

Progression of staining-type hypermelanosis on the blind side in normally metamorphosed juveniles and pigmentation progression in pseudoalbino juveniles of the Japanese flounder *Paralichthys olivaceus* using individual identification

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Abstract Abnormal flounder coloration frequently occurs in flounder hatcheries and diminishes the commercial value of the fish. To understand hypermelanosis, the progression of staining-type hypermelanosis in normally metamorphosed juveniles and ocular-side pigmentation in pseudoalbino juveniles were examined in the Japanese flounder *Paralichthys olivaceus*. Sixty-five days post hatching, juveniles (total length, 6 cm) were individually identified by color-marker implantation, and the darkened area of the body surface was examined for 10 weeks by image analysis of digital photographs of the fish taken from above or below the transparent tank. Staining was observed to mainly begin at the upper and lower bases of the tail fin, expanded anteriorly along the peripheral part of the trunk, and ceased after 2 months. The individuals in which staining occurred earlier expressed severe staining and small body size by the end of the experiment. Further, pigmentation of the ocular side in pseudoalbino juveniles ceased after 2 months, but the order of pigmentation was different from that on the blind side. In this case, darkening began from the posterior, but expanded from the center to the periphery of the trunk. Even at the end of the

experiment, ctenoid scales were exclusively found within the darkened area, together with cycloid scales.

Keywords Color anomaly · Ctenoid scale · Hatchery production · Hypermelanosis · Individual identification · Japanese flounder · Pseudoalbino · Staining

Introduction

The production of juvenile Japanese flounders *Paralichthys olivaceus* is successful on an industrial scale in hatcheries [1]. However, the occurrence of “staining” remains an uncontrolled problem. Staining is a type of color anomaly and is expressed as darkened areas on the blind side of fish after the completion of metamorphosis [2], which decreases the market price of the fish [3]. Previous studies on staining and related phenomena have shown that bottom sand is a preventive measure for staining [4–6]. In addition, information which suggests morphological similarity between staining and a normal ocular side has accumulated. For example, in the stained area of the blind side, there are adult-type melanophores, xanthophores, and ctenoid scales; those normally appear only on the ocular side during or after metamorphosis [7–14]. For “true ambicoloration”, a different type of darkening that is visible even just after the completion of metamorphosis, Seikai had proposed the idea that darkening of this type is due to an ocular side formation on the blind side [15]. In our previous study confirming the type and location of chromatophores and scale types (cycloid and ctenoid), we proposed the fundamental nature of staining as a “status change in the body surface conditions from the blind to the ocular side” [14].

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However, the detailed process and duration of staining expansion is still unclear. Furthermore, although the presence and local expansion of a ctenoid-scale area had been reported partially inside of the stained area [10, 14], quantitative analysis of ctenoid-scale area (cover ratio of ctenoid scales in stained area, for example) has not been accomplished yet. This is mainly because of the large individual variance in the extent of staining; the area of staining can vary from 0 to 50 % of the blind side of fish in a single rearing tank [14]. In the time-course samplings employed in most of the previous studies [4–6, 9–14], including ours, individual organisms were killed at each time point; thus, at each time point a different fish was assessed. Therefore, it was methodologically impossible to precisely reconstruct the actual staining process that occurred in an individual. Kang and Kim [13] used a large sample (60 individuals per sampling date), and first described the change in stained-area ratio with time on the blind side of a fish, giving a quantitatively precise indicator of the degree of staining. However, their final sampling was conducted at 3 months post-hatching, when the staining area was still increasing. Hence, the duration of staining progression has not been examined precisely enough yet to discuss the mechanisms of staining progression. To overcome the large individual variance in staining and determine the duration of staining progression, we used individual identification in this study, following Yamada et al. [16], and traced the staining expansion process without killing the target fish. Furthermore, by observing the process more frequently than Yamada et al. [16] and by adding detailed analyses between the stained area and various possible parameters, this method allowed us to retrace the characteristics of individuals that expressed severe staining at the end of the experiment.

Pseudoalbinism is another type of color anomaly, in which fish lack pigmentation on the ocular side, and this has been largely overcome by improvements in nutrition [15, 17, 18]. In pseudoalbino juveniles, it is known that pigmentation occurs after metamorphosis on the non-pigmented area on the blind side, and morphological similarity between the pigmented area and the normal ocular side has been indicated [8, 9]. This phenomenon is very similar to the staining observed on the blind side of normally metamorphosed juveniles. Moreover, the blind side of pseudoalbino juveniles tended to show severe staining [4, 8]. Therefore, we additionally examined pseudoalbino juveniles, focusing on the expansion process of the darkened area on the ocular side as well as on the blind side, by using our individual identification method.

Materials and methods

Samples

Rearing before experiments

Fertilized eggs of the Japanese flounder were obtained by natural spawning from mature adults maintained at Chiba Prefectural Farming Center, Chiba, Japan. After hatching, larvae were reared at natural water temperature, initially at 18 °C at hatching, 23 °C at the completion of metamorphosis (34 days post-hatching [DPH]), and 27 °C just before transportation to Kyoto University (62 DPH). The rearing density was 20,000 individuals/m³ at hatching, 8,000–9,000 individuals/m³ at 10 DPH, 5,000 individuals/m³ when metamorphosis was complete, and 500–1,000 individuals/m³ for settled juveniles. The average growth rate of the juveniles was 1.3 mm/day for the period of 55–60 DPH. At 63 DPH, 65 flounders without darkened areas and 29 pseudoalbino flounders, with total lengths of approximately 6 cm, were selected, and transported to Kyoto University through a parcel delivery service, packed with oxygen and at natural temperature. They arrived at 64 DPH.

Selection of individuals for experiments

Faint staining occurred on the blind side of some individuals during transportation. To examine the possible differences between unstained and faintly stained individuals, normally metamorphosed individuals were divided into two groups after arrival at Kyoto University: (1) clean-started, individuals with normal ocular side development and no darkened areas on the trunk of the blind side at the beginning of the experiment (ratio of darkening [described below in “Measurement of changes in darkened areas”]: 0.006–0.014, 13 individuals); (2) stain-started, individuals with normal ocular side development and darkened areas on the tail base (level 3 in Isojima et al. 2013) of the blind side at the beginning of the experiment (ratio of darkening: 0.012–0.077, 17 individuals). In addition, individuals with pseudoalbinism on the ocular side at the beginning of the experiment were classified as (3) pseudoalbino (ratio of darkening on the blind side: 0.015–0.035, 10 individuals). For each group, typical appearance on the blind side at the beginning of the experiment is shown in Fig. 1. In the pseudoalbino group, 7 individuals showed darkened areas on the blind side at the beginning of the experiment. The remaining individuals that were not included in the above groups and not used for this experiment were anesthetized in 0.1 % 2-phenoxyethanol (Nacalai Tesque Inc., Kyoto, Japan) and fixed in 10 % neutralized formalin (Nacalai Tesque Inc.) as the initial samples.

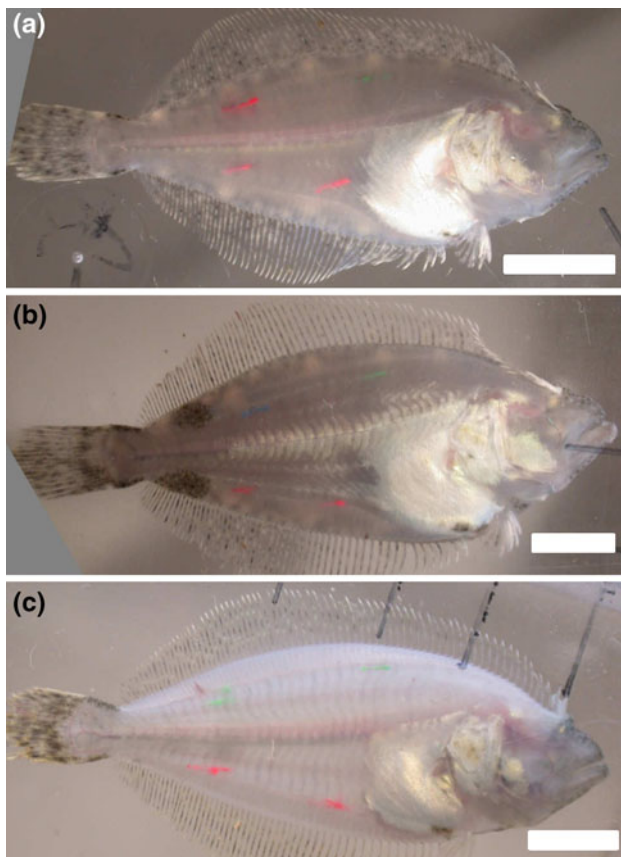


Fig. 1 Typical appearance of the blind side at the beginning of experiment. **a** Clean-started (51 mm, body length), **b** stain-started (49 mm), and **c** pseudoalbino (57 mm). White bars indicate 1 cm

At 65 DPH, the juveniles for the rearing experiment were marked with 3 colors (red, blue, and green) by using visible implant elastomer (Northwest Marine Technology, Inc., Shaw Island, WA, USA) at 4 points on the blind side to enable these individuals to be identified.

Rearing procedure and final sampling

All juvenile flounders of three groups (clean-started, stain-started, and pseudoalbino) were mixed after marking, and reared together in a 100-l polycarbonate tank (transparent, installed on a gray plastic plate) with a water temperature of approximately 25 °C, and were fed 4 times a day with artificial diets (Nagisa K1 [diameter, about 0.8–1.2 mm; 65–85 DPH] and Nagisa K2 [diameter, about 1.2–2.8 mm; 86–130 DPH], Oriental Yeast Co. Ltd., Tokyo, Japan; Otohime EP2 [diameter, about 1.9–2.3 mm; 131–142 DPH], Marubeni Nissin Feed, Tokyo, Japan).

Since previous studies have shown that the stained area is larger in flounders reared in tanks without bottom sand [4–6] and with longer periods of light irradiation per day [6], we reared flounders in tanks without bottom sand, with 16 h light irradiation per day. However, the density of

juveniles in the tank was not adjusted in response to their growth or decrease in numbers because staining occurs at a significant level in the tanks without bottom sand regardless of density [19].

At 143 DPH, all juveniles (except 8 individuals that were used for a different experiment) were anesthetized using 0.1 % 2-phenoxyethanol (Nacalai Tesque Inc.), fixed in 10 % neutralized formalin (Nacalai Tesque Inc.), and the distribution of ctenoid scales was examined.

Samples reared at Fukui Prefectural University

To examine the distribution of ctenoid scales on the blind side of juveniles after an extended period, flounders more than a year old were also examined. The flounders were originally purchased from Marinotech Co., Ltd. (Tahara, Japan) as juveniles and were reared in tanks without bottom sand at Fukui Prefectural University Research Center for Marine Bioresource, in accordance with the ordinary flounder-rearing procedure used in this laboratory. Six flounders of age about 1 year were randomly selected as samples, anesthetized using 0.1 % 2-phenoxyethanol (Nacalai Tesque Inc.), and fixed in 10 % neutralized formalin (Nacalai Tesque Inc.).

Measurement of changes in darkened areas

The blind side of each flounder was photographed once a week from 66 to 136 DPH without anesthetizing by using a digital camera (OLYMPUS Tough TG-615, Olympus Corp., Tokyo, Japan) installed under the transparent glass aquarium. The size of the darkened areas, the size of the blind side (excluding the fins), and body length were measured using NIH Image J (available online at <http://rsbweb.nih.gov/ij/>; National Institute of Health, USA). For pseudoalbino fish, the size of the darkened area on the ocular side and the size of the ocular side (excluding the fins and eyes) were measured after the first week. By using these results, the ratio of darkening on the blind side of each individual (size of the darkened area on the blind side/size of the blind side, excluding the fins) and the ratio of darkening on the ocular side of each individual (size of the darkened area on the ocular side/size of the ocular side, excluding the fins and eyes) were calculated. The measurements were terminated at 136 DPH (10th week) because the staining expansion appeared to cease in all individuals, and the ratio of darkening on the blind side at 136 DPH was regarded as the maximum ratio of darkening. Daily growth rate (mm/day) was calculated for the experimental period of 10 weeks.

Because an obvious increase in darkened area was observed during the period when the ratio of darkening on the blind side ranged from 10 to 90 % of the maximum ratio of darkening, this period was considered as the

darkening period. Further, the increase in the ratio of darkening with time (per week) during this period was calculated as the darkening speed as follows.

$$\frac{(\text{ratio of darkening at the end}) - (\text{ratio of darkening at the beginning})}{(\text{weeks between the beginning and end of the period})}$$

Examination of the distribution of ctenoid scales

To clarify the relationship between staining and shift from cycloid scales to ctenoid scales on the blind side of juvenile flounder, the distribution of ctenoid scales was extensively examined across the entire blind side by using the method described by Isojima et al. [14].

From the stain-started juveniles reared at Kyoto University, 6 individuals at 143 DPH with a typical degree of staining for the group at that age (ratio of darkening: 0.42–0.50, appearance similar to the illustrations of 4th–5th week in Fig. 3) were selected for this examination and were termed as “having finished staining.” In addition, all flounders reared at Fukui Prefectural University were used in this examination and were named “1-year-old” (ratio of darkening: 0.29–0.93).

Following the method of Isojima et al. [14], spines of ctenoid scales were visualized. In brief, formalin-fixed whole body samples were stained with Alizarin Red, immersed in 70 % ethanol, and air dried. Then, the spines of ctenoid scales reflect light irradiated from an anterior direction. On photographs taken of the whole blind side, the area of the blind side (excluding the fins), darkened areas, darkened areas occupied by ctenoid scales, and normal areas occupied by ctenoid scales were measured from the digital images using NIH Image J. By using these results, the ratio of darkening and the ratio of ctenoid scales in the darkened area (the ratio of areas occupied by the ctenoid scales in the darkened area) were calculated.

Statistical analysis

For statistical analyses, online tools provided by the Osaka University (available at <http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom>) were used. Student's *t*-tests followed by subsequent multiple comparisons using the Tukey–Kramer method were used to compare growth, daily growth rate, maximum ratio of darkening, and darkening speed among each group.

Results

Comparison of growth among the three groups

The increase in body length per day of the flounders reared at Kyoto University ranged from 0.7 to 1.4 mm/day, and

Table 1 Comparison of the initial and final body lengths (cm) and daily growth rate (mm/day) among the three groups

Group	0 week (cm)	10 weeks (cm)	Daily growth rate (mm/day)
Clean-started	5.5 ± 0.2 ^{ab} (13)	14.9 ± 0.3 ^a (11)	1.3 ± 0.04 ^a (11)
Stain-started	5.3 ± 0.1 ^a (17)	12.3 ± 0.3 ^b (16)	1.0 ± 0.04 ^b (16)
Pseudoalbino	5.7 ± 0.1 ^b (10)	13.4 ± 0.4 ^b (10)	1.1 ± 0.06 ^b (10)
Average	5.5 ± 0.1 (37)	13.4 ± 0.3 (37)	1.1 ± 0.04 (37)

Mean ± SE (*n*). Different characters indicate the presence of a statistical difference among groups in each week ($P < 0.05$)

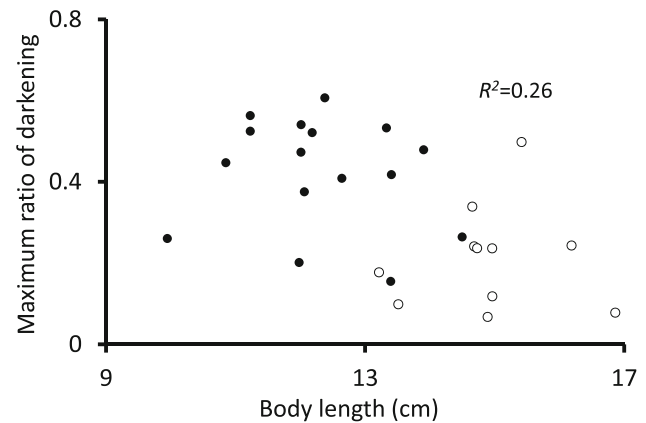


Fig. 2 Relationship between maximum ratio of darkening on the blind side and body length at the 10th week for individuals that had undergone normal metamorphosis. Open circles and closed circles indicate clean-started and stain-started individuals, respectively

the mean was 1.1 ± 0.03 mm/day. Although we attempted to select individuals of uniform body length before transporting the fish to Kyoto University, pseudoalbino fish were significantly larger than stain-started fish at the beginning of the examination ($P < 0.05$; Table 1). At the end of the measurements (10th week), however, clean-started fish were significantly larger than the other fish. It was the same for growth per day ($P < 0.05$; Table 1).

At the individual level, no obvious relationships were observed between body length and maximum ratio of darkening for individuals of both clean-started and stain-started groups ($R^2 = 0.26$, Fig. 2).

Qualitative and quantitative analysis of darkening enlargement on the blind side

Figure 3 shows the typical enlargement of staining on the blind side of a stain-started individual. In all the individuals, staining began from the base of the tail fin, extended anteriorly along the base of the dorsal and anal fins, and extended from the edge to the lateral line. Furthermore, in some individuals, after the darkening reached around the middle of the trunk, it began in three further places: the bases of the pectoral fin and pelvic fin, and the center of the

head. The former two spots expanded until the two areas were connected, and the spot on the head expanded concentrically. It was noticed that all the individuals at the end of experiment showed a similar pattern of darkened area to one of the figures shown in Fig. 3.

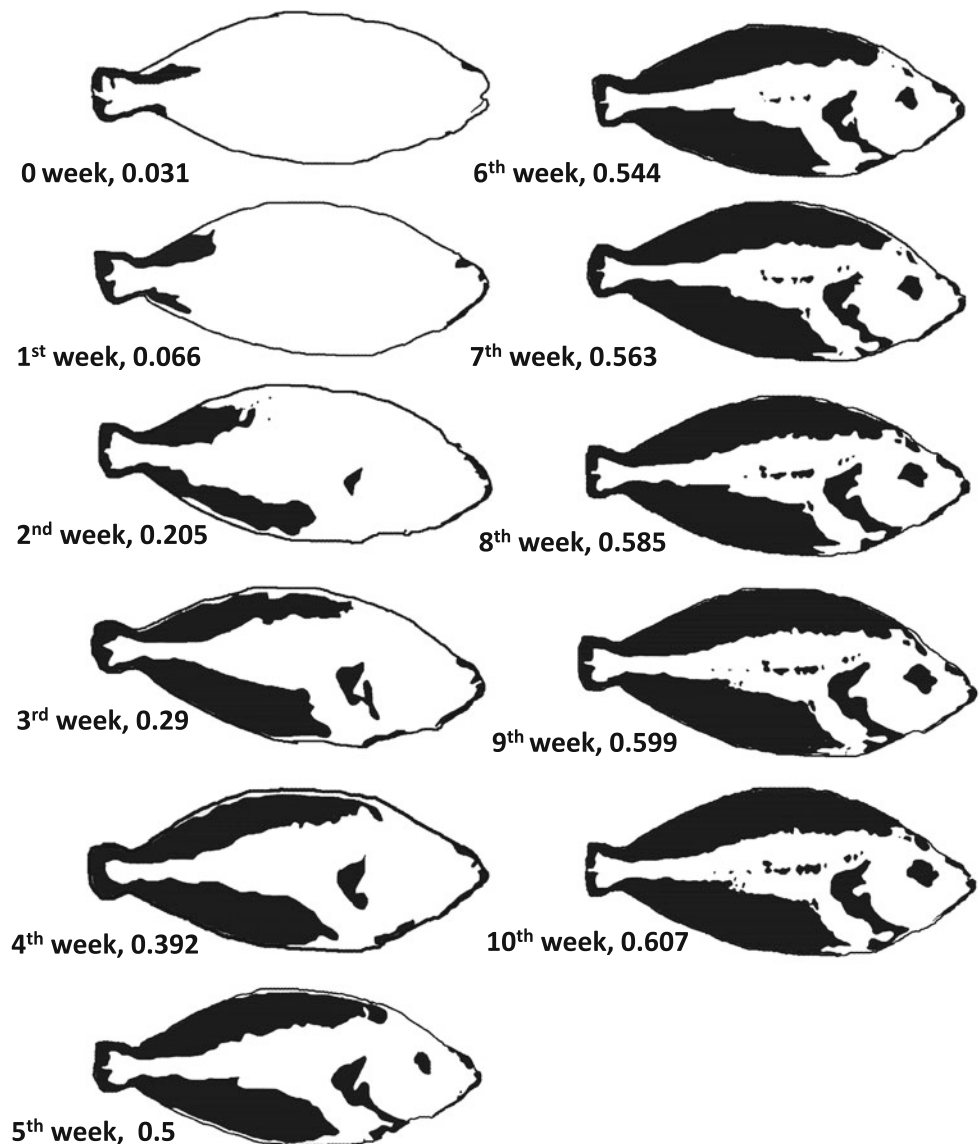
Regardless of the groups, the ratio of darkening on the blind side increased with time up to a certain value (Fig. 4). No obvious relationship was observed between the ratio of darkening at the start of the experiment and the maximum ratio of darkening (Table 2). However, in the measurements after the 5th week, the ratio of darkening showed a strong linear relationship with the maximum ratio of darkening and the contribution ratio of regression (R^2) was over 0.9 (Table 2).

However, as shown in Fig. 4, the increase in the ratio of darkening in each individual had ceased before the final

measurement, about 1–2 months after the beginning of the darkening period. The darkening period of clean-started fish tended to be longer and ceased later than that in the fish of the other groups.

The maximum ratio of darkening differed greatly, even among individuals in the same group. However, when comparing the groups, the maximum ratio of darkening of clean-started fish (0.21 ± 0.04) was significantly smaller than that of the stain-started (0.42 ± 0.03) or pseudoalbino fish (0.48 ± 0.05) ($P < 0.05$; Fig. 5a). A similar trend was observed for the darkening speed ($P < 0.05$; Fig. 5b). For individuals that had metamorphosed normally (clean-started and stain-started), a strong linear relationship was observed between the maximum ratio of darkening and the darkening speed (Fig. 6).

Fig. 3 Typical pattern of staining expansion. *Black area* indicates the darkened areas. The ratio of darkening on the blind side is indicated next to the weeks. Stain-started individual, 5.4-cm body length at 0 week and 12.4-cm body length at 10th week



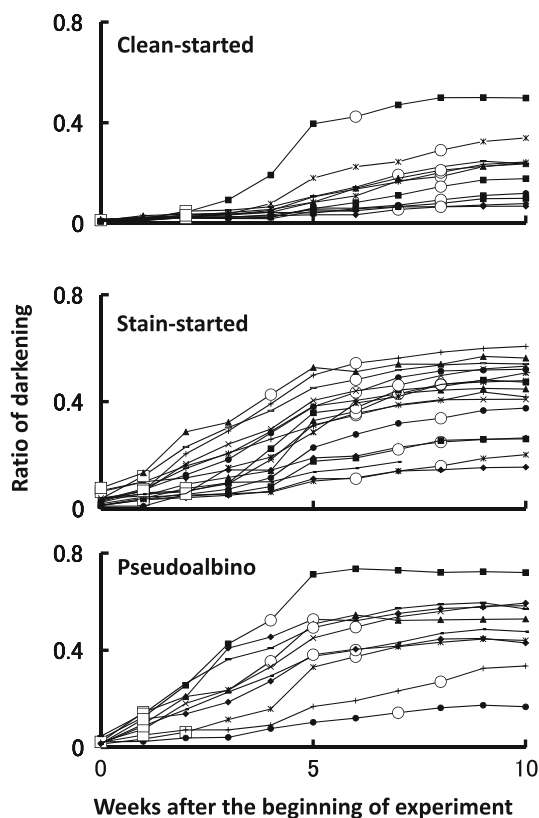


Fig. 4 Changes in the individual ratio of darkening on the blind side and the darkening period. *Open squares* and *open circles* indicate the beginning and the end of the darkening period, respectively

Table 2 Regression line and R^2 values of each week between ratio of darkening (x) and maximum ratio of darkening (y) of clean-started and stain-started fish

Week	Regression line ($y=$)	R^2
0	$4.66x + 0.26$	0.210
1st	$2.99x + 0.20$	0.509
2nd	$1.81x + 0.18$	0.700
3 rd	$1.28x + 0.19$	0.750
4th	$1.17x + 0.16$	0.855
5th	$0.95x + 0.10$	0.943
6th	$0.94x + 0.09$	0.965
7th	$0.95x + 0.06$	0.981
8th	$0.97x + 0.03$	0.990
9th	$0.99x + 0.01$	0.997

Darkening on the ocular side of pseudoalbino fish

There were two types of darkening order on the ocular sides of pseudoalbino fish (Fig. 7). In 6 individuals (type A), the darkening of the head occurred after complete darkening of the trunk. In the other 4 individuals (type B), the darkening of the head occurred simultaneously with the trunk darkening. In both the types, darkening hardly

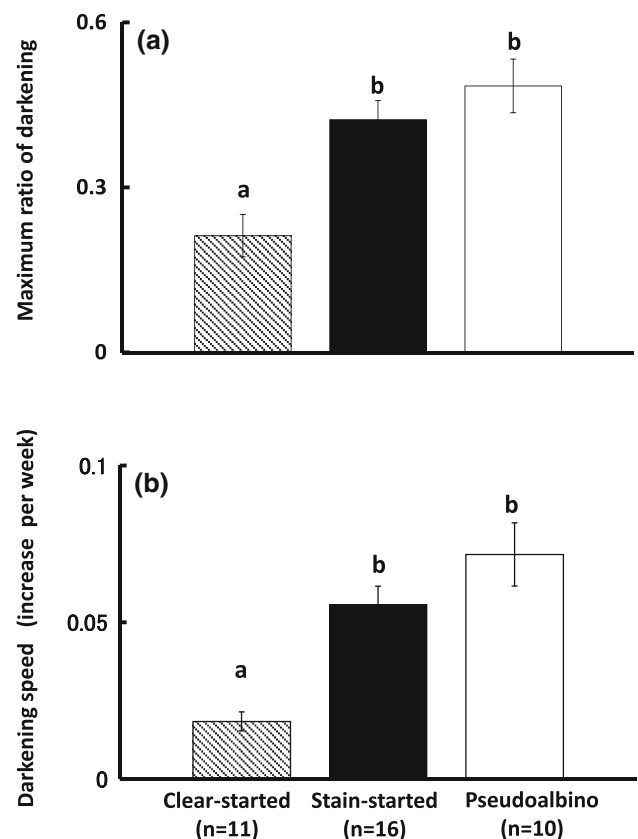


Fig. 5 Comparison of (a) maximum ratio of darkening and (b) darkening speed (increase in the ratio of darkening per week) among groups. Mean \pm standard error (SE). *Different characters* indicate the presence of statistical difference ($P < 0.05$)

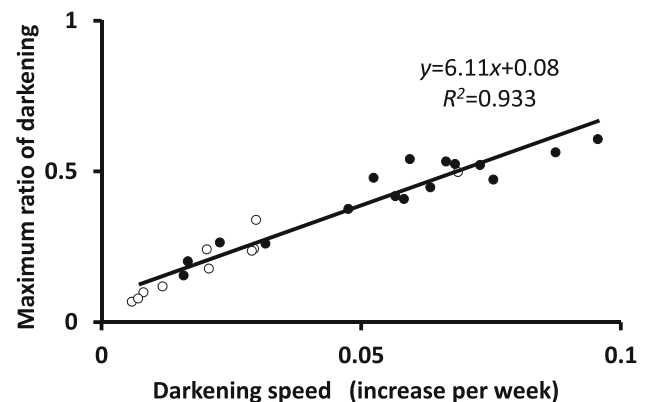


Fig. 6 Relationship between maximum ratio of darkening and darkening speed (increase in the ratio of darkening per week) for individuals that had undergone normal metamorphosis. *Open circles* and *closed circles* indicate clean-started and stain-started individuals, respectively

occurred at the anterior base of the dorsal fin. During the course of darkening, the darkened area on the trunk expanded from the tail to the head in a typical manner, as observed during the course of darkening on the blind side of the fish. However, in contrast, the darkening on the

Fig. 7 Typical two patterns of darkening expansion on the ocular side of pseudoalbino individuals. *Black areas* indicate darkened areas. Head darkened after trunk in type A, while simultaneously with trunk in type B

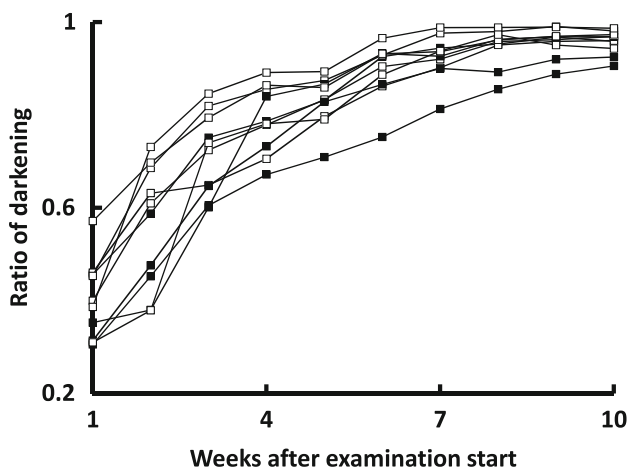
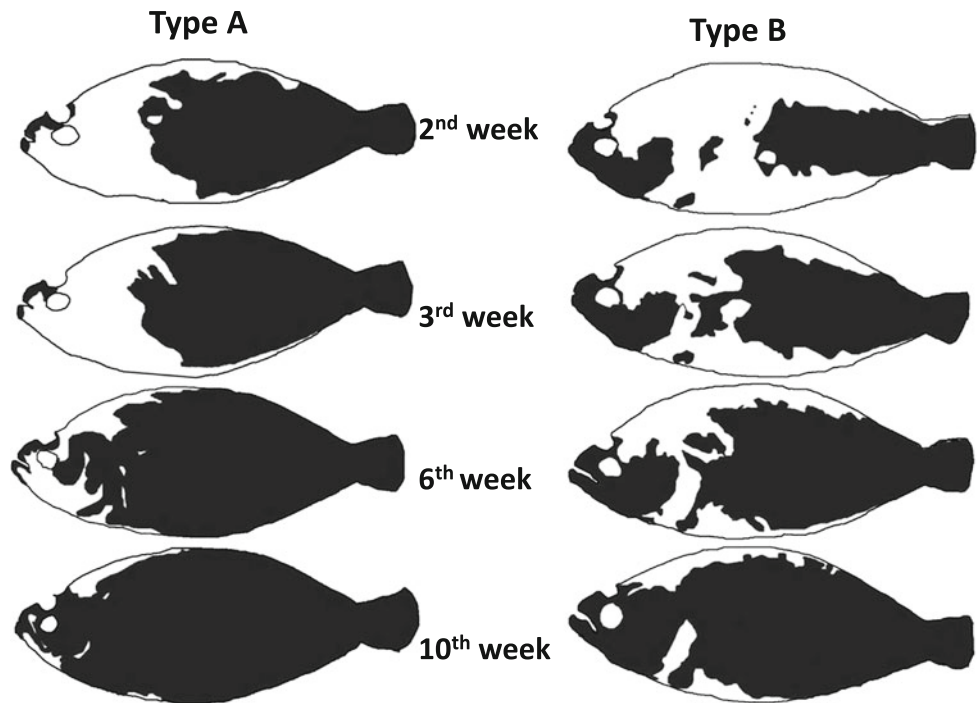


Fig. 8 Change in the individual ratio of darkening on the ocular side of pseudoalbino fish. *Open squares* and *closed squares* indicate “type A” and “type B” individuals, respectively

ocular side of pseudoalbino fish vertically expanded from the lateral line to the edge (Fig. 7). In addition, it was a characteristic of ocular side darkening that pale darkening occurred initially, and then the area gradually became darker and finally reached a similar coloration to the normal ocular side (data not shown).

The ratio of darkening on the ocular side of pseudoalbino fish increased with time (Fig. 8). Within 10 weeks, the increase in the darkened area stopped with the ratio of darkening greater than 0.9. There were no obvious differences in the ratio of darkening or darkening speed between type A and type B individuals (Fig. 8).

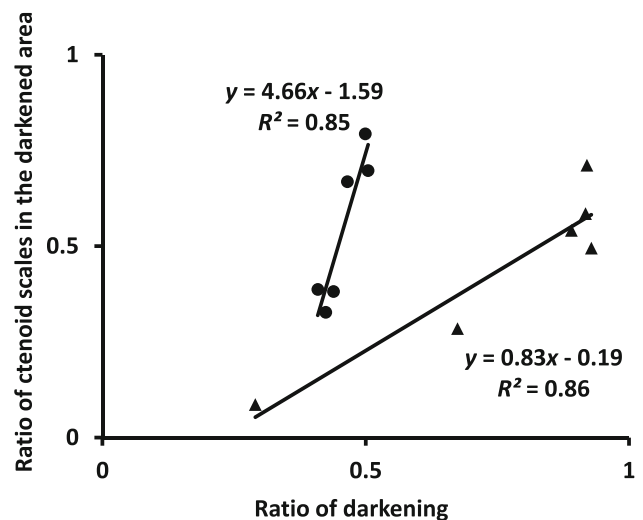


Fig. 9 Relationship between the ratio of darkening and the ratio of ctenoid scales in the darkened area. *Closed circles* indicate “having finished staining” individuals ($n = 6$), and *closed triangles* indicate “1-year-old” individuals ($n = 6$)

Distribution of ctenoid scales on the blind side

On the blind side of all examined individuals, the presence of ctenoid-scale-covered areas was confirmed almost exclusively to within darkened areas, together with cycloid-scale-covered areas, as observed on the normal blind side. The ratio of ctenoid scales in the darkened area varied from 10 to 70 %, even among individuals in a tank (Fig. 9). Examining the relationship between the ratios of

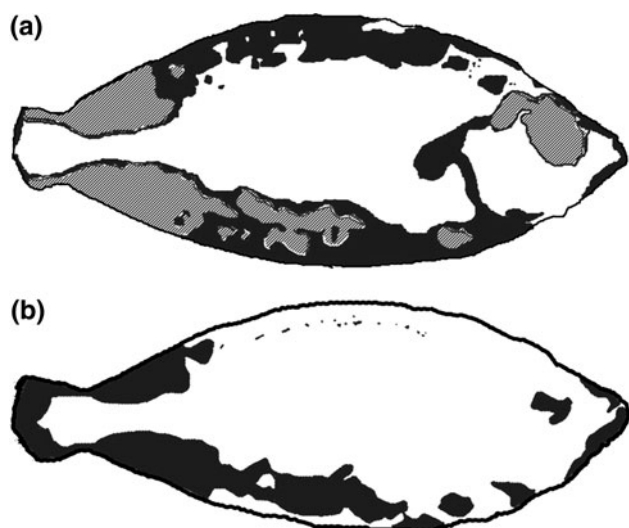


Fig. 10 Typical example showing the similarity between the ctenoid-scale area at the 11th week (**a**) and *darkened area* in the past (**b**; 3rd week). *Black area* indicates darkened area and *hatched area* indicates ctenoid-scale area at the 11th week

ctenoid and darkening, a strong linear relationship was observed, but only among individuals in the same rearing (Fig. 9).

For the location of the ctenoid-scale area at the end of the experiment, similarity to the darkened areas at a certain time point in the past was observed (Fig. 10 shows a typical example). The ctenoid-scale area at the 11th week (shaded area in the upper panel) resembles the stained area at the 3rd week (black area in the lower panel). Such similarities appeared stronger in individuals having smaller darkened areas. The time point in the past at which the strongest similarity was observed was different according to individuals, but was within the range of the 3rd to the 6th week.

Discussion

Changes in location and ratio of darkening on the blind side

From the results, almost all darkening on the blind side of juveniles in the present study occurred after the completion of metamorphosis and the beginning of the experiment, as shown in Fig. 4. Therefore, we have used “staining” for the darkening phenomenon on the blind side in the discussion section. Because the ratio of darkening increased gradually in all individuals and staining tended to appear next to existing stained areas, it is obvious that the stained area expanded gradually to the neighboring areas. In addition, as Fig. 3 indicates, the order of appearance was as follows: (1) starting from the tail base and expanding

anteriorly; (2) starting from the base of pectoral and pelvic fins and expanding until the two areas were connected; and (3) starting at the center of the head and expanding concentrically. So, although such a pattern of expansion was expected using observations from the time-course sampling [13, 14] and classifications of staining extent [19, 20], to our knowledge, this is the first report directly confirming the time-course of the expansion process in detail using individual identification.

Although, Kang and Kim [13] showed the increase in individual staining ratio with respect to time, the complete duration of staining progression was unclear. In the present study, the increase in the ratio of darkening decreased and ceased at about 8–10 weeks in all individuals, and the ratio of darkening differed extensively among individuals, as shown in Fig. 4. From this result, it is obvious that the progression of staining does not continue until adulthood, but only continues for about 2 months. This possibility was previously suggested [6, 14] and is further confirmed by the present study.

There are two possibilities for the stasis of staining expansion. One possibility is time limitation; the staining cannot progress longer than 2 months after the first appearance or 20 weeks after hatching. The second is area limitation; the maximum area of staining is individually prefixed, and no further progression of staining occurs after the individually prefixed maximum area is reached. Although we cannot conclude from the results of the present study, we consider that the latter is more probable. As Fig. 6 indicates, the maximum ratio of darkening was almost proportional to the darkening speed. Therefore, in the individuals that would have a larger stained area, the staining speed is fast from the beginning of the staining expansion. This suggests that the maximum ratio of darkening of each individual had been decided before the beginning of staining, and may support the presence of prefixed area of staining.

Comparison between clean-started and stain-started groups

The maximum ratio of darkening in stain-started fish was significantly larger than that in clean-started fish (Fig. 5a, $P < 0.05$). A similar result was observed for darkening speed as shown in Fig. 5b.

Interestingly, at the end of the experiment, the body length of stain-started individuals was significantly smaller than that of clean-started individuals (Table 1). Although stain-started individuals had a larger maximum ratio of darkening as shown in Fig. 5a, a direct relationship between stained area size and body length is not observed at the individual level (Fig. 2). This result suggests that the smaller final size of stain-started individuals was more

closely related to the early start of staining rather than the large size of the final stained area. Therefore, individuals showing a slow start to staining are not expected to develop severe staining, and at the same time, are expected to grow better. Although more confirmation is needed, the timing of the first appearance of staining on the blind side of juveniles may be utilized as an index of individual quality; the later appearance of staining indicates a better quality individual, for both the extent of staining and growth. To date, information on the causality between growth and staining is limited.

It was previously reported by Seikai [4, 8] that pseudoalbino flounders tended to have severe staining on the blind side. However, in our results, the extent of darkening on the blind side of pseudoalbino juveniles was similar to that observed in the stain-started individuals. In addition, no difference was observed in the process of staining on the blind side between the two groups.

Ocular side darkening of pseudoalbino individuals

In this study, more than 90 % of the ocular side of juveniles showed pigmentation by the end of experiment. Although darkening on the ocular surface was almost uniform and lacked black or white circle patterns, the darkness and hue was almost similar to that of normal fish (data not shown). This observation is in accordance with Seikai [8], who showed that the ocular side of pseudoalbino individuals eventually becomes similar to that of normal fish. Since staining of the blind side was considered to be the same as a normal darkening process of the ocular side skin [14], darkening of the ocular side of pseudoalbino flounders is possibly the same phenomenon as staining on the blind side. However, the direction of vertical extension was different between the two; pigmentation extended from the lateral line to the edge of the trunk in ocular side darkening of pseudoalbino juveniles (Fig. 7), while pigmentation extended from the edge to the lateral line in blind side staining of normal juveniles, as shown in Fig. 3, and as previously reported [14]. Therefore, the controlling systems of pigmentation are possibly different between staining on the blind side and darkening on the ocular side of pseudoalbino juveniles.

Ikuta [21] divided darkening of the ocular side of pseudoalbino flounders into two types according to the pattern of the darkened area. In the normal-color type (NC type), the darkened area formed a similar pattern to that of the normal ocular side, while in the abnormal-color type (AC type), the darkened area was uniformly dark brown with no characteristic patterns of the Japanese flounder. However, both type A and type B in the present study showed uniform coloration without specific patterns, suggesting that both A and B types are basically defined as AC

type. Further, Ikuta [21] reported the difference in the darkening process between the two types; the head quickly darkened in the NC type, while the head was barely darkened in the AC type. Accordingly, type A and type B fish in the present study resembled the AC type and NC type, respectively. Although not mentioning color pattern on the darkened area, nor the presence of the NC type, Terui [22] pointed out the similarity in darkening process to the AC type in their study using mud dab *Limanda yokohamae*. In addition, although the darkening speed of NC type fish was higher than that of AC type fish [21], such a tendency was not observed in this study (Fig. 7). The biological significance as well as the presence of the two types of darkening are not clear and require further information.

Process and regulation of ctenoid scale formation on the blind side

Normally, ctenoid scales are only present on the ocular side of flounder, but they have also been observed in the darkened area of the blind side of juveniles [9–11, 14]. In addition, it has been reported that ctenoid scales with fewer spines were found on the stained area near the boundary to the normal area [9, 14]. Although a significant part of the stained area was covered with cycloid scales, very little of the normal (white, not darkened) area of the blind side was covered with ctenoid scales, as previously reported [9–11, 14]. This is probably in accordance with Kikuchi et al. [7], who suggested that the putative factor(s) that induces ctenoid scale formation is only effective in the darkened area, based on the observation of the first formation of ctenoid scales on the normal ocular side.

Their idea was further supported, at least partially, from our observations. The ctenoid scale area at the end of experiment was similar to the stained area 3–6 weeks previously, as indicated in Fig. 10. Since staining gradually expands to neighboring areas, as shown in Fig. 3, it is suggested that the shift to ctenoid scales follows pigmentation. In addition, strong linear relationships were observed between the ratio of darkening and the ratio of ctenoid scales in the stained area within the group from the single rearing trial (Fig. 9). Therefore, it is expected that staining and a shift to ctenoid scales have a strong relationship, probably in terms of the blind side showing “a shift to the ocular side,” as previously suggested [14].

The expansion of staining stopped before 5 months after hatching (10 weeks after the beginning of the experiment) under rearing without bottom sand, as shown in Fig. 4. Therefore, a 1-year-old fish is considered to have had more than 7 months since the completion of staining. Although information is lacking on the period required for the completion of ctenoid scale formation in the stained area,

we consider that this period of 7 months is sufficient because this period is more than the period from hatching to staining stopping in the rearing experiment (less than 5 months, as above). However, no individuals were observed whose stained areas were completely occupied by ctenoid scales, suggesting that the shift to ctenoid scales stops independently from that of staining.

The strong linear relationship between the ratios of ctenoid scales and stained area was also reported by Isojima et al. [14], with a regression line ($y = 1.8812x + 0.0958$; $R^2 = 0.7162$) different from that in the present study, regardless of the sampling date (100 and 120 DPH). Therefore, it is possible that a certain factor unique to each rearing trial determines the regression line by determining the end-point of the shift to ctenoid scales.

These considerations suggest that darkening is regulated slightly differently from that of the final area of ctenoid scale formation. Since the black coloration, not the presence of ctenoid scales, on the blind side decreases the market price, the effort to clarify the factors and prevent the occurrence of black coloration is critical for industrial purposes. However, at the same time, studies on ctenoid scale formation on the blind side would contribute to the fundamental understanding of ocular–blind side differentiation from scientific point of view.

Possibility of staining degree prediction

In this study, expansion of staining ceased by 136 DPH (10th week), as shown in Fig. 4. In addition, by using individual identification, it was clarified for the first time that degree of staining was almost fixed among individuals by 101 DPH (5th week), as indicated in Table 2. Therefore, for the “comparison” of staining degree among experimental groups, it is possible to measure the stained area at approximately 4 months after hatching, although it is not clear from the present study whether age (DPH or days post metamorphosis completion) or period after the beginning of staining is critical. For the determination of the absolute degree of staining, measurements at 5 months after hatching seemed sufficiently long. We consider this information of good use for designing an experiment on staining.

The first appearance of staining has been proposed as a possible indicator of future staining degree in each individual because the maximum ratio of staining was significantly greater in the group where staining appeared earlier (Fig. 5a). In addition, examining the first appearance of staining in individual fish was not difficult. Thus, it might be possible to identify and exclude bad-quality juveniles (severe staining and slow growth in the future) by early and significant appearance of staining on the blind side.

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