Histological study of deformity in eye location in Japanese flounder *Paralichthys olivaceus*

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ABSTRACT: The internal structure of malformed Japanese flounder *Paralichthys olivaceus* juveniles in eye location was histologically examined and deformed bones were identified as the tissue important for eye relocation. A deformed pseudomesial bar (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.

KEY WORDS: deformity, eye relocation, Japanese flounder, left–right asymmetry, pseudomesial bar.

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 than in sea-caught adults.

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.

In contrast, information on AEL is very limited. Although the presence of AEL in sea-caught flatfish has been reported in many species, and the relation with the abnormality in body coloration has been reported in brown sole *Pleuronectes herzensteini* and Atlantic halibut *Hippoglossus hippoglossus*, 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. 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, we examined the asymmetrical development of bones and skin related to eye relocation at light microscopy level, and suggested that a membrane bone, pseudomesial bar (Pb), is the strongest candidate causing the asymmetrical relocation of the eyes during flounder metamorphosis.

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 *Paralichthys olivaceus* 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 by Okada et al.27

Abnormal fish, together with normal fish as control, were selected from the rearing tank at 48 days after hatching (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 h at room temperature, dehydrated through graded ethanol, and embedded in JB4 resin (Polysciences Inc., Warrington, PA, USA). The head part around the eyes was transversely sectioned at 2 μm thickness 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 based on Okada et al.27 and schematically summarized in Fig. 1. All samples were classified into four types depending on the degree of eye location, which is represented by the degree of θ (Fig. 1). When θ was approximately 90°, the fish was classified into Type I (normal). When θ was approximately 40–60°, the fish was classified into Type II (mild AEL). When θ was approximately 0–30°, the fish was classified into Type III (serious AEL) and when θ was approximately –90°, 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. 2) 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. 2b); the right one grew 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 parasphe- noid (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 retractor 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. 3). 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. 3b, 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. 4). 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 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

Fig. 3  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. 4  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.
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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 (Fig. 4).

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

### Table 2  Asymmetrical tendency of each tissue and organ found in different types of abnormal eye location (AEL) individuals of Japanese flounder

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Visceral handedness</th>
<th>Sc</th>
<th>Sob</th>
<th>Ps</th>
<th>Tc</th>
<th>Pb</th>
<th>Epidermis</th>
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+, 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.

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.27 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, 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, and since the AEL individuals in the study of brown sole were similar to the Type III individuals in the present study, our 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 Sb. 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. Higher occurrence of AEL was reported in albino fish of hatchery-reared mud dab *Limanda yokohamae* and frog flounder *Pleuronichthys cornutus,* 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. 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. 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 or after the metamorphosis in the fish with normal eye location. Primary ambicoloration was also reported in brown sole.

Norman divided ambicoloration into three categories, ‘staining’, ‘spotting’ and ‘true ambicoloration’. Seikai 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. 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 deter-
mine 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.

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