of Japanese flounder. Developmental stages are indicated by large characters (black square area shown in Fig. 4, red square shown in Fig. 3). Bars 0.5 mm



**Fig. 3** Transverse section of the retrorbital vesicle on the blind side at stage H (Bv blood vessel, C connective tissue, Mf Muscle fiber, Rv retrorbital vesicle). Bar 0.1 mm

**Fig. 4** Schematic drawings taken from the black square in Fig. 2. Bones are indicated in red, cartilages in blue, retrorbital vesicles in green and artificial cracks in gray. The skin beneath the eyes is in light red. (C connective tissue, Le lateral ethmoid, Pb pseudomesial bar, Ps parasphenoid, Rv retrorbital vesicle, Sc supraorbital canal, Sob supraorbital bar cartilage, Tc trabecular cartilage). Bars 0.5 mm



side, forming the blind side wall of the cranium, was the Pb. Pb was formed as a calcified bone from the beginning in the thick skin beneath the eyes. Ossification of parietal (Pa) occurred.

### Stage I (Figs. 11, 21, 41)

At the end of eye migration, stage I, both eyes were finally located on the same side of the head. The left eye had slightly migrated ventrally. The Sob of the ocular side grew larger and fused with Tc, while that of the blind side disappeared. The blind-lateral side of the migrated eye was fully covered by the Pb.

### Growth of the retrorbital vesicle and connective tissue

Figure 5 shows comparative changes in the volumes of the Rv and C between the eyes during development. The first definitive appearance of Rv was at stage F; the right (blind) one ( $0.005 \text{ mm}^3$ ) being larger than the left (ocular,  $0.001 \text{ mm}^3$ ). At stage H, the volume of the right Rv increased to  $0.025 \text{ mm}^3$ , about five times larger than at stage G. During the metamorphosis, from stage F to stage I, the Rvs significantly increased their volume ( $P < 0.001$  by two-way ANOVA), and the right Rv was always significantly larger than the left one ( $P < 0.05$  by two-way ANOVA). The volume of the C between the eyes was constant at a level of  $0.01\text{--}0.02 \text{ mm}^3$  during stages G through H, and no difference was observed between the right and the left C. At stage I, the volume of right C increased until it was two times larger than that of stage H and that of left C at stage I. However, there was no significant difference between the right and left C at any

stage. The volume of the eyeball was about  $0.2 \text{ mm}^3$  at stage G and about  $0.4 \text{ mm}^3$  at stage I (data not shown).

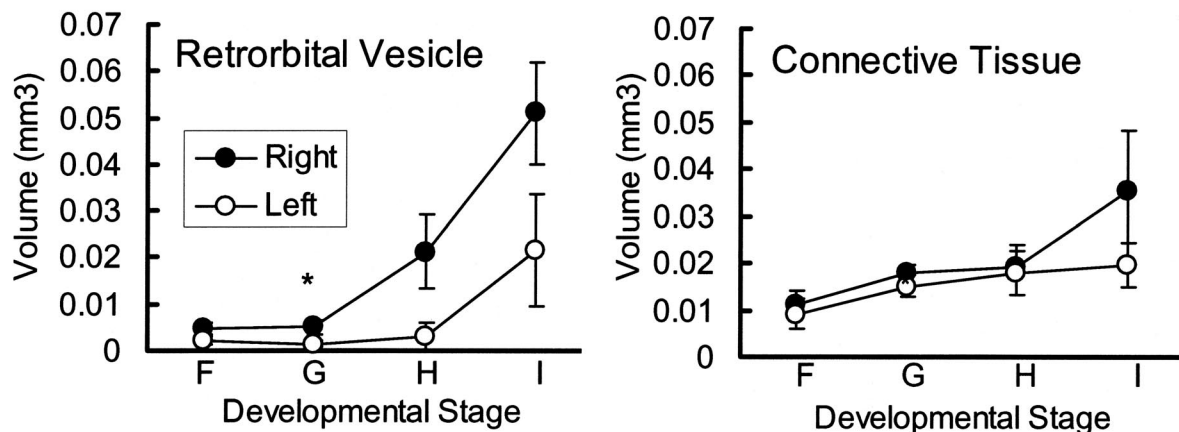
### Discussion

#### Development of the cranium

The development of tissues including bones during eye migration in the Japanese flounder was clarified in detail by the present study using stage-by-stage analyses of laboratory-reared larvae and early juveniles. Moreover, histochemical identification of ossified bones and cartilages on non-decalcified sections was performed by histo-resin embedding, providing the most powerful method to examine cranial development during metamorphosis.

In the past, Brewster<sup>19)</sup> observed the development of the cranium in several flatfishes in relation to eye migration based on the wild samples collected from the sea. He summarized his observations together with earlier literature as follows. The only cranial element ossified prior to eye migration was the Ps. After the beginning of eye migration, the ossification of the Frs took place from anterior to posterior, forming a wall ventral to the migrating eye. When the eye reached its new position, ossification of other cranial elements started. The asymmetry of the Pleuronectiformes cranium is mainly established by the relocation of the anterior part of the Fr from blind side to ocular side, and by the enlargement of the lateral ethmoid on the blind side.

My observations are mostly in accord with these of Brewster on the outline of the cranial development including the twisting of the Sc; however, his description of the "Le" is



**Fig. 5** Changes in volumes ( $\text{mm}^3$ ) of the retrorbital vesicle and the connective tissue from the anterior (cranial) to the posterior (caudal) edge of eyeballs. Solid circles represent values of the right (blind) vesicle, and open circles those of the left (ocular) vesicle (mean + SEM,  $n=5$ ). The retrorbital vesicles significantly increased their volume ( $P < 0.001$  by two-way ANOVA), and the right retrorbital vesicle was always significantly larger than that of the left ( $P < 0.05$  by two-way ANOVA).

not supported. Traquair<sup>7)</sup>, Kyle<sup>51)</sup>, Matsubara<sup>52)</sup> and Amaoka<sup>49)</sup> used a specific term "Pb" in flounders, for the bone corresponding to the "Le" of the blind side in Brewster's description. Since the Le and Pb developed from separate position each other, I used the name Pb, the specific name of the bone present only in flounders as in Traquair<sup>7)</sup>. The Tc, Ps, and Sc developed similarly and shared similar positions to those in other teleosts, but twisted toward the ocular side. A pair of Sob was first located symmetrically between the eyes at stage E, but one on the blind side disappeared at stage I. However, these bones do not seem to play an essential role in eye migration, because the distance between the eyes and these bones is relatively large.

On the other hand, the Pb, a specific and characteristic bone only present in flat fishes, exhibited the strongest asymmetry. This bone was present only on the blind side and became elongated intensively toward dorsal side. The formation of the Pb appears to push the right eye directly in the dorsal direction, or at least to prevent the right eye from the blind side from moving backward. However, ossification of the Pb started at stage G-H that is after the beginning of eye migration, at a time when the right eye had reached the margin of the dorsal line. Significant increase of skin thickness was observed in the future position of the Pb at the onset of eye migration: the first asymmetrical development observed in the flounder metamorphosis at all-or-none level. Whether the cell mass of the thick skin under the right eye produces a substrate strong enough to push the right eye at the beginning of metamorphosis needs to be clarified.

### Retroorbital vesicle

The Rvs are first identified and described in the present study. Since these structures were not bone or cartilage, the classical methods of double staining of whole-body samples could not detect their presence. Even microscopic observations of paraffin sections did not reveal them. They are illustrated by Kyle<sup>51)</sup> but difficult to distinguish from artificial cracks caused by sectioning or fixation. The possibility of their being artifacts is excluded by the presence of contents surrounded by a thin layer of cells stained positively with toluidine blue.

In Pleuronectidae, Brewster<sup>19)</sup> noted the presence of a sac-like structure in these terms: "a fluid-filled, sac-like structure situated posterior to the orbit", and suggested its involvement in the protrusion of the eyes at stages of metamorphosis and post-metamorphosis. It is possible that my Rv is the same

structure as Brewster's, but the lack of an illustration in his paper made the identification difficult. Brewster termed it the "ocular heart", following Agassiz's<sup>8)</sup> observation on Cynoglossidae.

The growth of the right Rv, located on the future blind side, was faster and greater than the left. There was no difference between the right and left volume of connective tissue around the eyes or among developmental stages. Inflation of the right Rv on the future blind side at the time of eye migration strongly suggests the involvement of the Rv in eye migration, probably by pushing the right eye to dorsal side. It is also possible that inflation of the right Rv is a "result" of right eye movement, filling up the empty space made by the migrating eye, since the volume increase in the right Rv ( $0.04 \text{ mm}^3$ ) does not seem to be large enough for pushing the eyeball ( $0.2\text{-}0.4 \text{ mm}^3$ ). Ultrastructural observation of the cells surrounding the Rvs will help me when considering the mechanisms of inflation, and the causal relationship between the inflation of the Rvs and eye migration.

### Asymmetry presented at metamorphosis

THs play essential roles in flounder metamorphosis (see review by Inui *et al.*<sup>5)</sup>). A sharp increase in T4 and T3 concentration begins at stages G-H<sup>35, 53, 54)</sup>. These stages are the times when most of the bones, cartilages, and Rvs begin asymmetrical development, which suggests contributions of THs to the asymmetrical developments of these tissue changes. However, the earliest asymmetry found in the future Pb place was as early as at stage F, much earlier than the T4 and T3 surges. Gradual elevation of T4 level at prometamorphic phase or a low level of T4 may still be important. TH sensitivity may appear earlier at the future Pb place where the increase of skin thickness occurred. As the order of formation of individual bones and cartilages was altered by T3 treatment in amphibians<sup>42, 43)</sup>, there may be differential responsiveness to T3 among individual bones and cartilages. Therefore, it is also possible that differential sensitivity to THs exists between right and left structures, resulting in asymmetry of development. As the cDNA of TH receptors has been cloned<sup>55-57)</sup>, the localization and ontogeny of the TH receptor, especially on the places of Pb and Rvs can now be investigated.

## Chapter 1.2

### Fine structure of soft and hard tissues involved in eye migration in metamorphosing Japanese flounder

#### Introduction

Substantial numbers of teleosts experience drastic changes in morphology and physiology from the larval to the juvenile stage<sup>47)</sup>. Flatfishes in particular change their body form dramatically, from symmetrical to asymmetrical. This form in adult flatfishes is the most dramatic right-left asymmetry among the vertebrates. The characteristic feature in flatfish metamorphosis is the migration of the eyes: one appears to migrate from one side to the other<sup>46)</sup>. In the aquaculture of flatfishes, abnormal eye location in juveniles is a serious problem because it leads to reduce production of normal juveniles and hence reduced survival rates after they are released into the sea<sup>58)</sup>. It is important to elucidate the mechanism of eye migration in order to improve rearing procedures and prevent the high occurrence of abnormal juveniles.

Brewster<sup>19)</sup> summarized the extensive literature regarding the external changes that occur during eye migration in flatfishes. In the Chapter 1.1, I described the internal process of eye migration in the Japanese flounder using light microscopy, and suggested that two tissues are especially important for this process. The Rvs, novel sac-like structures located under the eyes, grow asymmetrically, with the one on the right (blind) side being larger than that on the left (ocular) side. It was suggested that the expansion of the right Rv pushes the eyeball. However, the mechanisms of asymmetrical growth of the Rv (e.g., active or passive) were not clarified by light microscopy observations. Another characteristic structure was the Pb, a bone that is unique to flatfishes and is present on the right (blind) side<sup>7,49,51,52)</sup>. The calcification of the Pb occurs at the G-H stage, when the eye migrates drastically. At the beginning of eye migration (stage E), the thickness of the skin increases where the Pb will soon to be formed. Deformed Pbs have also been observed in juvenile Japanese flounders with abnormal eye location (Chapter 2.1). Thus, the Pb appears to be involved in eye migration. More detailed studies of the structure and development of the Pb are needed.

In this study, the developmental processes of eye migration were examined in detail using transmission electron microscopy (TEM), focusing on the Rv and the Pb.

#### Materials and Methods

##### Experimental Animals

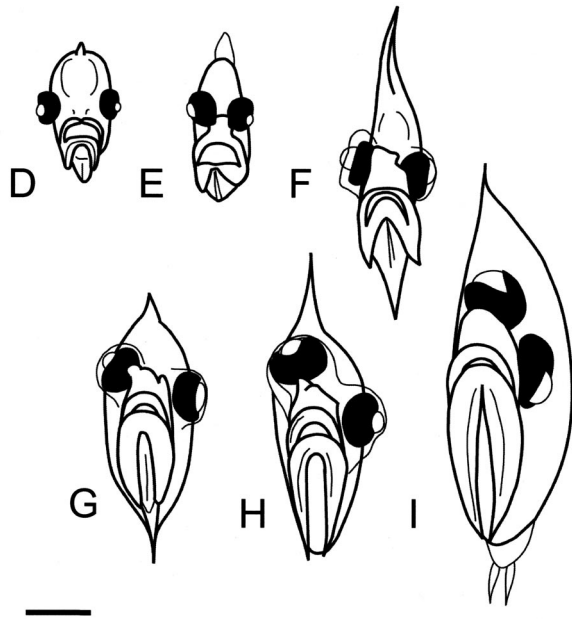
Fertilized eggs from Japanese flounders were obtained from the Miyako Station of the Japan Sea-Farming Association, Miyako, and transported to the Fisheries Research Station of Kyoto University. Sixteen thousand eggs were stocked in a recirculating 500 L tank at 14°C, under a photoperiod of 11L: 13D. About 50% of the eggs hatched 3 days later. Larvae hatched on that day were fed rotifer twice a day for the first 20 days. Newly hatched *Artemia* nauplii of the Utah strain were also provided 10 days after hatching. In this study, the developmental stages were classified according to Minami<sup>16)</sup> (e.g., right eye migration starts at stage E and is completed at stage I). To show an overview of eye migration during flounder metamorphosis, front views of larval flounder (stages D-I) were sketched after fixation using a stereomicroscope with a micrometer.

##### Electron Microscopic Study

Fish from stage D (before the start of eye migration) to stage I (end of eye migration) were anesthetized in MS-222 solution and decapitated. The heads were immersed in a mixture of 2% paraformaldehyde, 1% glutaraldehyde, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 0.1 M phosphate buffer (pH 7.4). The tissues were immediately irradiated with microwaves for 30 sec at 4°C, using a 500 W microwave oven (R-500T; Sharp, Osaka, Japan), and were further fixed for 5 hr at room temperature. The tissues were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). After dehydration in ethanol and then in propylene oxide, the tissue was embedded in Spurr's resin. Ultrathin sections were cut with an artificial-diamond knife, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM-100S (Tokyo, Japan) electron microscope.

#### Results

Figure 6 shows the process of eye migration during flounder metamorphosis. The larva with symmetrical eye location corresponds to stage D. The beginning and the final phase of eye migration correspond to stages E and H, respectively. Juveniles correspond to stage I. When the dorsal-ventral axis of the larval body was regarded as a reference point, it was observed that the whole head did not rotate; instead, the right eye migrated from the right side to



**Fig. 6** Schematic drawings of eye migration during the Japanese flounder metamorphosis. Bar 1 mm

the left side of the body during the metamorphosis. Figure 7 is a series of diagrams from the anterior side of the head section, based on light microscopy (Chapter 1.1), showing the Rvs, thickening of the dermis, Pb, and the location of electron micrographs described below.

#### Fine Structure of the Rv

The Rv sheath consisted of a single layer of flat cells about 0.5-1  $\mu\text{m}$  thick (Fig. 8). Inside the lumen of the Rv, amorphous material was found. The electron density of this material was intermediate, and not uniformly distributed. Fibroblast-like cells were present outside of, or attached to, the Rv sheath, as if constituting loose layers, nonuniformly, surrounding the Rv. The cell constituting the sheath had a flat nucleus and flat mitochondria. Little, rough endoplasmic reticulum (rER), and Golgi bodies or secretion granules were

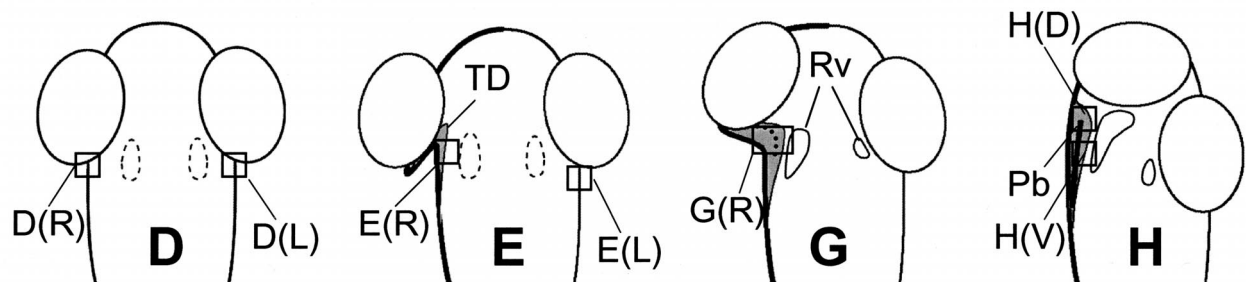
visible. Many vesicles were observed in the cytoplasm of the cell (Fig. 9). Some vesicles were isolated inside the cell. The membrane of some vesicles fused with the cell membrane, and these vesicles appeared as pits. The borders of adjoining cells overlapped with each other at cell junctions where cell membranes were closely placed (Fig. 10). A small number of white blood cells were found within the lumen of the Rv (Fig. 11), but there were no red blood cells.

#### Development of the Pb

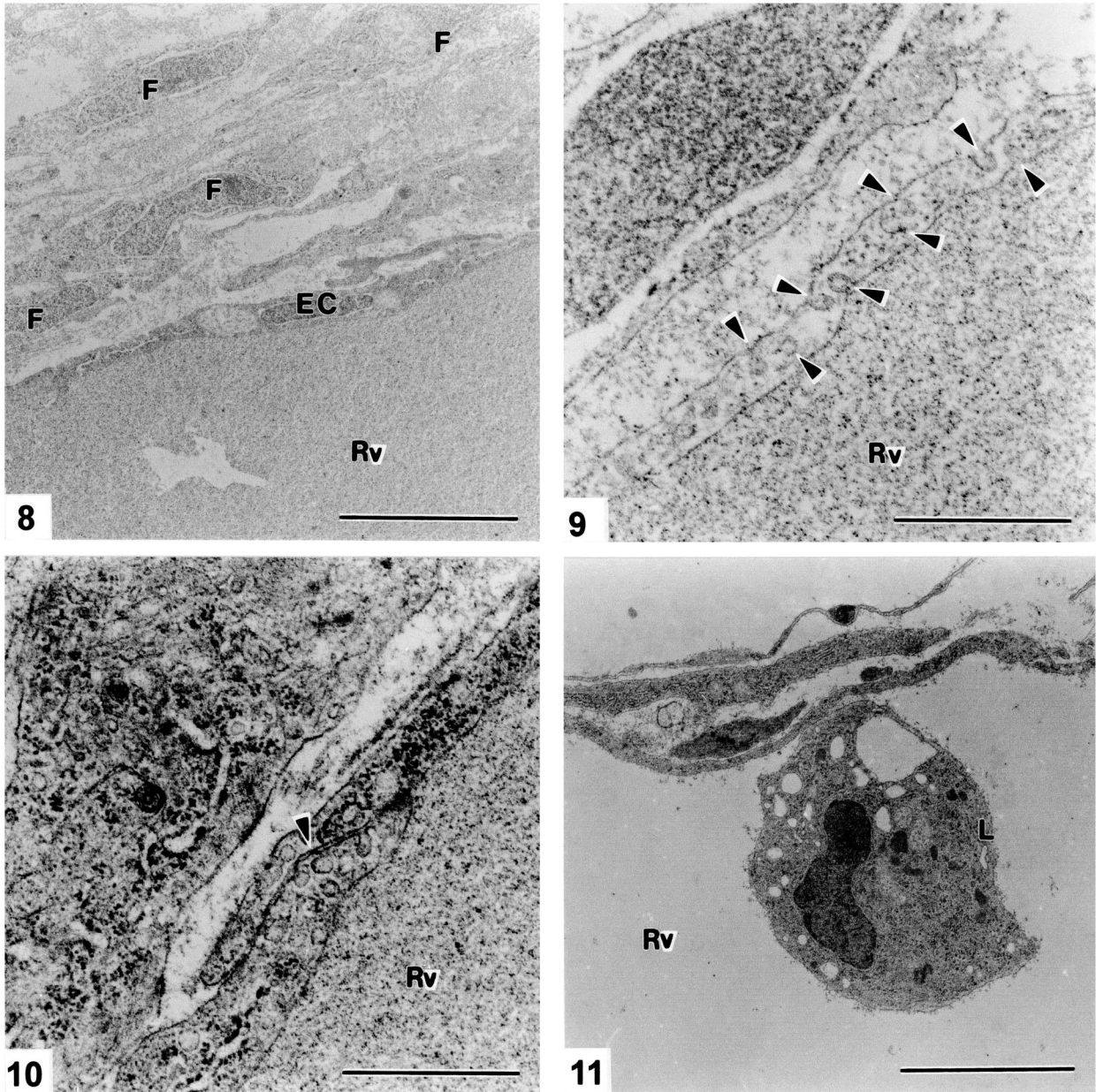
Before eye migration (stage D) there was no obvious difference, at the ultrastructural level, in skin structure between the right and the left eyes (Fig. 12D(R) and 12D(L)). An electron-dense basal lamina was present under the epidermis, together with collagen fibrils and a few fibroblastic.

At the beginning of eye migration (stage E), the skin beneath the right eye was thicker than that under the left (Figs. 7, and 12E(R) and 12E(L)). Only on the right side was there mesenchymal condensation of fibroblast-like cells with lobulated nuclei under the collagen fibrils. rER was developed in the fibroblast-like cells, and several layers of collagen fibrils were present around the cells. No similar thickening was visible on the left side. There was no remarkable change at stage F (data not shown). The structures were intermediate between stage E and G.

At stage G, the eye migration progressed, and thickened skin was found at the ventral side of the right eye (Fig. 7). At the cephalic part of the right eye, fibroblast-like cells aggregated under the epidermis, and the Pb was first observed as a piece of osteoid among the fibroblast-like cells. Osteoblasts were not distinguished as a distinct cell type. The fibroblast-like cells were polymorphic, but lobulated nuclei, abundant rER, and mitochondria were commonly observed in all cells (Fig. 13G(R)). Collagen fibrils were densely



**Fig. 7** Schematic drawing indicating the location of the Pb (thick black bar), Rv, and thickening of the dermis (TD, gray area) at stages D, E, G and H. The locations of the photograph in Figs. 12 and 13 are indicated.

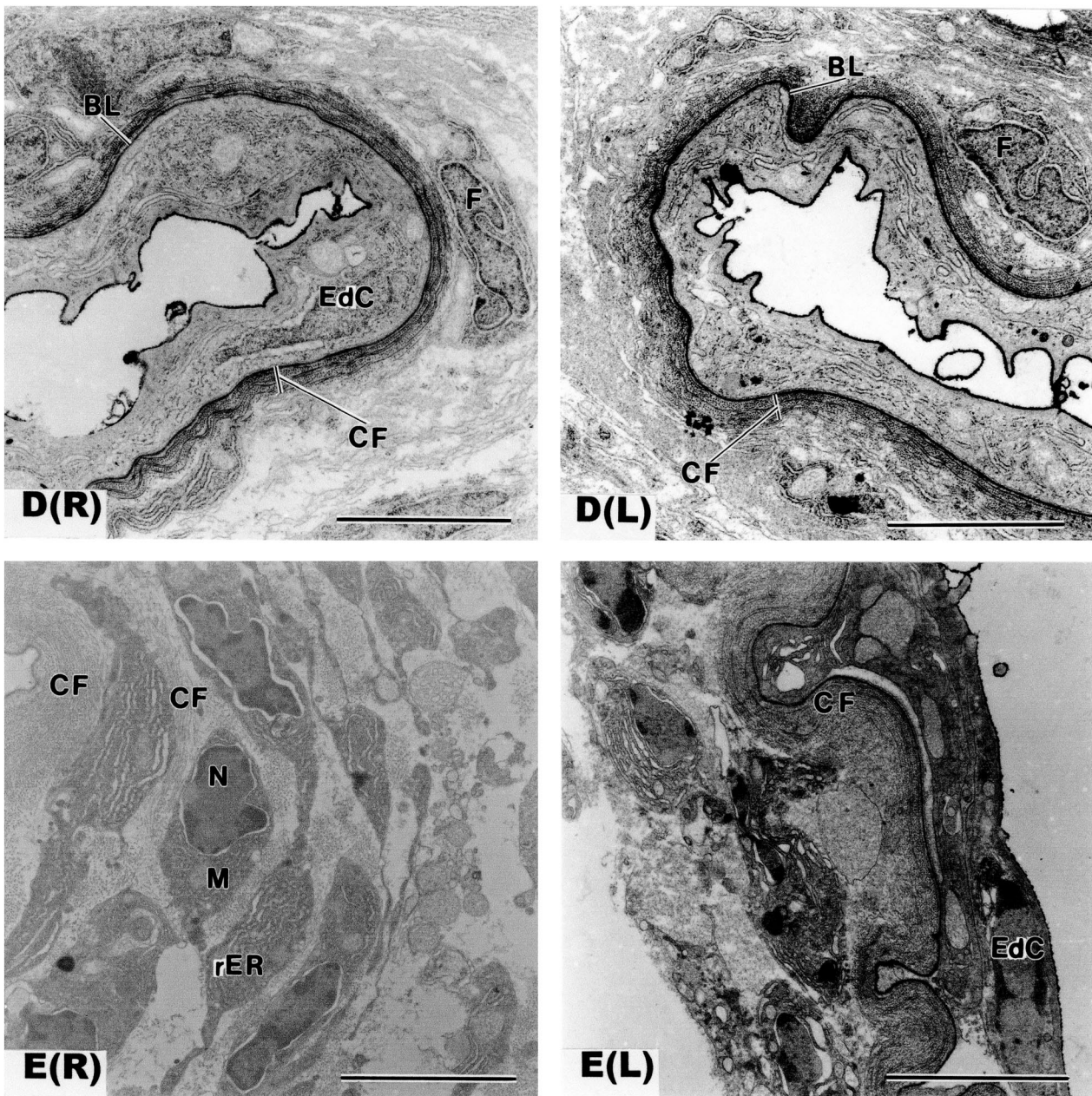


**Fig. 8** Electron micrograph of the Rv in the head region of Japanese flounder at stage H. The Rv sheath consists of a single layer of thin cells. F, fibroblast; EC, endothelial cell. Bar 5  $\mu$ m

**Fig. 9** Electron micrograph of the Rv in the head region of Japanese flounder at stage H. Pinocytotic vesicles (arrow heads) have openings on the endothelial cells of the Rv sheath. Bar 1  $\mu$ m

**Fig. 10** Electron micrograph of the Rv in the head region of Japanese flounder at stage H. There is a junction (arrow head) between the endothelial cells of the Rv. Bar 1  $\mu$ m

**Fig. 11** Electron micrograph of the Rv in the head region of Japanese flounder at stage H. A white blood cell (L, leukocyte) is present inside the Rv. Bar 5  $\mu$ m



**Fig. 12** Electron micrographs of transverse sections of skin beneath the eyeball of a metamorphosing Japanese flounder. D (R), stage D - right side; D (L), stage D - left side; E (R), stage E - right side; E (L), stage E - left side. The specific location of each photograph was shown in Fig. 7. BL, basal lamina; CF, collagen fibril; EdC, epidermal cell; F, fibroblast; M, mitochondrion; N, nucleus; rER, rough endoplasmic reticulum. Bars 5  $\mu$ m

distributed among the fibroblast-like cells. On the left side, as in stage E, the epidermis remained thin, and cells beneath the epidermis were rare (data not shown).

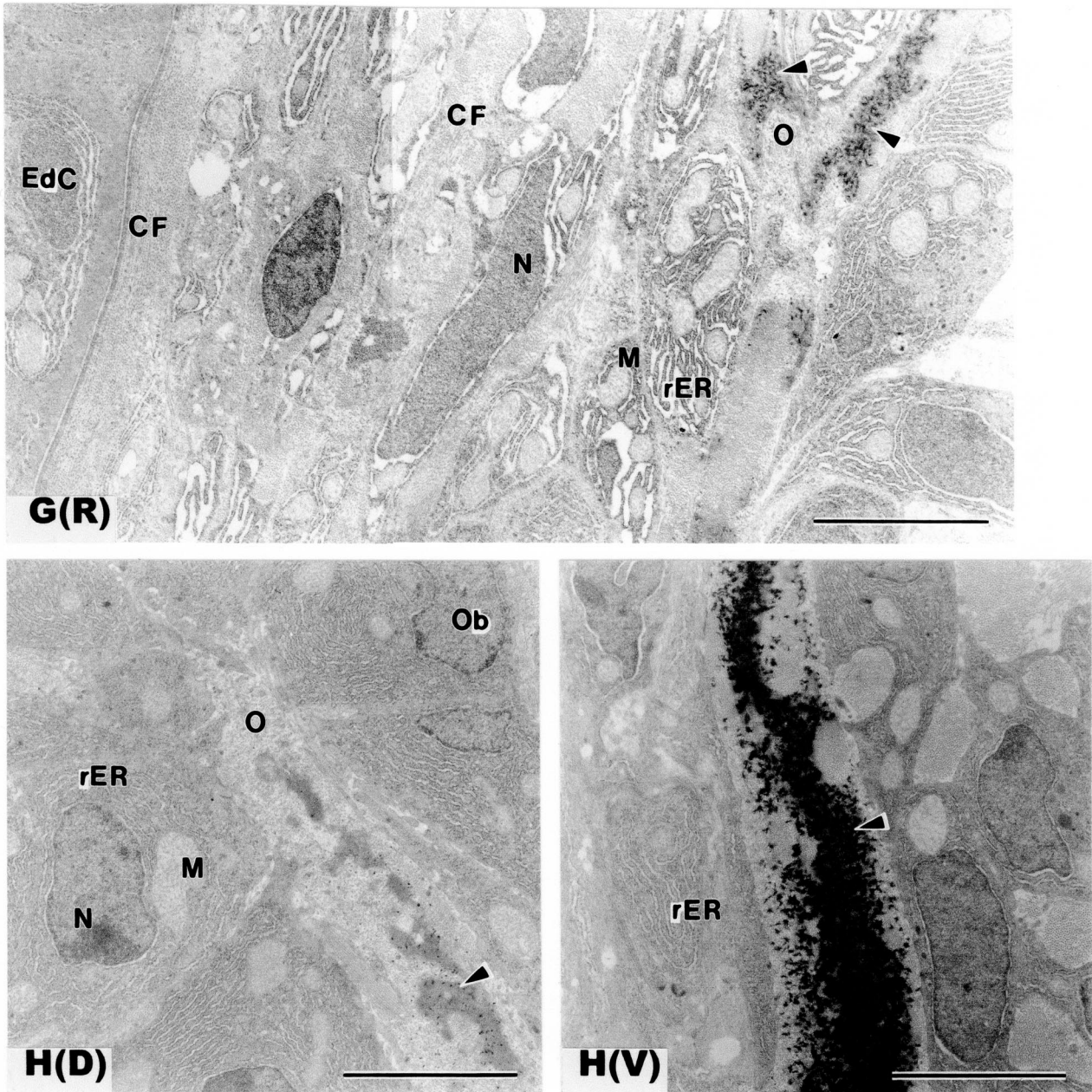
At stage H, the uncalcified osteoid was observed on the dorsal tip of the Pb (Fig. 13H(D)), when the right eye was located on the midline (the climax of the eye migration). The osteoblasts around the osteoid were cuboidal, had lobulated nuclei, and contained abundant rER and mitochondria. Calcified bone matrix was observed at the ventral part of the

Pb (Fig. 13H(V)). The surrounding cells were flat and had extensive rER.

### Discussion

#### Asymmetric Growth of the Rvs

I previously reported (Chapter 1.1) that the expansion of the Rvs coincided with eye migration, with the right (blind) Rv being bigger than the left, and suggested that the



**Fig. 13** Electron micrographs of transverse section of the Pb region of metamorphosing Japanese flounder. G (R), stage G - right side; H (D), stage H - dorsal side; H (V), stage H - ventral side. CF, collagen fibril; EdC, epidermal cell; M, mitochondrion; N, nucleus; O, osteoid; Ob, osteoblast; rER, rough endoplasmic reticulum. Arrowhead indicates crystals of calcium carbonate. Bars 5  $\mu\text{m}$

expansion of the Rv is the driving force behind eye migration.

The present study indicates that the Rv sheath cells are flat and have many pinocytotic vesicles, with a scant rER and a small Golgi apparatus. These ultrastructural features are reminiscent of endothelial cells, which represent the inner lining of blood and lymphatic vessels. However, since no red blood cells were observed in the Rv, the Rv is not considered to be a blood vessel. Bony fishes have subocular lymph sacs in the head<sup>59</sup>. Both the location and the ultrastructure of the

Rv lead me to consider this structure as part of the subocular lymph sacs. In fishes, the lymph system is connected to the arteries by capillaries (the term "secondary vascular system" has been used for this lymphatic system in fishes<sup>60</sup>). Kampmeier<sup>59</sup> described a lymph system with a closed sac-like structure in the head of rainbow trout.

Moreover, the Rv consisted of endothelial cells alone, without fenestrae, smooth muscles, or pericytes. These characteristics are common to the lymphatic capillaries of

mammals. Although the secondary vascular system has not been confirmed in the Japanese flounder, the Rv is considered to be a lymphatic structure that originates from that system.

If I assume that the Rv sheath has a high permeability to liquid, similar to the mammalian lymphatic capillaries, it is possible that the body fluid can move freely in and out of the Rv. Therefore, even if there are any active mechanisms to take up fluid into the Rv, it is probably difficult to expand the Rv enough to push the right eye. I speculate that the Rv is expanded passively to fill the empty place left by the eye migration.

### Development of the Skin and the Pb in Relation to Right Eye Migration

The Pb is a bone that is characteristic of flatfishes<sup>7)</sup>, and there is a possibility that it provides the driving force for eye migration. The Pb rudiment is first observed at stage E. Formation of osteoid and calcification starts from a point touching the trabecular cartilage at stage G. Kyle<sup>51)</sup> described the process of eye migration in *Glyptocephalus*, *Pleuronectes*, *Rhombus*, and *Bothus* in detail, and pointed out the putative contribution of a "ligament" that grows up like a wall on the outer side of the migrating eye, and is ossified from anterior to posterior. Based on the description and figures in that work, it is clear that his "ligament" has the same structure as the Pb rudiment described herein. However, since the Pb rudiment is an aggregation of fibroblast-like cells with numerous collagen fibrils around them, the word "ligament" seems improper.

Generally, in development of dermal bones, cells aggregate first and then an osteoid is formed inside of the aggregation<sup>61)</sup>. In the Pb rudiment of the metamorphosing flounder, the aggregated fibroblast-like cells are active (judging by the lobulated nuclei, extensive rER, and mitochondria) and are surrounded by many collagen fibrils. Therefore, these fibroblast-like cells are considered to produce collagen fibrils, and some of the mesenchymal stem cells, which are also the origins of the fibroblast-like cells, may differentiate into osteoblasts to form the Pb.

The transformation of the Pb rudiment appears to be an important factor in initiating right eye migration. If I assume that the rudiment is becoming straight from the vented form, it is possible that the right eye is being pushed up to the top of the head by a force from the ventral side. To confirm the contribution of the Pb rudiment to eye migration, further studies of malformed juveniles with abnormal eye location will be necessary to correlate the absence of a rudiment with

the failure of eye migration.

The Rv sheath consisted of a single layer of flat cells about 0.5-1  $\mu\text{m}$  thick (Fig. 8). Inside the lumen of the Rv, amorphous material was found. The electron density of this material was intermediate, and not uniformly distributed. Fibroblast-like cells were present outside of, or attached to, the Rv sheath, as if constituting loose layers, nonuniformly, surrounding the Rv. The cell constituting the sheath had a flat nucleus and flat mitochondria. Little, rough endoplasmic reticulum (rER), and Golgi bodies or secretion granules were visible. Many vesicles were observed in the cytoplasm of the cell (Fig. 9). Some vesicles were isolated inside the cell. The membrane of some vesicles fused with the cell membrane, and these vesicles appeared as pits.

## Chapter 2.1

### Histological study of deformity in eye location in Japanese flounder

#### Introduction

Flatfish are important species for commercial fisheries in the world. In addition, aquaculture and stock enhancement of these fish are extensively and intensively attempted, especially in Japan. However, in the large-scale hatcheries for seed production, a much higher occurrence of abnormal juveniles has been observed<sup>20,21)</sup> than in sea-caught adults<sup>6,22-26)</sup>.

There are two major types of abnormality in flatfish, abnormality in body coloration and abnormality in eye location (AEL). A large volume of information has been accumulated on body coloration, and technical progresses, based on the understanding of the mechanism of abnormal coloration, have enabled fish farmers to reduce the occurrence of this abnormality<sup>27,28)</sup>.

In contrast, information on AEL is very limited. Although the presence of AEL in sea-caught flatfish has been reported in many species<sup>30)</sup>, and the relation with the abnormality in body coloration has been reported in brown sole<sup>29)</sup> and Atlantic halibut<sup>33)</sup>, effective methods for reducing AEL, together with basic mechanisms of the occurrence, remain to be studied.

Apart from the needs of aquaculture, the process of eye relocation during flatfish metamorphosis has attracted attention since the 19th century<sup>7-19)</sup>. However, since most of those studies only describe the morphological changes in the



head tissues, only fragmented information has been accumulated for understanding the mechanisms of eye relocation. Using histological techniques, I examined the asymmetrical development of bones and skin related to eye relocation at light microscopy level, and suggested that a membrane bone, Pb, is the strongest candidate causing the asymmetrical relocation of the eyes during flounder metamorphosis (Chapter 1.1).

By using the occurrence of abnormal individuals in mass production, it is possible to survey a tissue(s) that is absent or deformed only in AEL fish. In the present study, AEL individuals of the Japanese flounder at the early juvenile stage were examined with light microscopy, and a positive correlation was found between the deformity in the Pb and the extent of AEL. Induction cascade among hard tissues, skin and body color is also discussed.

### Materials and methods

#### Experimental animals

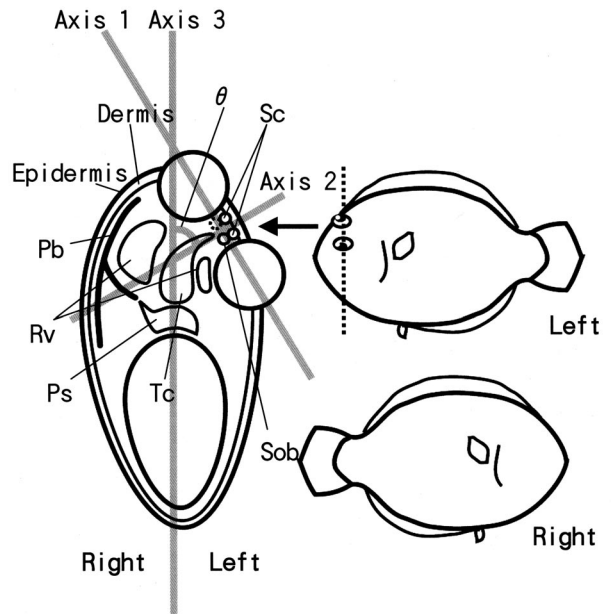
Fertilized eggs of the Japanese flounder were transported from the Kumamoto Prefectural Fisheries Research Center, from the Miyako Station of the Japan Sea-Farming Association, and from the Fukui Prefectural Sea-Farming Center to the Fisheries Research Station of Kyoto University, Maizuru. They were reared following the standard protocol for flounder culture as described in Chapter 1.1.

Abnormal fish, together with normal fish as control, were selected from the rearing tank at 48 DAH for eggs from Kumamoto, 58 DAH for eggs from Miyako, and 47 DAH for eggs from Fukui when most fish completed the metamorphosis and settled.

#### Light microscopic study

Sampled fish were fixed in 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3-6 hours at room temperature, dehydrated through graded ethanols, and embedded in JB4 resin (Polysciences Inc., Warrington, PA, USA). The head part around the eyes was transversely sectioned at 2  $\mu$ m thick and stained with silver nitrate-toluidine blue. Cartilage undergoes metachromasy with staining, becoming blue violet, whereas calcified bone becomes dark brown by the silver-nitrate reaction. The adjacent sections were checked to ensure that the difference between the left and the right was not due to tilted sectioning.

Identification and names of cartilages and bones were



**Fig. 14** Schematic drawings of the transverse section in the head part of the Japanese flounder juvenile. See abbreviations in Table 1. The straight line connecting the center of two eyes is Axis 1, the straight line crossing Axis 1 vertically is Axis 2, and dorsal-ventral axis of the body is Axis 3. The angle between Axis 2 and Axis 3 is  $\theta$ .

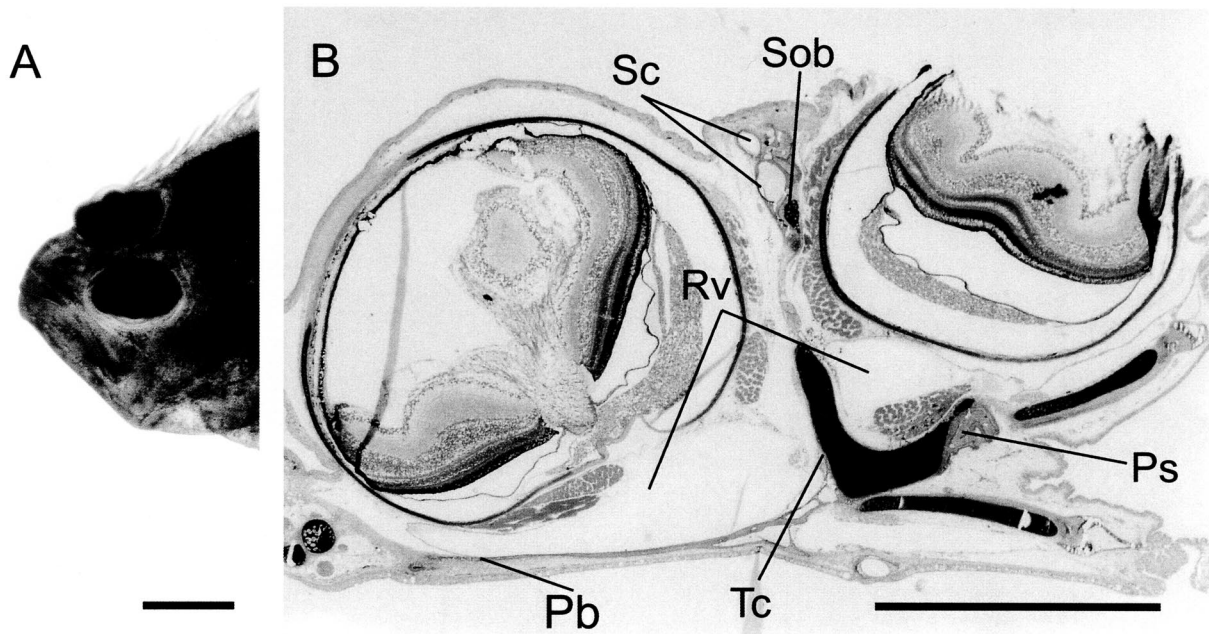
based on Chapter 1.1 and schematically summarized in Fig. 14. All samples were classified into four types depending on the degree of eye location, which is represented by the degree of  $\theta$  (Fig. 14). When  $\theta$  was approximately  $90^\circ$ , the fish was classified into Type I (normal). When  $\theta$  was approximately  $40\text{--}60^\circ$ , the fish was classified into Type II (mild AEL). When  $\theta$  was approximately  $0\text{--}30^\circ$ , the fish was classified into Type III (serious AEL) and when  $\theta$  was approximately  $-90^\circ$ , the fish was classified into Type IV (reversed).

### Results

#### Normal (Type I)

The asymmetrical structures of juvenile flounder with normal eye location were examined under a light microscopy (Fig. 15) and are summarized in Table 1.

Normal juveniles were pigmented on the left (ocular) side and not pigmented on the right (blind) side of the body. The visceral handedness was determined by the sidedness of larger liver lobe and the direction of the gut looping. The left lobe of the liver was larger than the right. When the specimen was placed with its head toward the left, the rectum was located deeper than the stomach. A pair of supraorbital canals (Sc) twisted to left (clockwise on Fig. 15B); the right one grew



**Fig. 15** Normal (Type I). (A) External appearance of the head part. Bar 2 mm. (B) Transverse section of the head through eyeballs. Bar 1 mm. See abbreviations in Table 1.

dorsal, and the left one ventral. The supraorbital bar (Sob) of the left side was present, but that of the right was absent. Asymmetrical formation was also observed in the trabecular cartilage (TC) and the parasphenoid (Ps).

The Pb, a unique bone only present in flatfish, was present only the right side and fully developed. The epidermis and dermis of the right side were thicker than those of the left side. The retrorbital vesicle (Rv) on the right side was larger than on the left side. Because there were abnormal-colored individuals (albino, having non-pigmented body on both sides) in Type I, three albino individuals were also examined on the above-mentioned asymmetry. However, as shown in Table 2, there was no structural difference between normal and albino individuals except for the color of the left side.

#### Mild AEL (Type II)

In the individuals with mild AEL, the right eye migrated over the dorsal line but incompletely (Fig. 16). The left eye migrated ventrally to a similar extent as in Type I. Because there were also albino individuals in Type II, three individuals of each color were examined (Table 2). The deformity generally found in Type II was the insufficiently developed Pb and Rv. As shown in Fig. 16B, the Pb in this group were shorter and the size difference between right and left Rv was smaller than that in Type I fish. In addition, II-N-1 had one supraorbital canal only, and II-W-3 had a deformed

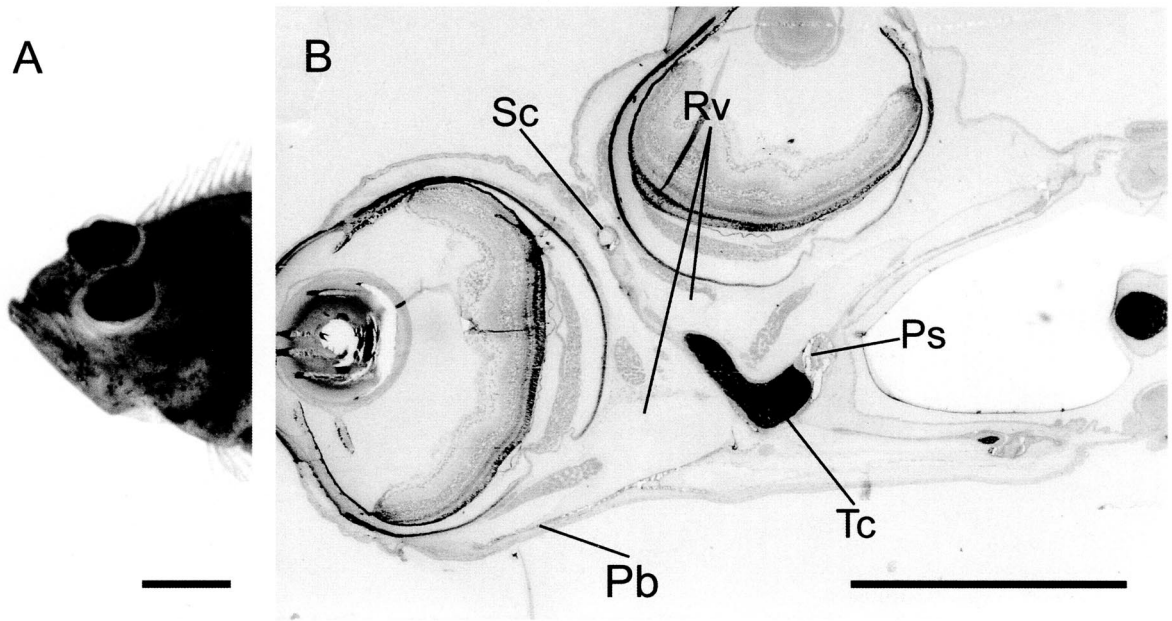
parasphenoid (Table 2). However, other individuals in Type II had normal bones, and all polarities of tissue development were the same as in normal individuals. There was no difference between normal and albino individuals except for the pigmentation.

#### Serious AEL (Type III)

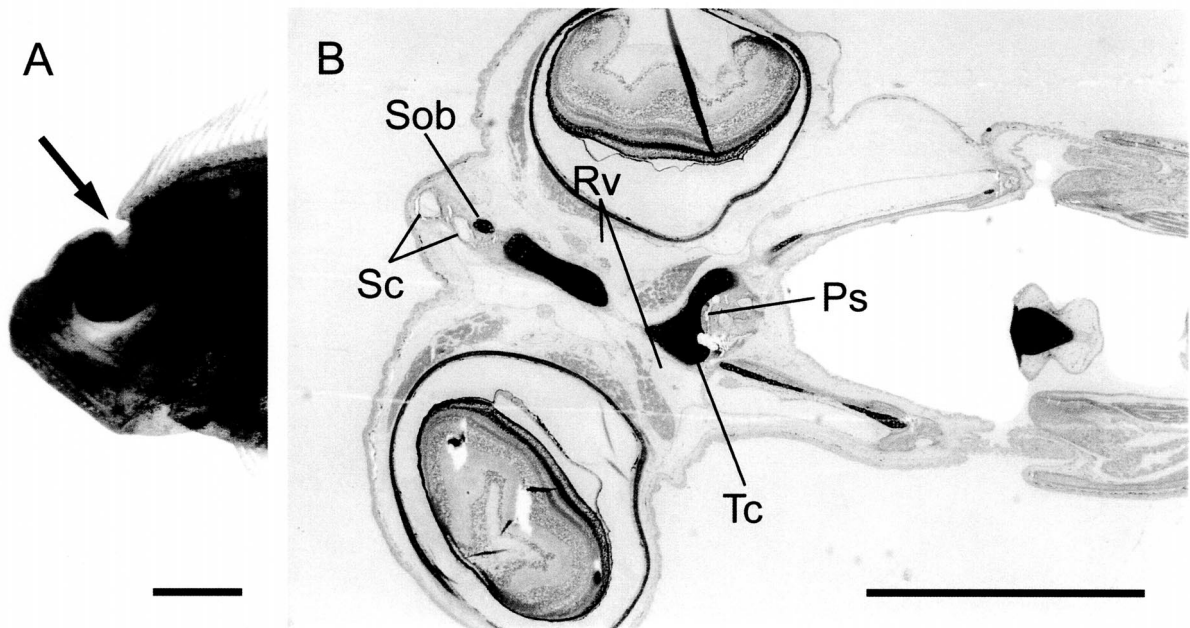
In the individuals with serious AEL, the locations of the eyes were almost symmetrical (Fig. 17). Although there were three types of pigmentation: normal, albino and ambicolored (black color on both sides of the body), there was no clear correlation between the pigmentation patterns and bone

**Table 1** Asymmetrical feature of each tissue and organ in normal individuals of Japanese flounder.

Tissue	Right	Left
Body color	Not pigmented	Pigmented
Liver	Small	Large
Supraorbital cana (Sc)	Twisting to left	Twisting to left
Supraorbital bar (Sob)	Absent	Present
Parasphenoid (Ps)	Twisting to left	Twisting to left
Trabecular cartilage (Tc)	Twisting to left	Twisting to left
Pseudomesial bar (Pb)	Fully developed	Absent
Epidermis	Thick	Thin
Dermis	Thick	Thin
Retrorbital vesicle (Rv)	Large	Small



**Fig. 16** Mild abnormal eye location (Mild AEL; Type II). (A) External appearance of the head part. Bar 2 mm. (B) Transverse section of the head through eyeballs. Bar 1 mm. See abbreviations in Table 1.



**Fig. 17** Serious abnormal eye location (Serious AEL; Type III). (A) External appearance of the head part. Arrowhead shows a dent. Bar 2 mm. (B) Transverse section of the head through eyeballs. Bar 1 mm. See abbreviations in Table 1.

deformity (Table 2).

The Pb was absent in all individuals except for III-W-2, which has Pb on both sides. Moreover, the polarities of individual bones (Table 1) were randomized within individuals. In most individuals, the sizes of Rv were almost the same between right and left. The left Rv of III-B-1 was bigger than the right, but the eye did not move. In half the individuals without Pb, dermal and epidermal thickness did

not differ between right and left sides, and neither the dermis nor the epidermis was thickened. In III-N-1 and III-W-3, the epidermis was thicker on the right side as in normal individuals, but thinner on the right side in III-B-2.

In the individuals having Pb on both sides (III-W-2), the epidermis and dermis of both sides were thickened. Some individuals had a dent in front of the pterygiophore of the dorsal fin rays where the right eye is located in normal fish

**Table 2** The asymmetrical tendency of each tissue and organ found in different types of AEL individuals of Japanese flounder.

	Left Body	Right Body	sample ID	Visceral handedness									
	Color	Color		Sc	Sob	Ps	Tc	Pb	Epidermis	Dermis	Rv		
Type I (normal)	++	++	I-N-1	++	++	++	++	++	++	++	++	++	
	++	++	I-N-2	++	++	++	++	++	++	++	++	++	
	++	++	I-N-3	++	++	++	++	++	++	++	++	++	
	albino	--	++	I-W-1	++	++	++	++	++	++	++	++	++
		--	++	I-W-2	++	++	++	++	++	++	++	++	++
		--	++	I-W-3	++	++	++	++	++	++	++	++	++
Type II (slight AEL)	++	++	II-N-1	++	deformed *1	++	++	++	++	++	++	+	
	++	++	II-N-2	++	++	++	++	++	++	++	++	+	
	++	++	II-N-3	++	++	++	++	++	++	++	++	+	
	albino	--	++	II-W-1	++	++	++	++	++	++	++	++	+
		--	++	II-W-2	++	++	++	++	++	++	++	++	+
		--	++	II-W-3	++	++	++	++	+(deformed) *2	++	++	++	+
Type III (serious AEL)	++	++	III-N-1	++	++	++	++	++	++ 0 *3	++	0 *5	0	
	++	++	III-N-2	++	++	++	0	++ 0 *3	0 *5	0 *5	0		
	albino	--	++	III-W-1	++	--	--	--	++ 0 *3	0 *5	0 *5	0	
		--	++	III-W-2	++	--	--	0	0 0 *4	0 *6	0 *6	0	
		--	++	III-W-3	++	++	++	0	++ 0 *3	+	+	0	
	ambicolored	++	--	III-B-1	++	--	deformed *7	++	-- 0 *3	0 *5	0 *5	-	
++		--	III-B-2	++	++	0	--	-- 0 *3	--	--	0		
++		--	III-B-3	++	++	++	0	++ 0 *3	0 *5	0 *5	0		
Type IV (reversed)	--	--	IV-N-1	++	--	--	--	--	--	--	--	--	
	--	--	IV-N-2	++	--	--	--	--	--	--	--	--	
	--	--	IV-N-3	++	--	--	--	--	--	--	--	--	

++, Normally developed tissue; --, reverse direction; 0, symmetrical tissue.

\*1 (deformed), absence of one of the Sc; \*2 (deformed), the form is distorted; \*3, absence of the Pb; \*4, existence of two Pb on both sides; \*5, both sides are thin; \*6, both sides are thick; \*7 (deformed), absence of both of the Sob. See Table 1 for abbreviations.

(Fig. 17).

All samples of Type III had the similar handedness of visceral organs to those of Type I (Table 2).

### Reversed (Type IV)

In the individuals with reversed location of eyes, there were only normal color individuals; the ocular side was pigmented with melanophores, and the blind side was not. As shown in Table 2, the localizations of all structures were completely opposite to those in Type I (normal) except for the visceral handedness.

## Discussion

### Relation between the Pb and the eye relocation

To find important tissue(s) for eye relocation in flounder metamorphosis, the identification of common deformities present in all AEL individuals was the major purpose of this study. The results show a close relation between the AEL and abnormal formation of the Pb. The Pb is the bone that is present only in flatfish and formed only on the right side, covering the right eyeball from the blind side in the Japanese flounder (Chapter 1.1). The Pb was present on the opposite side in the reversed individuals (Type IV). In Type II individuals, in which the eyes relocated incompletely, the Pb was on the normal side but the length in the section on a slide

glass was shorter. In Type III individuals, the Pb was either totally absent or present on both sides. It was also noticeable that the eye did not move when the Pb did not exist, although all bones except for the Pb formed appropriately in III-N-1 (Table 2). These results would suggest that normal formation of the Pb is indispensable for normal eye relocation.

In brown sole<sup>29)</sup>, the blind side is characterized by the presence of the lateral bone (Le) and the frontal bone (Fr) in normal individuals. Interestingly, however, these bones were present on both sides, or absent on either side in some AEL individuals of this species. Since the Pb had been considered as part of the Fr<sup>52)</sup>, and since the AEL individuals in the study of brown sole<sup>29)</sup> were similar to the Type III individuals in the present study, my results of the symmetrical presence of Pb in Type III is in accordance with the results in brown sole.

In addition, the polarity of each bone formation is first described in the present study, and the randomized polarities of individual bones are pointed out in Type III (Table 2). As typically shown in III-B-2, normal asymmetry was found in the Sc, reversed in the Ps and the Tc, and no asymmetry was seen in the Sob. The presence of single Pb may determine the polarity of other bones to express normal left-right asymmetry.

### Relation between the body color and the eye relocation

Body color is one of the asymmetrical characteristics in flatfish. Several studies relate malpigmentation (ambicoloration) to incomplete eye relocation<sup>6,23,62,63</sup>. Higher occurrence of AEL was reported in albino fish of hatchery-reared mud dab<sup>31</sup>) and frog flounder<sup>32</sup>), while a direct relation was questioned between coloration and eye relocation from the results of several rearing trials of Atlantic halibut. It is indispensable to distinguish two types of malpigmentation, primary (eye-location dependent) and secondary (eye-location independent). Secondary albino is frequently seen in seed production of the Japanese flounder and other flatfish. In this type of albinism, collapse of the chromatoblast occurs also on the ocular side and there is no increase in mucous cell density, which is characteristic of the ocular side of normal fish<sup>64</sup>). In contrast, primary albino is found in individuals having undergone abnormal eye relocation; both eyes tend to move and therefore both sides tend to be blind sides. Examples in brown sole have been reported by Aritaki<sup>29</sup>). Ambicoloration also has two types. Primary ambicoloration has two "ocular sides" after the abnormal metamorphosis; both eyes do not move and both body sides are pigmented. In secondary ambicoloration, the body of the blind side is pigmented during<sup>65</sup>) or after<sup>20,66</sup>) the metamorphosis in the fish with normal eye location. Primary ambicoloration was also reported in brown sole<sup>29</sup>).

Norman<sup>6</sup>) divided ambicoloration into three categories, "staining", "spotting" and "true ambicoloration". Seikai<sup>66</sup>) regarded "staining" as "secondary" coloration, and "true ambicoloration" as "primary" ambicoloration because of the absence of eye relocation and presence of two "ocular skins" on both sides. In the present study, the albino individuals of normal (Type I) and mild AEL (Type II) in Table 2 can be regarded as secondary albino, because of the correct determination of left-right asymmetry with the existence (complete or incomplete) of the Pb on the right side. An albino individual (III-W-2) and ambicolored individuals of Type III can be regarded as primary malpigmentation, because the Pb-present sides were white (III-W-2) and the Pb-absent sides were pigmented. There seem to be double malformations, lack of Pb and secondary albinism, in the cases of III-W-1 and III-W-3. Although both sides of the individuals were to be pigmented due to the absence of Pb, the pigmentation did not occur probably due to the secondary albinism.

### Development of the Pb and the skin

In Type II individuals, the length of the Pb was shorter in the section than in the normal individuals, in spite of the presence of thick dermis, which is the aggregation of fibroblasts, and normally appears before the Pb formation (Chapter 1.1). This observation is interesting, because the thick dermis is considered to be the site that produces the Pb. Therefore, the presence of more than one controlling step is suggested between the formation of thick dermis and the full development of the Pb. In Type III, most individuals did not have thick dermis in both sides, and the Pb was totally absent. In III-W-3, with weak left-right asymmetry, the slightly thickened dermis was observed on the right side but no Pb had developed. These observations suggest that full thickening of the dermis is required for Pb formation. The opposite case was found in III-W-2, in which fully thickened dermis was present on both sides and the Pb were formed on both sides. Other fish in Type III (III-W-3 and III-B-2) had thick dermis but no Pb.

In summary, the important role of Pb was suggested from the possible contribution to determine the polarity of bone development, and therefore, the direction of eye relocation. In addition, left-right asymmetry that first occurred on the epidermis and dermis possibly controls the development of the Pb.

## Chapter 2.2

### Insufficient ability to synthesize thyroid hormone in abnormal juveniles during seed production of the Japanese flounder

#### Introduction

Since flatfish are widely distributed throughout the world, and regarded as an important commercial resource, much attention has been directed to the occurrence of deformed individuals. From the first half of the 19th century, many deformed specimens in pigmentation and/or eye location have been reported from wild flatfish<sup>6,22-26,63</sup>) and from hatchery reared flatfish<sup>20,21</sup>). Improved rearing techniques to reduce abnormal coloration during large-scale seed production, have been, at least, partially achieved<sup>27,28,67</sup>). Other than coloration, valuable information concerning the ability to reduce deformity is limited<sup>20,33,58</sup>).

The effects of TH on flatfish metamorphosis have been extensively demonstrated at the whole body level<sup>34,35</sup>), the

individual tissue level; erythrocytes<sup>36)</sup>, skeletal muscle<sup>37)</sup>, gastric glands<sup>38-40)</sup>, chloride cells in the gill<sup>41)</sup> and specific bones essential for eye relocation (Chapter 3.1). Therefore, if insufficient or excess amounts of the hormone are produced at a critical time for metamorphosis, abnormal development of individual organs is predicted.

However, very little information has been obtained concerning the thyroid system in abnormally developed fish, for example, the TH concentration in whole body or serum, or the status of the thyroid gland. In this study, using the abnormal fish, which accidentally occurred at a rate of about 4% in a hatchery comprising Japanese flounder, thyroidal status and responsiveness to exogenous TH in peripheral tissues, was examined.

## **Materials and Methods**

### **Experimental Animals**

A high incidence of abnormal Japanese flounder individuals was found at the Obama Station of the Japan Sea-Farming Association in Fukui Prefecture, during routine seed production from March to May 2000 as follows. Artificially fertilized eggs (700,000 eggs) were stocked in a tank (18 m<sup>3</sup>) with a flow through system of filtered seawater at 14°C. 94.8% of the eggs hatched three days later, and the temperature was increased to 18°C gradually. The larvae were fed on the rotifer for the first 21 days, and newly hatched brine shrimp, *Artemia* nauplii were also provided twice a day from 17 DAH.

Artificial diets were also offered from 25 DAH. On 13 DAH, the water temperature was accidentally increased to 28°C at night. When most of the fish completed metamorphosis (about 40 DAH), about 4% of the fish remained in the larval form (stage G) and were swimming in the tank. On 49 DAH, the abnormal fish and normal juveniles were selectively captured by hand net and cup, transported to the Fisheries Research Station of Kyoto University, and stocked in two separate tanks. They were further reared for 16 days following the ordinary rearing protocol of flounder juveniles in the laboratory until the experiment.

### **Comparison between the normal and abnormal fish**

Five to ten fish each were taken from the stock tanks on 65 DAH, anesthetized in 0.02% MS-222, and fixed in 4% paraformaldehyde for 72 hours, and kept in 70% ethanol. The external appearance of sampled fish was observed, focusing

on eye location. For bones and cartilage, three fish of each group were cleared and double-stained with alcian blue and alizarin red 'S', following the standard method described by Dingerkus and Uhler<sup>45)</sup>. The classification of developmental stages followed that of Minami<sup>16)</sup>, in which right eye migration starts at stage E and finishes at stage I. Names of cartilages and bones were based on Amaoka<sup>12)</sup> and Matsuoka<sup>50)</sup>. Another five fish were fixed in Bouin's solution, embedded in paraffin wax, and the lower jaw region including the thyroid gland was frontally sectioned into 4 µm sections. The epithelial cell heights of thyroid follicles were measured using computer software (NIH Image, a public domain program, available at <http://rsb.info.nih.gov/nih-image/>) after taking photographs. In addition, the size and shape of erythrocytes in the ventral aorta were examined.

### **TH responsiveness of the abnormal fish**

On 65 DAH, thirty individuals each were transferred from the stock tanks to three experimental tanks (10 fish/tank, 6 L), and reared in seawater (control), seawater containing T4 (0.1 ppm of L-thyroxine sodium salt), or TU (30 ppm). Half of the rearing water was changed everyday and T4 and TU were added to keep the concentrations constant. The treatment was terminated 14 days later (79 DAH). All fish were sampled for morphological and histological examination as described above.

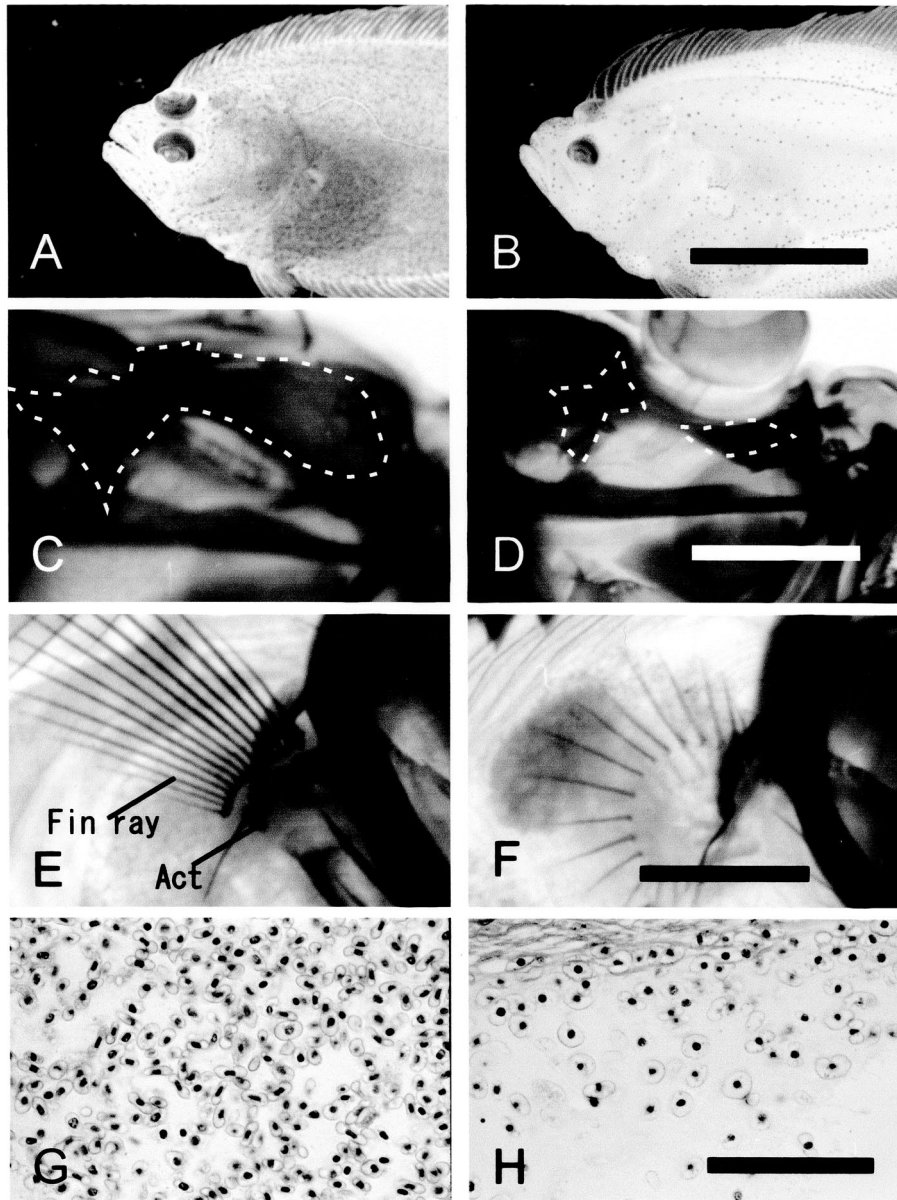
### **Serum TH concentration**

Measurement of TH was carried out when the fish grew large enough for blood sampling (115 DAH). More than five fish in each group were taken from the stock tanks and anesthetized in 0.02% MS-222. The blood was collected directly from the caudal artery by capillary tubes, and serum was separated by centrifugation at 12,000 rpm for 5 min. T4 and T3 were measured by radioimmunoassay (RIA) according to Tagawa and Hirano<sup>68,69)</sup>.

## **Results**

### **Comparison between the normal and abnormal fish (65 DAH)**

Although there were individual differences in the growth and development of the abnormal fish, retardation of metamorphosis was the most common characteristic as follows. All normal fish completed metamorphosis by 65 DAH, and were judged to be stage I (Fig. 18A). While, eye

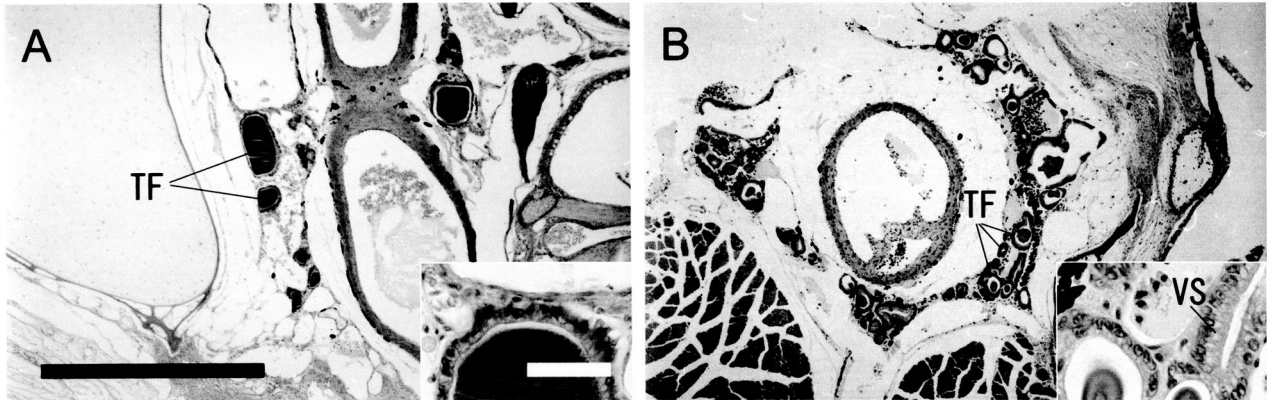


**Fig. 18** Comparison between normal (left column) and abnormal (right column) individuals of Japanese flounder at 65 DAH. A, B: external appearance of the head part. Bar 1 cm. C, D: the pseudomesial bar of double stained samples. The outline of the pseudomesial bar is indicated by a white dashed line. Bar 2 mm. E, F: pectoral fins on the blind sides. Act, actinost. Bar 2 mm. G, H: erythrocytes. Bar 50  $\mu$ m.

location of the abnormal fish was equivalent to stage G; the right eye was visible from the left side of the fish (Fig. 18B). The difference was also found in the internal organs. The Pb fully developed and covered the right eye from the blind side in the normal fish (Fig. 18C), but only a small and thin Pb was present in the abnormal fish (Fig. 18D). The actinosts of the pectoral fin were present in the normal fish (Fig. 18E), but were absent in the abnormal fish (Fig. 18F). Moreover, the fin rays of the pectoral fin became parallel in the normal fish (Fig. 18E), while they remained radially spread in the abnormal fish (Fig. 18F). The normal fish had small and

elliptical erythrocytes (Fig. 18G), while large and round erythrocytes were found as the major form in the abnormal fish (Fig. 18H).

The thyroid gland of the normal fish showed an image of inactivation as typically found in juveniles just after metamorphosis; flattened epithelial cells, intensively stained colloid, and an absence of vacuoles in the colloid (Fig. 19A). The thyroid follicles in the abnormal fish were unique (Fig. 19B); many numbers of small follicles were present instead of larger ones, and a small amount of colloid was contained. The epithelial cells possessed vesicle-like structures in their



**Fig. 19** High magnification images of transverse sections of thyroid glands of Japanese flounder. (A), normal; (B), abnormal. Black Bar 500  $\mu\text{m}$ . White Bar 30  $\mu\text{m}$ .

cytoplasm and the height of the cells was relatively high. There were no vacuoles in the colloid.

#### TH concentrations in serum

Figure 20 shows T4 and T3 concentrations on 115 DAH. The average T4 concentration of the normal fish (1.11 ng/ml) was significantly (*t*-test,  $P < 0.01$ ) and more than 10 times higher than that of the abnormal fish (0.08 ng/ml). Furthermore, T3 concentrations in the normal fish (0.75 ng/ml) were significantly higher (*t*-test,  $P < 0.01$ ) than those of the abnormal fish (0.38 ng/ml).

#### TH responsiveness of the abnormal fish

In the normal fish, there was no difference among T4-treated, TU-treated, and control fish (data not shown), except for the thyroid gland.

In the abnormal fish, the relocation of the right eye advanced to that of stage H, 14 days after T4 treatment; the cornea of the right eye became visible from the left side (Fig. 21A). The right eye in control fish also moved, but to a smaller degree than in T4-treated fish (Fig. 21B). There was no change in eye location in TU-treated fish (Fig. 21C).

The Pbs in the T4-treated (Fig. 21D) and control (Fig. 21E) fish fully developed and covered the right eye 14 days after the treatment on the abnormal fish, while the Pb did not show remarkable changes in TU treated fish (Fig. 21F). The actinosts of the pectoral fin were formed in T4 treated (Fig. 21G) and control fish (Fig. 21H) after 14 days. In addition, the fin ray became parallel in T4 treated fish (Fig. 21G). No change was observed in TU-treated fish in the actinost and fin rays (Fig. 21I).

The major erythrocytes became elliptical and were of small

type in T4-treated (Fig. 21J) and control (Fig. 21K) fish. Although the total number of erythrocytes was small, round and larger erythrocytes remained as the major type in TU-treated fish (Fig. 21L).

Figure 22 shows the changes in the epithelial cell height of thyroid follicles. When T4 was provided, the cell heights significantly decreased both in the normal and abnormal fish. In the case of TU treatment, an increase in the cell height was only observed in the normal fish, and no change was observed in the abnormal fish.

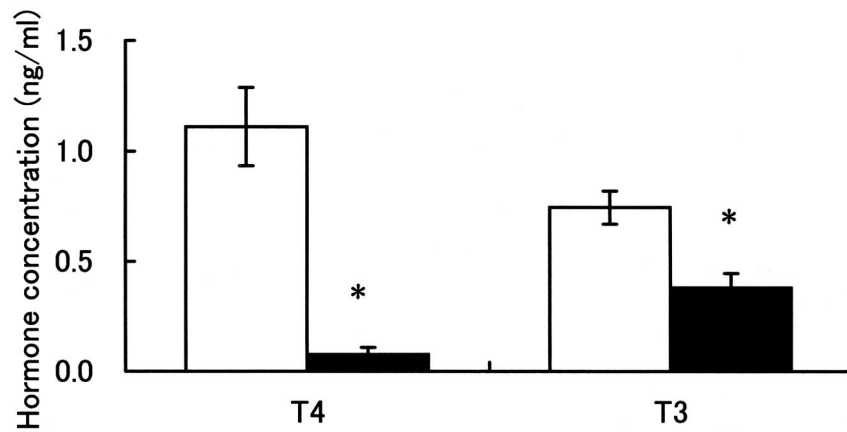
#### Discussion

##### The morphology and malfunction of the thyroid system in the abnormal fish

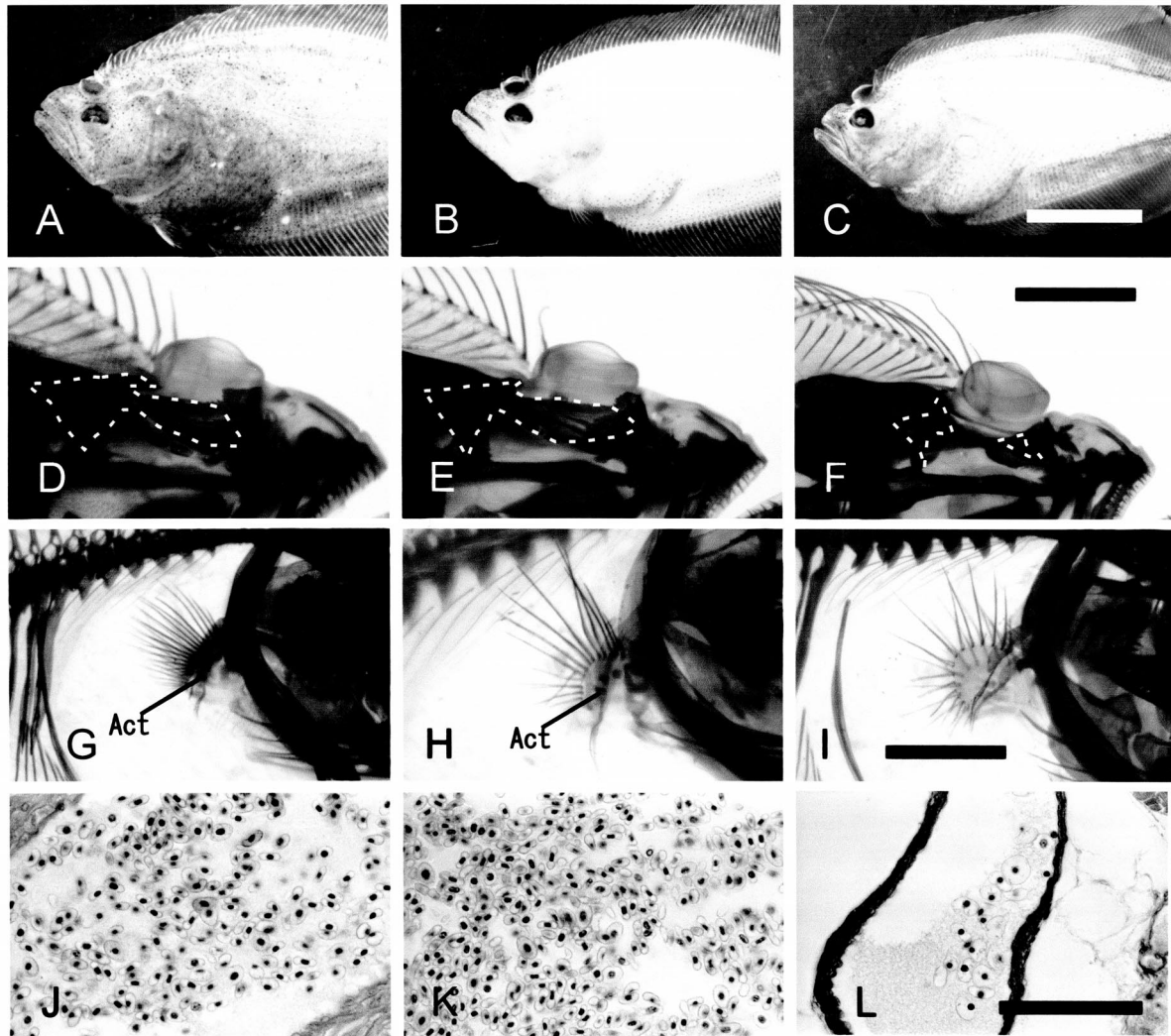
In this study, I examined the morphology of abnormally developed individuals, which occasionally occurred during ordinary seed production of the Japanese flounder. From Table 2, the characteristics of abnormal and normal individuals on 65 DAH, as well as the result of T4/TU treatment, was classified into larval type or juvenile type, based on previous studies (eye location (Minami<sup>16</sup>), Pb (Chapter 1.1), pectoral fin (Matsuoka<sup>50</sup>, Chapter 3.1), erythrocytes (Miwa and Inui<sup>36</sup>)). In this way, the characteristics of the abnormal individuals retained larval aspects, suggesting that the fundamental phenomenon of the abnormality is the retardation of development, especially during metamorphosis.

The morphology of the thyroid gland was totally atypical; follicles were small or lobulated with vesicle like structures in the cytoplasm of epithelial cells and containing lower amounts of colloid without vacuoles (Fig. 19B).

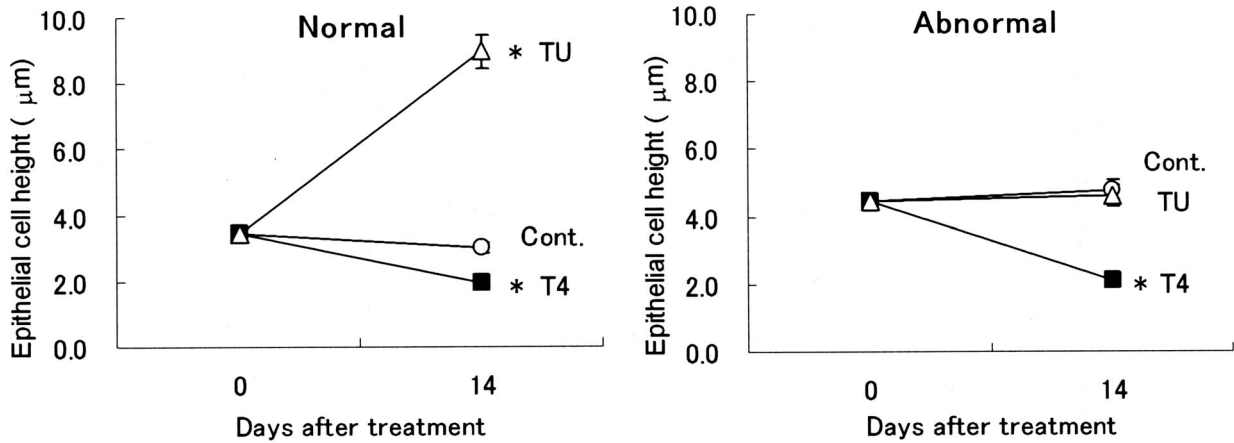




**Fig. 20** T4 and T3 concentrations in blood serum of normal and abnormal Japanese flounder on 115 DAH. White bar, normal; black bar, abnormal. Vertical lines represent mean  $\pm$  SEM (normal, n=28; DR, n=16 pools). \* indicates significant difference from the normal (P<0.01 by t test).



**Fig. 21** Comparison among T4 treated (left column), control (middle column), and TU treated (right column) abnormal fish on 79 DAH. A, B, C, external appearance. Bar 1 cm. D, E, F, the pseudomesial bar of double stained samples. The outline of the pseudomesial bar is indicated by a white dashed line. Bar 3 mm. G, H, I: pectoral fins on the blind sides. Act, actinost. Bar 3 mm. J, K, L: erythrocytes. Bar 50  $\mu$ m.



**Fig. 22** Changes in the epithelial cell height of thyroid follicles in normal (left panel) and abnormal (right panel) individuals treated with T4 or TU for 14 days. \* indicates significant difference from Day 0 ( $P < 0.01$  by t test).

**Table 3** Summary of results from the comparison between normal and abnormal individuals on 65 DAH and from the T4 and TU experiment on 79 DAH. The individual characteristics were classified into larval (L) type or juvenile type (J), with L-J being between the two types.

Tissue and organ	Abnormal fish				Normal fish	
	Start of the exp. 65 DAH	End of the exp. 79 DAH			Start of the exp. 65 DAH	End of the exp. 79 DAH
		T4	cont.	TU		
Eye migration	L	J	L-J	L	J	J
Pseudomesial bar	L	J	J	L	J	J
Pectoral fin	L	J	L-J	L	J	J
Erythrocyte	L	J	J	L	J	J

Moreover in older individuals (115 DAH), serum T4 and T3 concentrations of the abnormal fish were significantly lower than in the normal fish. It is suggested that TH production was depressed in the follicles of the abnormal fish. When considering that TH is essential for metamorphosis of the Japanese flounder (see review by Inui *et al.*<sup>5)</sup>) and that the appearance of abnormal individuals resembled larval flounder (Table 3), deficiency of and/or irresponsiveness to the hormone is probably the main cause of delayed metamorphosis.

To examine the responsiveness to TH in peripheral tissues, the administration of TH was conducted as the next step. The exogenous T4 induced changes in abnormal individuals, in a similar manner, as would be found during the normal course of metamorphosis (Table 1). Therefore, TH responsiveness was present. In addition, it is considered that a small amount

of TH was secreted in the abnormal fish because several juveniles were observed to progress forward, even in the control group, which had been completely inhibited by TU treatment. Body color also drastically changed during larval to juvenile transformation (metamorphosis) in flatfish. However, the occurrence of abnormal body coloration (pseudoalbinism) was so high (more than 80%) in this study, that it was difficult to specifically discuss the effect of delayed metamorphosis on body color based on quantitative data. Therefore, qualitative observation was attempted on a small number of individuals having normal coloration. The melanophores of juvenile types developed on the body surface of TH treated abnormal individuals; meanwhile the melanophores of larval type remained on those of TU treated (data not shown), suggesting the possible responsiveness to TH as in other peripheral tissues.

On the other hand, the responsiveness of thyroid follicles was different between the normal and abnormal fish. In the normal fish, the activation of the thyroid gland was observed by TU administration, probably through the possible increase of thyroid stimulating hormone (TSH) induced by the lowered TH concentration, whereas such activation by TU was not observed in the abnormal fish (Fig. 22). The reduction of TH concentration was expected in the abnormal fish, because TU inhibited the changes that occurred in the control group. However, responsiveness to T<sub>4</sub> was present as shown in Fig. 22, indicating the normal control of TSH on the thyroid gland. I speculate that the follicles of abnormal individuals were already producing hormones at their maximum level. No more activation was possible as the TH concentration becomes lower, and therefore inactivation was possible by increased T<sub>4</sub> concentration. The formation of the thyroid gland may be irreversibly damaged in the abnormal individual during early development. As such, the thyroid follicle produces hormones at their maximum level, and therefore it is reasonable that the amount was not sufficient for normal metamorphosis.

#### **Abnormal development and deformity in relation to thyroid function**

As described, the thyroid function has not been examined in abnormal or deformed individuals, which could occur in hatcheries, although TH is known to be extensively involved in morphological changes during metamorphosis in teleosts. Recently, the significance of TH in flatfish deformity has been suggested. For example, the occurrence of albinism was directly affected by the timing of TH treatment in Japanese flounder<sup>70</sup>. Malpigmentation was decreased by feeding wild zooplankton to Japanese flounder<sup>71</sup> and Atlantic halibut<sup>72,73</sup>. The reason could be attributed to the higher iodine concentration in wild zooplankton than in *Artemia*<sup>74</sup>, since iodide is an essential component of THs.

In Brown sole, deformity including eye location was reduced by controlling the rearing temperature<sup>58</sup>, and the rearing temperature was revealed to affect the secretion of TH<sup>75</sup>. The present study is the first direct example indicating thyroid malfunction as a main cause of abnormal development that occurred in a hatchery.

The abnormal fish used in this study were collected by selecting the floating fish from the majority of normal settled fish on 49 DAH. This timing of selecting abnormal fish was about 10 days later than that of average settlement during

ordinary rearing at 18°C. Although the possibility cannot be ruled out that I had simply selected the individuals belonging to the slower portion from the wide variation of developmental rates, the timing of settlement of the abnormal fish seems to be independently distributed from the majority of the normal fish. In addition, the abnormal fish occurred in a tank accidentally exposed to 28°C for one night. It is possible that formation of the thyroid gland may be vulnerable to high temperatures at a certain period during development. However, causality between the elevation of water temperature and malformation of the thyroid gland is still unclear, because the abnormal fish similar to the ones in this study are sometimes observed in the hatchery, even when no accidental increase in water temperature occurred, although the ratio was less than 0.1% (Morita, personal communication). The real cause of thyroid malformation could be different from the temperature increase. Then, formation of the thyroid gland may be vulnerable to unknown factor(s) present in hatcheries, and therefore it is possible that thyroidal malformation is potentially present at a significant rate in hatcheries, which may cause abnormal development and deformities under certain circumstances.

In conclusion, malformation of the thyroid gland was the strongest candidate for the cause of the abnormal development in this specific case of the Japanese flounder, and I propose the possible vulnerability of the thyroid gland during development as a potential problem for hatchery reared seedlings.

### **Chapter 3.1**

#### **Bone development during metamorphosis of the Japanese flounder: differential responses to thyroid hormone**

##### *Introduction*

A large number of teleosts undergo metamorphosis by changing their bodies from larval to juvenile forms so as to adapt to new habitats<sup>47</sup>. With regard to the study of dramatic changes in morphology, extensive research has been conducted on flatfish metamorphosis. For natural populations of the Japanese flounder, pelagic larvae migrate to shallow water and settle on the sea-bed. During the inshore migration the body changes from a symmetrical to an asymmetrical form, which includes the relocation of right eye and pigmentation of left side<sup>16</sup>. As in amphibians, THs induce

flounder metamorphosis at the whole body level<sup>34,35</sup>, and at the individual tissue level such as erythrocytes<sup>36</sup>, skeletal muscle<sup>37</sup>, gastric glands<sup>38-40</sup>, and chloride cells in the gill<sup>41</sup>. Therefore, eye migration, one of the most characteristic changes in flounder metamorphosis, is also expected to be under the control of THs.

In the Japanese flounder, it was revealed that the development of the Pb, a bone present only in flatfish, is important for eye relocation (Chapter 1). Changes in body proportion occur during flatfish metamorphosis<sup>4</sup>, and are also related to bone development<sup>46</sup>. Although the role of THs in skull development during amphibian metamorphosis has been studied (anurans<sup>42</sup> and urodeles<sup>44,45</sup>), no research has been done on flatfish metamorphosis focusing primarily on bones and their control, despite the extreme morphological changes.

The presence of a stage-specific response to TH is indicated at the whole body level during metamorphosis of the summer flounder<sup>41</sup>. Since the progress of metamorphosis was assessed using a stage index based on the location of the eyes, it is possible to study if some of the bones relating to eye relocation are directly controlled by THs and have specific response timings. To clarify these points, the development of individual hard tissues were examined under TH-deficient conditions, and the extent of recovery was tested by THs supplied to the Japanese flounder at different timings.

## Materials and Methods

### Experimental Animals

Fertilized eggs of the Japanese flounder were transported from the Fukui Prefectural Sea-Farming Center to the Fisheries Research Station of Kyoto University, Maizuru, Kyoto. They were reared following the standard protocol for flounder culture as described in Chapter 1.1.

The classification of the developmental stages followed that of Minami<sup>16</sup>, in which right eye migration starts at stage E and finishes at stage I.

### Experiment 1.

On the 16th day post hatching (DAH), 2 groups of 1,000 larvae were transferred to 100 L transparent tanks with or without TU (30 ppm, an inhibitor of TH synthesis). Two-fifths of the seawater was changed every two days, and TU was added to keep the concentration constant.

For the analysis of THs, larvae (1 g wet weight) were obtained from each tank every 5 days and frozen at -40°C. T3

and T4 were extracted from whole-body homogenate and measured by radioimmunoassay following the procedure of Tagawa and Hirano<sup>54,68</sup>.

The larvae on 16, 19, 22, 29, and 35 DAH in each group were anesthetized in MS-222 solution and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4). Body length, body height, and length of dorsal fin were then measured. Three to five samples were double stained with alcian blue and alizarin red 'S', following the standard method described by Dingerkus and Uhler<sup>48</sup>. The bones and cartilages of the cranium and body were observed under a stereomicroscope. Bones and Cartilages were identified following the descriptions of Amaoka<sup>49</sup> and Matsuoka<sup>50</sup>.

Differences in body length at each age in Experiment 1 were analyzed by t-test (Excel, Microsoft).

### Experiment 2.

Fish reared with TU in Experiment 1 were further divided into two groups (n=40 per group) and transferred to 30 L tanks at 38 DAH and 67 DAH. The TU-pretreated and metamorphosis-retarded fish were further reared with only TU (30 ppm, control group), or with both TU and T4 (30 ppm and 0.1 ppm, respectively, T4 group). Two-fifths of the seawater was changed every two days, and TU and T4 were added to keep the concentrations constant. After 14 days of treatment, the fish were harvested and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4). The external appearance was noted and the bones and cartilage were observed after undergoing the above-mentioned double staining method.

## Results

### Experiment 1. Effect of TH deficiency on development

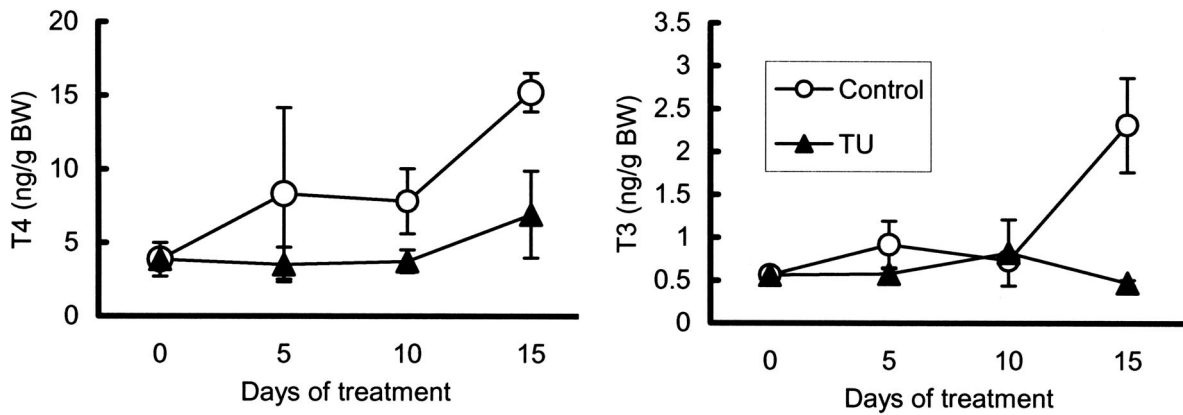
Figure 23 shows the changes in tissue T4 and T3 levels of the TU-treated and the control fish. The T4 concentration in the control fish tended to increase during metamorphosis from 3.8 ng/g to 15.2 ng/g, while the T4 level in the TU fish remained lower during the first 10 days and increased slightly to 6.9 ng/g by day 15. The T3 level of the control fish was about 0.5 - 1.0 ng/g during the first 10 days and increased to 2.5 ng/g by day 15. No increase in T3 concentration was observed in the TU-treated fish.

At the beginning of the experiment, at 16 DAH, the fish had symmetrical bodies, elongated fin rays, and no pigmentation (stage D, Fig. 24A). After 20 days, the fish

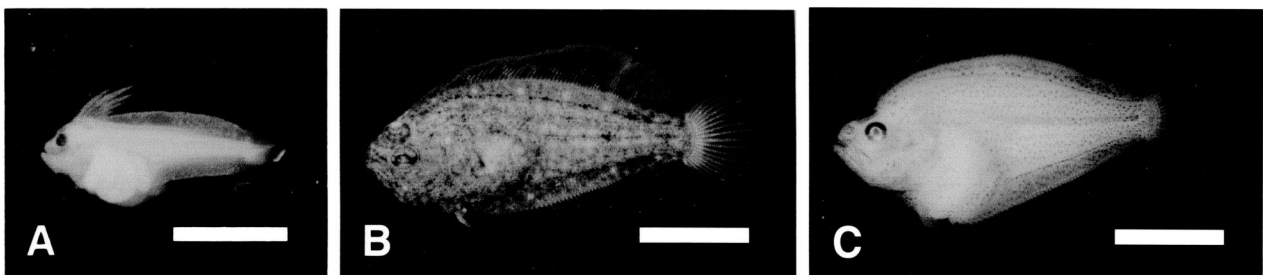
without TU were found to be completely metamorphosed (stage I, Fig. 24B), while eye migration, resorption of the dorsal fin rays, and asymmetrical pigmentation of the body in the TU-treated fish was prevented (Fig. 24C). As shown in Fig. 25A, the body length of both groups increased similarly up to 29 DAH, but the TU-treated fish were significantly larger at 35 DAH (at the end of metamorphosis in the control

group).

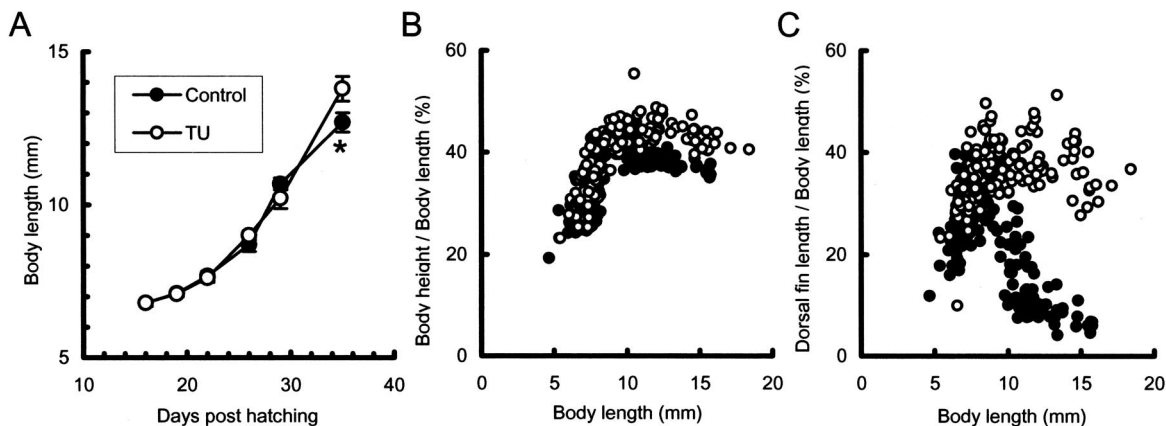
Figures 25B and 25C show the relative increases in body height, and dorsal fin length against body length. In both cases, changes in the relative growth were observed at body lengths of about 9 to 10 mm. The percentage of body height compared to body length was higher in the TU-treated fish after the flexion point of the relative growth curve (Fig.



**Fig. 23** Changes in whole body T4 and T3 concentrations in Japanese flounder without (Control) or with TU (30 ppm). Vertical lines represent mean  $\pm$  SEM (n=3 pools).



**Fig. 24** Effect of TU on external appearance of Japanese flounder. (A) initial larva of stage D (16 DAH), (B) larva in control group reared in ordinary seawater (35 DAH), (C) larva in TU group reared in 30 ppm TU for 20 days (35 DAH). Bars 5 mm



**Fig. 25** Growth of larvae and early juveniles of Japanese flounder reared without (control) or with TU (30 ppm). (A) body length. Vertical lines represent mean  $\pm$  SEM (n>28), \* indicates significant difference from control (P<0.05 by t test). (B) Relative growth of body height against body length. (C) Relative growth of dorsal fin ray against body length.

25B). Resorption of the dorsal fin rays was prevented in the TU-treated fish (Fig. 25C).

Significant differences were found in several bones between the control and the TU-treated fish at 35 DAH (Fig. 26). The Pb formed beside the relocated eye in the control fish (Fig. 26A), but was lacking in the TU-treated fish (Fig. 26B). No difference was observed in other parts of the cranium between the two groups. Development of the pectoral fins into juvenile type was also prevented by TU. The pectoral fin of the TU-treated fish did not shrink (Fig. 26C, D), and the actinost and the distal radials were not formed (Fig. 26E, F). The pterygiophore of the anal fin elongated in an anterior manner towards the pelvic fin and calcified in the control fish (Fig. 26C); while a weakly calcified pterygiophore was ventrally elongated in the TU-treated fish (Fig. 26D), resulting in a protruding abdomen for this group. No other notable differences were observed in hard tissues including vertebrae between the two groups.

## Experiment 2. Response to T4 at different timings

The second experiment examined the difference in timing of T4 responsiveness among various tissues. External appearance is shown in Fig. 27, and the internal skeleton is shown in Fig. 28. At the beginning of the experiment, at 38 DAH (Fig. 27A, 28A), the right eye was located on the right side, and pigmentation of the body was symmetrical, as in the TU-treated fish at 35 DAH in Experiment 1.

At 51 DAH, the fish receiving only TU treatment continued to grow larger. Although the right eye relocated slightly to the left side, there were no major differences from the fish at 38 DAH (Figs. 27B, 28B). T4 treatment starting from 38 DAH succeeded in inducing complete metamorphosis by 51 DAH. The right eye was relocated completely, the dorsal fin rays were absorbed, and the body color was differentiated asymmetrically as in adults (Fig. 27C). The formation of the Pb, the actinost and the distal radials of the pectoral fin, and the pterygiophore of the anal fin were all complete (Fig. 28C).

At 81 DAH, the fish receiving only TU continuously grew longer, without showing any remarkable changes in external and internal structure (Figs. 27D, 28D). The fish receiving T4 starting from 67 DAH failed to produce juveniles of normal appearance (Fig. 27E). Resorption of the dorsal fin rays, as well as formation of the actinost and the distal radials of the pectoral fins, and the pterygiophore of the anal fin, were induced by T4 (Fig. 28E). However, the changes in

body color were incomplete (Fig. 27E). Moreover, the right eye moved only slightly to the dorsal edge of the head where the cornea was visible from the left side of the fish (Fig. 27E). There was an absence of Pb, and the presence of the pterygiophore of the dorsal fin in the place where the right eye would pass through (Fig. 28E).

## Discussion

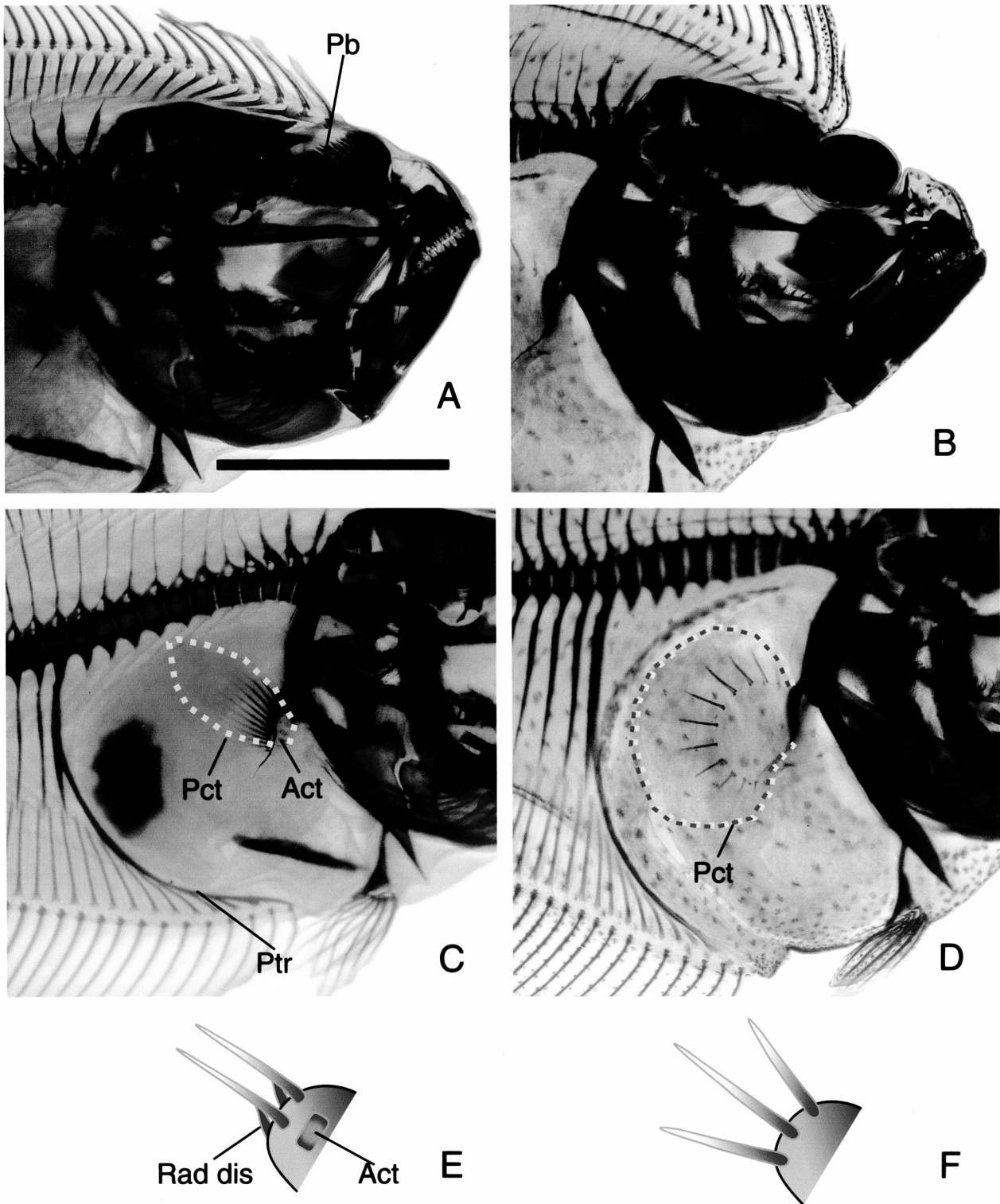
THs are one of the major regulators of bone development and remodeling in vertebrates (e.g., Mosekilde *et al.*<sup>76</sup>), including teleosts<sup>77-80</sup>. Since bone development and remodeling are essential aspects of morphological change that occurs during flatfish metamorphosis, particularly in eye relocation (Chapter 1.1), the involvement of TH in the specific bones essential for metamorphic changes in flatfish has been implicated.

In the present study, TU treatment resulted in TH deficiency in the larval flounder, as shown in Fig. 23. Since the differences between the TU-treated and the control fish disappeared following exogenous T4 treatment provided at early stages (from 38 DAH) (Figs. 27C, 28C), these differences were considered to be induced by hormone deficiency, not by the nonspecific (toxic) effect of TU.

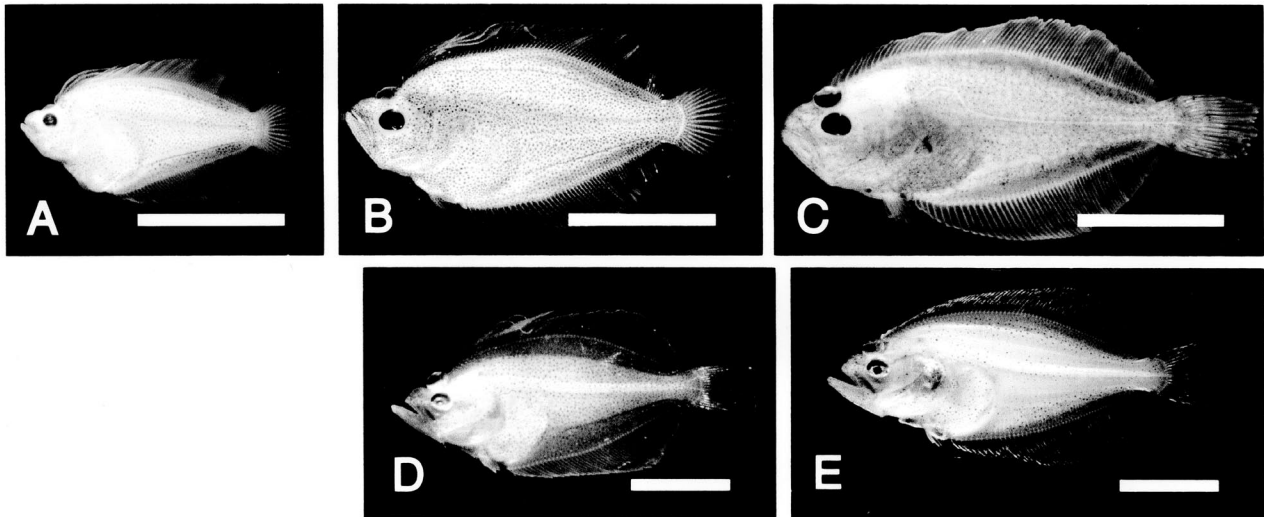
The effects of TH deficiency are summarized as follows:

- 1) Inhibition of pigmentation on the left side of the body (Fig. 24C);
- 2) Inhibition of Pb formation (Fig. 26B) and right eye relocation (Fig. 24C);
- 3) Inhibition of the development of the anal fin pterygiophore (Fig. 26D) and inhibition of body height reduction (Fig. 25B);
- 4) Inhibition of the absorption of the dorsal fin ray (Fig. 25C);
- 5) Inhibition of the formation of actinost and distal radials of the pectoral fin, and inhibition of pectoral fin shrinkage (Fig. 26D, F).

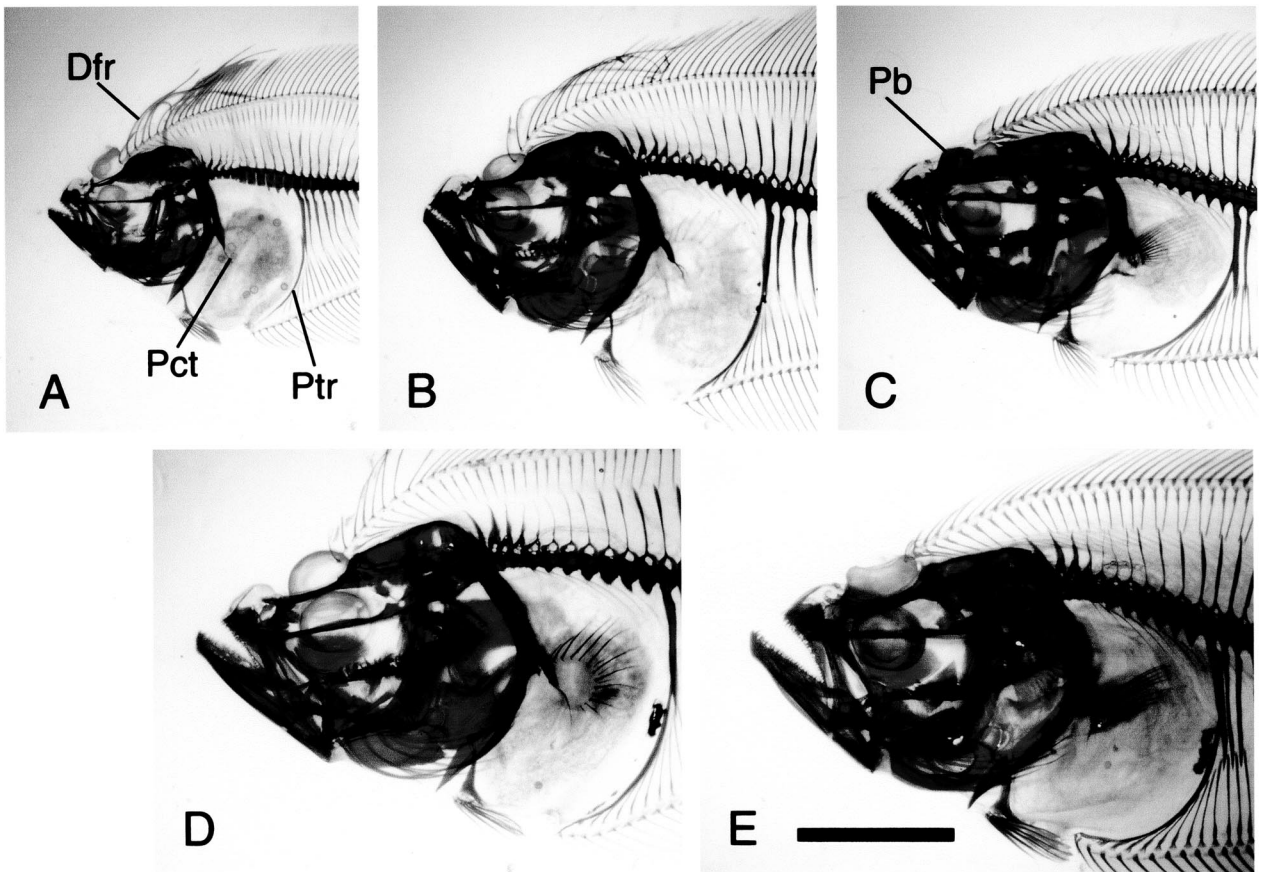
Increase in body length, however, was unaffected (Fig. 25A), suggesting little or no effect of TH deficiency on the growth of vertebrae. Consequently, there is a differential response to TH among the bones, at least at the early stages of development in the Japanese flounder. Therefore, I have tentatively classified the bones into two types; TH-dependent and TH-independent during early developmental stages.



**Fig. 26** Anterior part of Japanese flounder at 35 DAH reared 20 days; (A), (C) in ordinary seawater Control, and (B), (D) in 30 ppm TU. Schematic drawings of the pectoral fin of Control fish (E) and TU-treated fish (F). Photographs were taken on cleared and double stained samples from the blind side. The outline of the pectoral fin is indicated by a yellow broken line. Act, actinost; Pb, pseudomesial bar; Pct, pectoral fin; Ptr, pterygiophore of anal fin; Rad dis, distal radial. Bar 3 mm



**Fig. 27** Differential responsiveness of external morphology of TU-treated fish to exogenous T4 provided at different timings. All the fish including control were reared under the presence of TU (30 ppm) from 22 DAH. (A) 38 DAH, initial control; (B) 51 DAH, control; (C) 51 DAH, T4 (0.1 ppm, 38 to 51 DAH); (D) 81 DAH, control; (E) 81 DAH, T4 (0.1 ppm, 67 to 81 DAH). Bars 1 cm



**Fig. 28** Differential responsiveness of internal morphology of TU-treated fish to exogenous T4 provided at different timings. All the fish including controls were reared under the presence of TU (30 ppm) from 22 DAH. Photographs were taken on cleared and double stained samples. (A) 38 DAH, initial control; (B) 51 DAH, control; (C) 51 DAH, T4 (0.1 ppm, 38 to 51 DAH); (D) 81 DAH, control; (E) 81 DAH, T4 (0.1 ppm, 67 to 81 DAH). Dfr, dorsal fin ray; Pb, pseudomesial bar; Pct, pectoral fin; Ptr, pteriophore of anal fin. Bar 1 cm



Predictably, the TH-dependent bones were all related to the functional and morphological changes that occur during metamorphosis. TH-dependent changes were observed in the elongated dorsal fin rays and large pectoral fins, as well as in a tall body height, which helped the larvae to float easily during the pelagic period and are only required in the larval period, disappearing during metamorphosis. One of the TH-dependent bones, the Pb, is an important bone for eye relocation during metamorphosis (Chapter 1.1). This finding explains the suspension of metamorphosis by TU treatment at specific stages; at stage G (Japanese flounder, in this study) and from prometamorphosis to early climax of metamorphosis in the summer flounder<sup>81)</sup>, the last stages before the drastic relocation of one eye. On the other hand, the growth of vertebrae is TH-independent, at least during the early stages. Although other bones were not compared in detail, most of the bones other than the above-mentioned TH-dependent bones are considered unaffected by TH deficiency at the early stages. In addition, in the summer flounder, the inhibitory effect of TU appeared at 40 DAH for metamorphosis, but did not appear before 53 DAH for growth<sup>81)</sup>. Ordinary body growth during early development may be on a "steady state" time scale (degree days, for example), and the metamorphosis is on a time scale determined by an event-specific trigger (other than absolute growth) mediated by THs. The larvae migrate to the near-shore shallow areas to settle passively or actively<sup>82)</sup>, and it is well known that there is size variation when larvae settle<sup>2)</sup>. The possible presence of two time scales, one for growth and one for metamorphosis, may explain, at least partially, size variation at the time of metamorphosis and settlement.

TH is known to activate osteoblasts in teleosts<sup>80)</sup> and to accelerate osteoblastic differentiation in mammals<sup>83)</sup>. It is possible that bones or their rudiments are originally only responsive to THs at a specific stage during bone formation, and therefore the bones categorized as TH-independent are just "mature" bones and no longer require THs for growth when TU treatment is given.

In the second experiment, the effect of the timing of T4 supplementation was examined first in TU-induced developmentally inhibited larvae. As shown in Figs. 27 and 28, supplementation of T4 from 38 DAH induced complete metamorphosis, but supplementation from 67 DAH failed to induce pigmentation and the formation of Pb, resulting in juveniles with abnormal coloring and eye location. Therefore,

it is clear that there is a critical time point between 38 and 67 DAH when the TH responsiveness of the pigment cells and Pb (or their rudiments) expires. On the other hand, the dorsal fin rays or pectoral fin-related bones are considered to maintain responsiveness until 67 DAH. Even among tissues that are modified during metamorphosis by THs, each tissue seems to have a different timing of TH responsiveness. Concerning skull development during anuran metamorphosis, T3 responsiveness is expressed earlier in chondrogenesis and later in ossification<sup>42, 43)</sup>, indicating a different timing for the commencement of the response among different tissues. The present study showed that bones, which are important for morphological changes during flounder metamorphosis, require TH, and a specific expiration time of TH responsiveness was suggested for each bone. It is expected that the different expression of TH receptors (TRs) in each tissue is the fundamental phenomenon concerning different responsiveness to TH. In the Japanese flounder, TRs have been identified<sup>55, 56)</sup>, and TR transcripts shown in most of the tissues<sup>57)</sup>. As the definition of metamorphosis is complicated in teleosts<sup>47)</sup>, it may be possible to determine the individual "metamorphic change" from the TH responsiveness of larval fish.

## Chapter 3.2

### Effect of stage-specific thyroidal stasis on the eye migration of Japanese flounder

#### Introduction

A large number of teleosts undergo metamorphosis from larval to juvenile forms in order to adapt to new habitats<sup>47)</sup>. In flat fishes, eye migration is one of the most drastic changes occurring during metamorphosis, and has attracted the attention of many researchers from aspects of morphology, ecology, and physiology (see review by Brewster<sup>19)</sup>. In addition, the significance of the Pb, a bone unique to flat fish, has been recently demonstrated for eye migration (Chapter 1)).

TH plays an essential role during flatfish metamorphosis (see review by Inui<sup>5)</sup>), as is well known in anurans. Induction of metamorphic changes by TH was demonstrated at the whole body level<sup>34, 35)</sup>, and at individual tissue level in erythrocytes<sup>36)</sup>, skeletal muscle<sup>37)</sup>, gastric glands<sup>38-40)</sup>, and chloride cells in the gill<sup>41)</sup>. Therefore, it is very likely that TH also controls eye migration. However, no research has

focused on the relationship between TH and the development of specific tissue(s) involved in eye migration.

In the present study, I regarded eye migration to be the result of the individual development of various tissues and attempted to specify the tissues that are essential for eye migration. By starting to depress thyroid status at different developmental stages, the timing of TH requirements of the tissues were histologically clarified.

## **Materials and Methods**

### **Experimental Animals**

Fertilized eggs of the Japanese flounder were transported from the Fukui Prefectural Sea-Farming Center to the Fisheries Research Station of Kyoto University, Maizuru, Kyoto. Fertilized eggs were stocked in a 100 L tank at 17°C, with running seawater under a photoperiod of 11L: 13D. More than 50% of the eggs hatched three days later, and the larvae that were collected over a 24-h period were fed on the rotifer twice daily until the beginning of the experiments (22 DAH). Newly hatched *Artemia* nauplii (Utah strain) and an artificial diet were provided starting from 16 DAH.

The following developmental stages were classified after Minami<sup>16)</sup>, Keef<sup>84)</sup> and Schreiber<sup>81)</sup>, and each stage was assigned a score as indicated in parentheses for use in statistical analyses: stage D (0), bilaterally symmetric eye placement; stage E (0.5), initiation of right eye translocation; stage F (1.0), right eye further translocates, but is not visible from the left side; stage G (1.5), right eye reaches the dorsal mid-line and is partially visible from the left side; stage H- (2.0), cornea of the right eye is visible from the left side; stage H (2.5), most of the right eye has crossed the dorsal mid-line and the pupil is visible from the left side; stage H+ (3.0), the entire right eye has crossed the dorsal mid-line; and stage I (3.5), completion of right eye translocation.

At stages D, E, F and G, subpopulations of larvae were transferred from the 100 L stock tank to 25 L experimental tanks. Since survival rate is variable depending on the starting stages, the number of individuals was different among groups: 1,200 individuals (divided into two tanks) at stage D (22 DAH), 480 individuals at stage E (26 DAH), 360 individuals at stage F (30 DAH), and 240 individuals at stage G (30 DAH). After a 1-day acclimation, TU (30 ppm, an inhibitor of TH synthesis) was provided to each of the tanks with the exception of the stage D tank (started at 22 DAH, control tank). When the control fish developed into each stage, more

than twenty larvae were sampled from each tank. The experiment was terminated at 46 DAH when the control fish completed metamorphosis. Sampled fish were fixed in 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3-6 hours at room temperature, and then immersed in 70% ethanol.

Differences in developmental stage at each age were analyzed by Mann-Whitney test, and differences in body length were analyzed by t-test (Excel, Microsoft, Redmond, WA, U.S.A.).

### **Light microscope study**

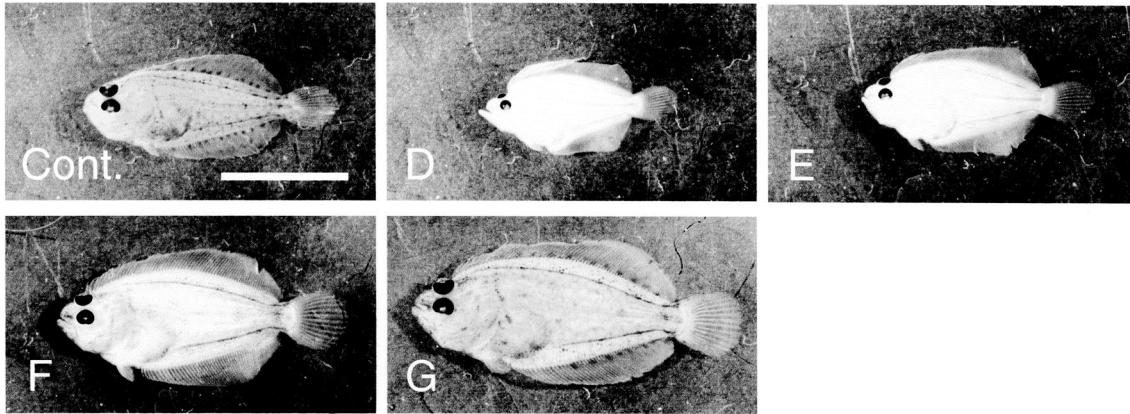
Samples were dehydrated through graded ethanol and embedded in JB4 resin (Polysciences, Inc. Warrington, PA, U.S.A.). The portion of the head around the eyes was transversely sectioned (2 µm thick) and stained with silver nitrate-toluidine blue. Cartilage undergoes metachromasy upon staining, becoming blue violet, while calcified bone becomes dark brown during the silver-nitrate reaction. The adjacent sections were checked to ensure that differences between left and the right portions of the section were not due to tilted sectioning.

## **Results**

### **Effects of TU on growth and development**

Figure 29 shows the external appearance of the fish at the end of experiment (46 DAH). Eye migration was more severely inhibited when TU treatment started at earlier stages. In the larvae treated with TU from stages D and E, the right eye migrated slightly, but was still located on the blind (right) side. However, when treatment started in stages F and G, the right eye almost migrated to the ocular (left) side but was not complete when compared to the migration in the control fish.

Figure 30 shows the changes in developmental stages and body length when TU administration was started at different stages. When TU was administered from stages D and E, development was halted at stage G, and did not reach to stage H, even at the end of the experiment (46 DAH). The body length of those fish receiving TU from stages D and E was most similar to those of the control group. On the contrary, starting TU treatment at stage G had no effect on development. The larvae receiving TU from stage F showed intermediate development; eye migration was delayed, but not halted at stage G and continuously developed toward to stage H+ or further. Body length for fish receiving treatment from



**Fig. 29** Effect of 30 ppm TU treatment started at different stages on the external appearance of Japanese flounder at 46 DAH. (Cont.) control group in ordinary seawater, (D) TU treatment started at stage D (22 DAH), (E) TU treatment started at stage E (26 DAH), (F) TU treatment started at stage F (30 DAH) and (G) TU treatment started at stage G (30 DAH). Bar 1 cm

stages F and G was significantly larger than that of control fish from 42 DAH and 35 DAH, respectively.

#### Effect of TU on development of tissues related to eye migration

At the end of the experiment on 46 DAH, the Pb was absent in the larvae treated with TU starting at stages D and E (Fig. 31D1, 31D2, 31E). In these larvae, only the rudiment of a Pb was present as an aggregation of the fibroblast-like cells at the place where the Pb would have developed. Lateral ethmoid was formed adjacent to the trabecular cartilage. On the other hand, Pb formation was observed in larvae treated with TU starting at stages F and G (Fig. 31F, 31G), though the length was shorter than that of control fish (Fig. 31 Cont). The length of Pb and thickened skin were too short to completely cover the right eyeball from the right side.

Although the torsion of the trabecular cartilage was incomplete in the larvae treated with TU starting at stage D, other bones of the cranium did not differ among the groups, irrespective of the presence of TH (control group) or timing of TU treatment. Results of bone observation are summarized in Table 4.

After the first demonstration of TH inducing metamorphosis in Japanese flounder<sup>34</sup>, the presence of thyroidal controls in the metamorphic changes of various tissues has been well studied (see Introduction, Inui and Miwa<sup>34</sup>, Miwa *et al.*<sup>35</sup>, Miwa and Inui<sup>36</sup>, Yamano *et al.*<sup>37</sup>, Miwa *et al.*<sup>38</sup>, Huang *et al.*<sup>39</sup>, Soffientino and Specker<sup>40</sup>, Schreiber and Specker<sup>41</sup>). However, relatively little attention has been given to the timing of thyroidal control. While Schreiber and Specker<sup>41</sup> first showed stage-specific responses

to TU treatment at whole body level in summer flounder, the individual bones related to eye migration were not examined. I have focused on the individual bones related to eye migration and the stage-specific responses to TU.

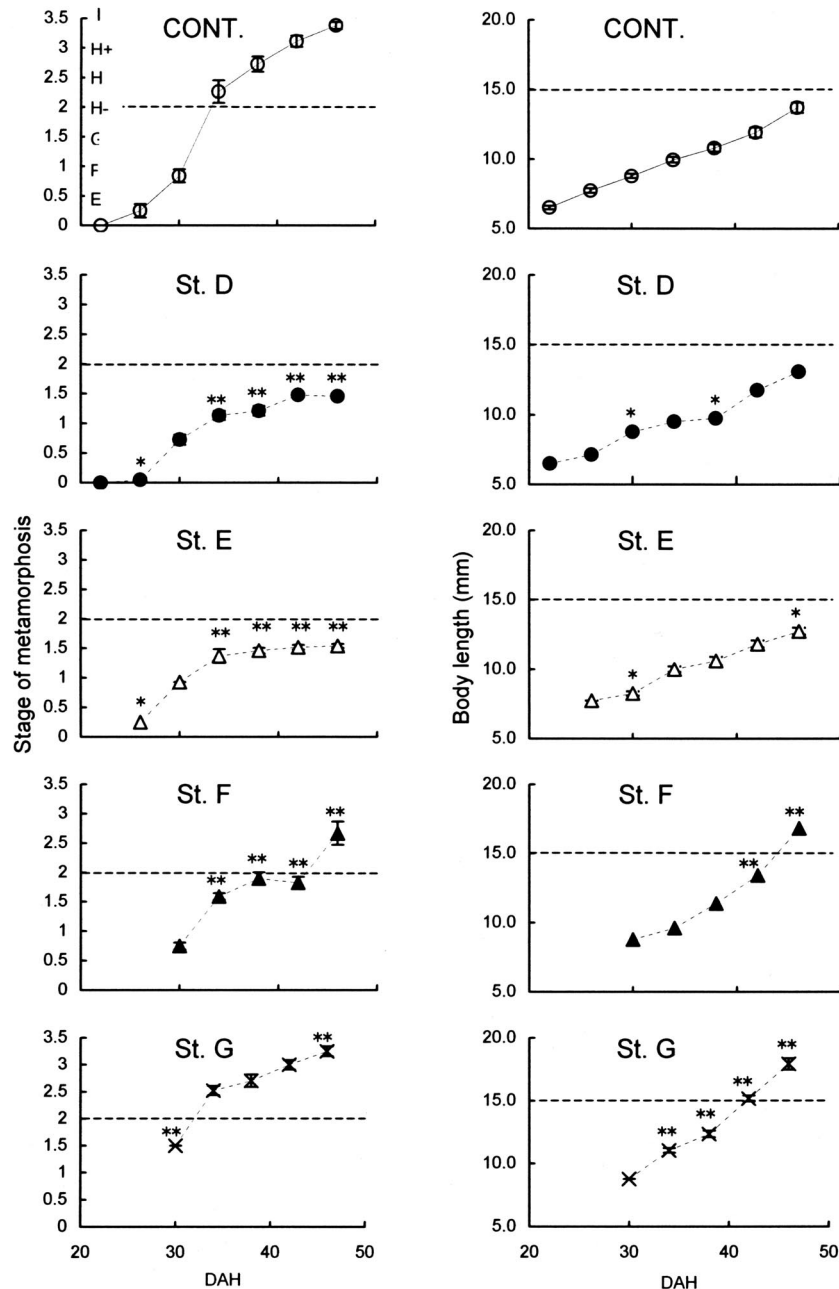
TU administration inhibited eye migration to a different extent among the groups (Fig. 29). I categorized the TU treatment groups based on the extent of eye migration. In larvae treated with TU starting at stages D and E (early start group), the right eyes were located on the blind side and the cornea was not visible from the ocular side. On the other hand, larvae receiving TU from stages F and G (late start group), the right eyes migrated to the ocular side, though migration was not complete when compared to the control group. The difference between the two groups is also clear in Figure 30. Eye migration progressed continuously until the end of the experiment in late start group, but that in early start group was halted at a stage equivalent to development stage G. The development of the Pb was also different between the two groups. The Pb was formed, though not completely, in the late start group, but was absent in the early start group (Fig. 31). Based on these results, it is clear that the location of the right eye at the end of the experiments is affected by the starting time of TU treatment and is evident in the extent of the Pb formation.

Even in the early start group, the right eye migrated slightly up to the location equivalent to that in stage G. The incomplete inhibition of eye migration by TU was reported by Miwa and Inui<sup>35</sup>. They proposed the following two explanations: 1) eye migration may be very sensitive to TH and 2) right eye migration may be induced without stimulation of TH. In the present study, the body was

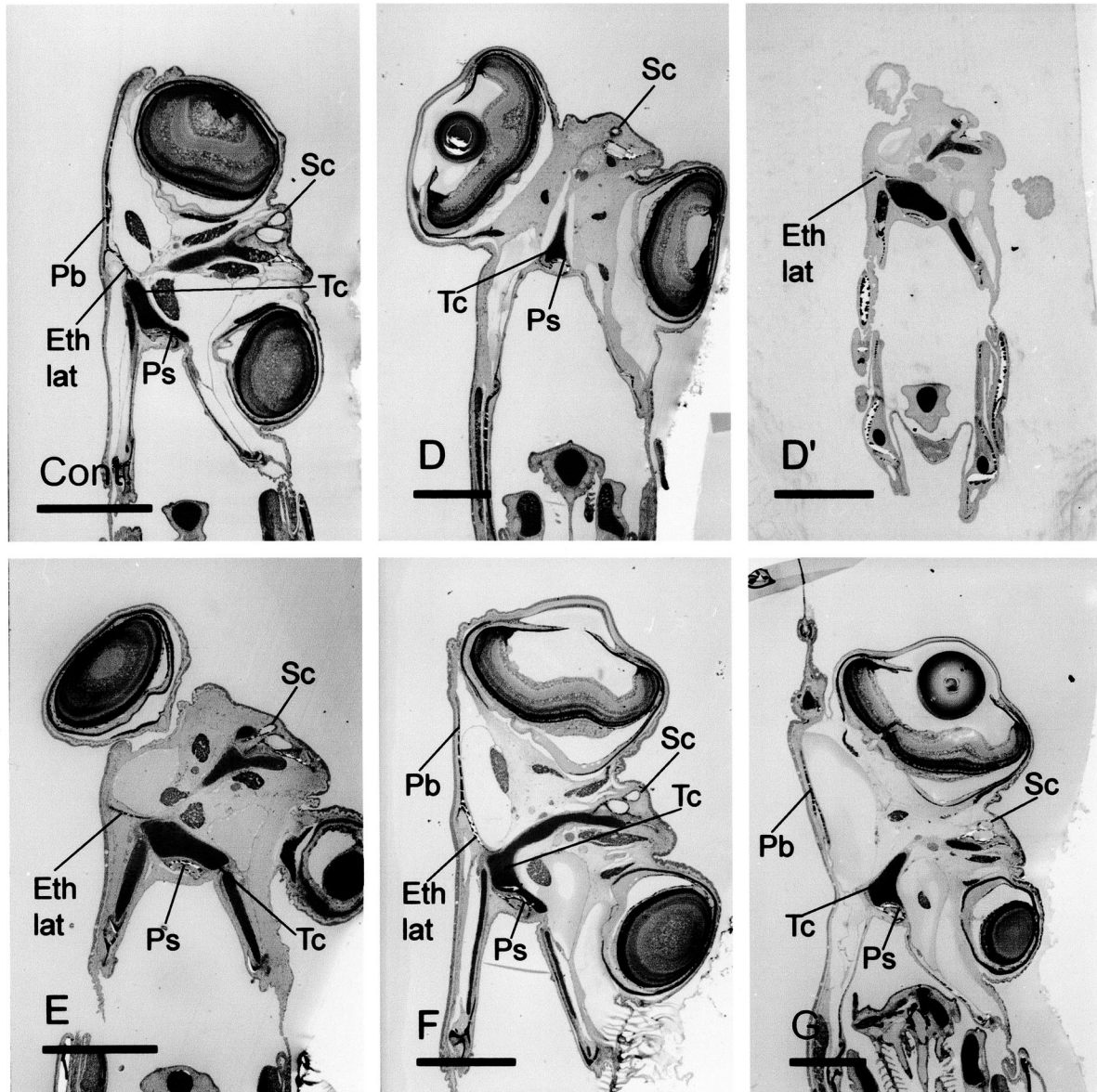
symmetrical in stage D of normal development (Chapter 1.2). However, in the larvae treated from stage D, the skin beneath the right eye and some bones and cartilage structures (lateral ethmoid, parasphenoid, supraorbital canal) were developed completely and asymmetrically at the end of the experiment (Table 4), indicating the presence of slight but significant progress of metamorphosis in bones other than the Pb.

Therefore, it is possible that only the development of the Pb requires a significant amount of TH while other tissues may require very small amounts of or no TH.

Although the right eye in the late TU group (stages F and G) moved to the ocular side, the relocation was not complete. In summer flounder, TH elevation during stages H- through H seemed to be necessary for completion of metamorphosis<sup>81)</sup>.



**Fig. 30** Changes in developmental stage (left column) and total length (right column) of Japanese flounder reared without (Control) or with TU (30 ppm) treatment starting from different development stages. (Cont.) control group in ordinary seawater, (D) TU treatment started at stage D (22 DAH), (E) TU treatment started at stage E (26 DAH), (F) TU treatment started at stage F (30 DAH) and (G) TU treatment started at stage G (30 DAH). Vertical lines represent mean + SEM (n>9). Left column; \*, \*\*: significantly different from control at P<0.05 and P<0.01, respectively, by Mann-Whitney test. Right column; \*, \*\*: significantly different from control at P<0.05 and P<0.01, respectively, by t test).



**Fig. 31** Transverse sections of bone and cartilage around the eyes of the Japanese flounder (46 DAH) treated without (Control) or with TU (30 ppm) from different stages. (Cont.) control group in ordinary seawater, (D) TU treatment started at stage D (22 DAH), (E) TU treatment started at stage E (26 DAH), (F) TU treatment started at stage F (30 DAH) and (G) TU treatment started at stage G (30 DAH). To show the presence of lateral ethmoid in the fish treated with TU from stage D, a more anterior section was presented as D'. Eth lat, lateral ethmoid; Pb, pseudomesial bar; Ps, parasphenoid; Sc, supraorbital canal; Tc, trabecular cartilage. Bars 250  $\mu$ m

**Table 4** Development of the bones and cartilage around the migrating eye. +, present and completely formed;  $\pm$ , present but incompletely formed; -, absent

	Cont.	St. D	St. E	St. F	St. G
Pseudomesial bar (Pb)	+	-	-	$\pm$	$\pm$
Lateral ethmoid (Eth lat)	+	+	+	+	+
Parasphenoid (Ps)	+	+	+	+	+
Supraorbital canal (Sc)	+	+	+	+	+
Trabecular cartilage (Tc)	+	$\pm$	+	+	+

In my observation of histological sections, the cause of incomplete eye migration was attributed to insufficient growth of the Pb. Therefore, the TH probably not only initiates Pb formation, but also enhance Pb growth to complete the eye migration. In this point, however, body growth seems to follow a different pattern. When larvae in the control group reached stage H, the larvae of the late TU group (treatment from stages F and G) were larger than those in the control group (Fig. 30). Energy costs for completing metamorphosis in control group may be attributed to the body growth.

Since a noticeable difference in Pb formation was present between the early (D-E) and late (F-G) TU treatment groups (Table 4), it was considered that normal Pb development requires TH addition between stages E and F. In other words, because TH already exerts a stimulatory effect on the Pb before reaching a point in development between stages E and F, TU treatment starting from stage F cannot inhibit development of the Pb. During the course of Pb formation, the rudiment first appears as an aggregation of fibroblast-like cells at stage E and an osteoid forms at stage G (Chapter 1.2). The presence of the osteoid form of Pb at stage G suggests the presence of functional osteoblasts at stage F. Since THs accelerate osteoblastic differentiation in mammals<sup>83)</sup>, it is possible that the role of TH in Pb formation involves the functioning of osteoblasts.

In the metamorphosis of South African clawed frog (*Xenopus laevis*), many of the TH responsive genes were identified<sup>86)</sup>, and developmental programs (e.g. tail resorption) have been understood as complex changes of up-regulated and down-regulated genes by TH<sup>87)</sup>. In the Japanese flounder, TRs have been cloned<sup>55, 56)</sup> and the presence of TR transcripts has been demonstrated in most tissues<sup>57)</sup>. However, other TH responsive genes have not yet been identified in flounders. It is expected that TH responsive genes that are critical for Pb formation are expressed by the time in development between stages E and F.

In conclusion, TH was shown to be essential for the initiation of right eye migration by a point in development between stages E and F. In addition, the full development of the Pb was suggested to be the principle process for the completion of eye migration and strictly requires TH. Since metamorphosis is a complex change occurring in various tissues and is orchestrated by the thyroid, more study on individual tissues as receptors of TH stimuli is needed for a more complete understanding of the mechanisms involved in

metamorphosis.

## Concluding Remarks

Metamorphosis is widely observed in large number of fishes<sup>47)</sup>. Although the involvements of various environmental and hormonal cues are demonstrated for the initiation and progress of metamorphosis, TH is considered to be the most important. In my study, the mechanisms of asymmetrical eye migration were physiologically examined using the Japanese flounder.

At the first step of the research, the development of various tissues in head region was examined in detail during flounder metamorphosis by using stage-by-stage analyses of laboratory-reared larvae and early juveniles (Chapter 1.1). The strongest asymmetry was found in the Pb, a specific and characteristic bone only present in flatfishes. This bone was present only on the blind side and became elongated intensively toward dorsal side. The manner of Pb formation appeared to push the right eye directly in the dorsal direction, or at least prevented the right eye from moving backward. A significant increase of skin thickness was observed in the future position of the Pb at the onset of eye migration: the first asymmetrical development observed in flounder metamorphosis at an all-or-none level.

Another significant asymmetry was observed in the retrorbital vesicle (Rv). The growth of the right (future blind side) Rv was faster and greater than that on the left (future ocular side). Inflation of the right Rv at the time of eye migration strongly suggested the involvement of the Rv in eye migration, probably by pushing the right eye to the dorsal side.

The electron microscopic study indicated that the Rv sheath cells were flat and had many pinocytotic vesicles with scanty rER and a small Golgi apparatus (Chapter 1.2). These ultrastructural features were reminiscent of endothelial cells, which represented the inner lining of blood and lymphatic vessels. Both the location and the ultrastructure of the Rv lead me to consider this structure as part of the subocular lymph sacs. When the Rv sheath was assumed to have a high permeability to liquid like the mammalian lymphatic capillaries, it was possible that the body fluid moves freely from inside to outside or outside to inside of the Rv. Therefore, even if there were any active mechanisms to take up fluid into the Rv, it was probably difficult to expand the Rv to push the right eye. I speculated that the Rv was

expanded passively to fill the empty place "caused by" the eye migration.

The Pb was also a candidate for providing the driving force for eye migration. In the Pb rudiment of the metamorphosing flounder, the aggregated fibroblast-like cells were also observed by electron microscopy and appeared active judged from the lobulated nuclei, extensive rough endoplasmic reticulum and mitochondria. In addition, many collagen fibrils surrounded the cells. Therefore, these fibroblast-like cells were considered to produce collagen fibrils. For the initiation of right eye migration, the formation of the Pb rudiment appeared to be important.

To find important tissue(s) for eye relocation in flounder metamorphosis, common deformities present in individuals with abnormal eye location (AEL) was examined in the next step of this study (Chapter 2.1). As the result, a close relation between the AEL and abnormal formation of the Pb was pointed out. Since the eye did not move when the Pb did not exist, although all other bones formed appropriately, the normal formation of the Pb is considered indispensable for normal eye relocation. In addition, the significance of the epidermis and dermis was suggested as the upper control for the Pb development.

On the abnormally developed individuals that appeared among ordinary seed production of Japanese flounder, morphological examination was carried out (Chapter 2.2). For this specific case, the fundamental phenomenon of the abnormality was attributed to the retardation of development, especially metamorphosis. The abnormal morphology of the thyroid gland, as well as significantly lower serum T4 and T3 concentrations on 115 DAH and the differential responsiveness of thyroid follicle to TU, suggested the insufficient function of the thyroidal system. Since the responsiveness to TH was demonstrated in peripheral tissues by the administration of TH to the abnormal fish, the insufficient production of TH was pointed out as the main cause of the abnormal fish of this case. If formation of thyroid gland is vulnerable to unknown factor(s) present in hatcheries, it is possible that thyroidal malformation (as found in this case) is potentially present at a significant rate in hatcheries, which may cause abnormal development and deformities at a certain circumstances.

The significance of the timing of TH secretion was also suggested as a potentially important cause of abnormal juveniles in this study. From the TU and T4 treated experiment (Chapter 3.1), the bones important for

morphological changes during flounder metamorphosis was shown to require TH, and presence of a specific expiration time of TH responsiveness was first suggested for each bone. In Chapter 3.2, the stage specific responses to TH deficiency were examined in detail for individual bones. As the result, it was possible that only the development of the Pb requires significant amount of TH, and other tissues may require very small amount of, or totally free from, TH. Since essential difference in Pb formation was present between the TU treatment starting from E and F stages, it was considered that the normal Pb development requires TH by the time between stage E and F.

From the present study, the essential contribution of the Pb and its rudiment in eye migration was first revealed using Japanese flounder. In addition, the significance of tissue and stage specific responsiveness to TH was strongly suggested for the eye migration during flounder metamorphosis. In the large-scale hatcheries for flatfish seed production, a much higher occurrence of abnormal juveniles has been observed<sup>20, 21)</sup> than in sea-caught adults<sup>6, 22-26)</sup>. To solve this problem, as suggested in this study, the basic research on the development of the Pb, the responsiveness to the TH of individual tissue, and secretion system of the TH, need more attention.

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