Formation process of the staining-type hypermelanosis in Japanese flounder juveniles revealed by the examination of chromatophores and scales

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Abstract The staining-type hypermelanosis, defined as the blind side melanosis occurring after the completion of metamorphosis, reduces commercial value in hatchery-produced flatfishes. Detailed characterization was performed on the stained area of juvenile Japanese flounder *Paralichthys olivaceus* to physiologically understand this phenomenon. From 80 to 120 days after hatching (DAH), juveniles were reared in sandy and sandless tanks. By classifying the staining degree into 7 levels, about 2 times higher occurrence of middle-level staining was reconfirmed in sandless tank (about 80%) than in sandy tank (about 40%). In the stained area, we found 3 types of chromatophores (melanophore, xanthophore, and iridophore) and ctenoid scales, which would be typically observed on the normal ocular side. Detailed examination on the melanophores revealed further similarity between the stained area and the normal ocular side, in terms of the distribution at 2 layers (shallower and deeper than scale), and the densities in both layers (about 1000 cells/mm^2 above scale and 200 cells/mm^2 beneath scale). These results strongly suggest that the staining is a status change in the body surface conditions from the blind side to that on the ocular side, and not a simple darkening caused by disordered proliferation of melanophores on the blind side.

Keywords coloration, domestication, hatchery production, hypermelanosis, Japanese flounder, staining

Introduction

Japanese flounder *Paralichthys olivaceus* is an important species in Japanese both fishery and aquaculture. In these days, the artificial rearing of Japanese flounder in aquaculture has been very successful such that the production of market-sized individuals was equivalent to that of wild fish caught from the sea [1]. This is mainly because color anomaly, particularly pseudoalbinism, which had been a serious problem in the early days of flatfish seed production for stock-enhancement, was overcome largely by improvements in food nutrition [2-4]. However, even now, "staining" remains a major problem in seed production of Japanese flounder.

By citing a review of Norman (1934), Seikai [5] explained "staining" as a phenomenon in which darkening occurs on the blind side of fish following metamorphosis. In contrast, blind side darkening that occurs before the completion of metamorphosis is a different phenomenon called "true-ambicoloration." True-ambicoloration is only a minor problem in Japanese flounder hatchery production because of its rare occurrence in this species [5]. The dirty appearance caused by staining significantly decreases the market value of the fish to between 20 and 70 % of the normal flatfish price [6]. Therefore, an effective protocol to prevent the occurrence of staining in aquaculture is needed.

Although the causes and process of staining have not been thoroughly investigated, extensive attempts have been conducted to prevent the occurrence of staining. For example, sand introduced to

56 the bottom of rearing tanks [7-9] and delayed timing of feeding artificial diet by increasing period of live
57 food availability before metamorphosis [10] were shown to prevent staining. However, from a practical
58 view, sand at the bottom of the tank makes cleaning difficult, and offering live food for extended time
59 periods increases the rearing cost, because of higher cost of live feed than of artificial diet. Thus, an
60 alternative method for preventing staining is required in which efficient mass production of juveniles and
61 effective prevention of staining is simultaneously achieved. However, it is not sufficient to only try to
62 find alternative rearing methods in which the occurrence of staining is low, but it is imperative to
63 determine the essential nature of staining, as well as the fundamental mechanisms that cause it.

64 In the normal course of development, larval type melanophores first appear on both sides of the
65 fish during the larval stage, and adult type melanophores only appear on the ocular side of fish after the
66 completion of metamorphosis [11, 12]. In normal juveniles, both the blind and ocular sides of the fish
67 are first covered with cycloid scales, but the scales on the ocular side quickly change into ctenoid scales
68 [5, 13]. Other than the presence of melanophores and ctenoid scales on the stained area, much less is
69 known about the stained area [13-15]; for example, to our knowledge, the characteristics of
70 chromatophores have not been examined. All that is known about the stained area is that it is simply
71 "darkened." In addition, the processes involved in stain appearance and progression have not been
72 described.

73 Therefore, to develop a method to reduce staining, we aimed to understand the staining process

74 itself, from both morphological and physiological aspects, by accumulating basic information about
75 staining and the stained area at the organism and tissue levels. It is suggested that the
76 true-ambicoloration is a formation of ocular side characteristics on blind side [5]. Similarly, it is
77 possible that staining is a local formation of ocular side skin on blind side, since several characteristics of
78 ocular side have been reported in pigmented area of blind side [15-17]. However, there is no report
79 clearly proposing such an idea for staining. Therefore, we aimed to verify the hypothesis whether
80 staining is a status change in the body surface conditions from the blind side to that on the ocular side, by
81 reconfirming previous information and examining more in detail the characteristics of pigment cells and
82 scales, using samples those are definitely staining. As a result, we revealed that the manner of
83 melanophore increase on the blind side is not disorganized, but organized, just like that on the ocular side.
84 Furthermore, scales and other chromatophores were observed to show developments that indicated a
85 change towards conditions typically observed on the ocular side of fish. In conclusion, staining is
86 attributed to local reconstruction of ocular side skin occurring on the blind side of the fish
87
88 Material and Method
89 Procedure for rearing experiment
90
91 Fertilized eggs of the Japanese flounder were obtained by natural spawning from mature adults

maintained at the Chiba Prefectural Sea Farming Center, Chiba, Japan, and transported to Maizuru Fisheries Research Station, Kyoto University. After arrival, eggs were preliminarily reared in two 500-l polycarbonate stock tanks at 18°C.

At 50 days after hatching (DAH; total length ± standard error [SE] = 13.60 ± 0.37 mm; H stage, 20%, I stage, 78%; G stage, 2%), 1000 larvae were randomly removed from the stock tanks and distributed into 4 experimental tanks (200 l, transparent) placed on light-gray floor. Two of the experimental tanks had artificial sand at the bottom (Micros ceramic, MS-1, 0.5–1.5 mm; Norra Co. Ltd., Japan) at a depth of approximately 3 cm (sandy tank), and artificial sand was not supplied to the other 2 tanks (sandless tank). It has been confirmed that Japanese flounder at the size at which they were transferred to the experimental tanks have no staining on their blind side [18]. The bottom of the tanks was cleaned once a day, and water temperature was maintained at 18°C.

The larvae were fed the rotifers *Brachionus plicatilis* (0–23 DAH) and then *Artemia* sp. nauplii (15–63 DAH). The rotifers and *Artemia* nauplii were enriched with nutritional supplement (Marine Gross, Nisshin Marinetec Co., Ltd, Japan) for 6–8 h. From 23 DAH juveniles, artificial diets (Otohime S1 [about 1.0 mm in diameter, 23–77 DAH] and Otohime S2 [about 1.4 mm in diameter, 77–130 DAH]; Marubeni Nisshin Feed, Tokyo, Japan) were also supplied to the tanks to satiation twice a day.

Sampling

At the beginning of the experiment (50 DAH), 50 juveniles were taken from the stock tank as initial samples. During the daily care, the blind side of juveniles was roughly observed with the naked eye for the beginning of staining. Since the appearance of a significant dark area was first observed at 80 DAH, periodical sampling was started from this point onward. Fifty juveniles each were randomly sampled from sandy and sandless tanks at 80, 90, 100, 110, 120 DAH. At 130 DAH, however, 50 juveniles were sampled only from the sandless tanks because there were not enough juveniles in the sandy tanks, and the experiment was terminated. Density of juveniles was not adjusted in response to the decrease caused by sampling because of the minor effects of rearing density on the occurrence of staining [18].

After removal from the tanks, juveniles were immediately anesthetized in 0.1% 2-phenoxyethanol (Nacalai Tesuque Inc.). Forty fish were then fixed in 10% neutralized formalin (Nacalai Tesuque Inc.). For 10 juveniles, chromatophores on the body surface were examined under anesthesia as described below. After measurements of total and body length, these 10 juveniles were fixed in 10% formalin.

Chromatophore observation under anesthesia

By using a digital camera system (DV-Vi1-L2, Nikon, Japan) equipped to microscope (BHT323,

Olympus, Japan), 5 photographs of the stained area of an individual fish was captured, particularly focusing on the various darkening intensities. The mirror-image locations on the ocular side against the target areas of blind side were also photographed.

Classification of the degree of staining

Because the degree of staining was extremely variable among individuals, we classified the degree of staining into 7 categories by first using samples fixed at 120 DAH from the sandless tanks. The classification was conducted by 2 investigators independently, followed by discussions to decide the category of the sample if the judgments differed. Forty juveniles were classified twice and the results only differed for 2 samples, suggesting sufficient reproducibility of the classification method.

Estimation of melanophore depth relative to scales

As there were both shallow and deep melanophores relative to scales in the stained areas, the depth of the melanophores was estimated using fixed samples. To visualize the scales and confirm scale removal, the body surface of formalin-fixed juveniles was stained with alizarin red solution (0.5 g alizarin red S, 5 ml acetic acid, 10 ml glycerol, 60 ml 1% chloral hydrate) for 10 min. After capturing photographs of the

target area, the scales in these areas were removed using a fine forceps. If melanophores disappeared after scale removal, the melanophore was judged to exist above the scale (shallower than scale), while those melanophores that did not disappear were judged to be underneath the scale (deeper than scale). Similar examination was also conducted on the ocular side and normal blind side of juvenile fish.

To confirm whether wild fish also has dense melanophores under scales on ocular side, the ocular sides of 3 wild individuals were examined. The wild fish that were captured from Wakasa Bay, Sea of Japan, by using a dredge net between May and August 2011 showed no darkening area on the blind side. Although we cannot exclude the possibility that the artificially reared and released fish could be contained in the wild-caught fish, it is highly possible that the individual fish we used had spent early life stages (especially around and shortly after the metamorphosis) in the sea, because they did not have staining on blind side. The total lengths of wild individuals A, B, and C, were 99, 166, and 257 mm, respectively.

Determination of melanophore density in stained areas, and on the ocular and normal blind sides

Using specimens at 80, 90, 100, 110, 120, and 130 DAH from sandless tanks, the number of melanophores was counted in the areas near the dorsal and caudal fins under a dissection microscope. Because melanophore density of these areas was relatively uniform among individuals, 1 area per

164 individual was examined, and 6–10 individuals per age were used.

165 Where only deeper melanophores existed, the number of melanophores on the body surface
166 was directly counted for a 0.3–2 mm^2 area. Where both deeper and shallower melanophores existed, the
167 number of melanophores was counted after removing the stained scales. For deeper melanophores, the
168 melanophores remaining on the body surface were counted as above. For shallower melanophores, the
169 melanophores on 6 scales from one individual were counted. Because the area where only shallower
170 melanophores existed was very limited we did not examine melanophore density in this area.

171

172 Examination of ctenoid scale distribution

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174 To examine the possible overlap of the stained area and the area covered by ctenoid scales, the
175 distribution of ctenoid scales was extensively examined across the entire blind side of the fish. From
176 100 DAH and 120 DAH samples, 6 individuals, each with a typical degree of staining, were selected in
177 each age. After staining the scales with alizarin red solution and immersing them in 70% ethanol for
178 more than 1 night, the specimens were air-dried at room temperature for approximately 30 min. The
179 spines of the ctenoid scales were clearly visible when irradiated from the anterior direction.

180 Photographs of the whole blind side were captured using a digital camera. The size of the
181 stained area, stained area covered with ctenoid scales, white areas covered with ctenoid scales, and whole

182 blind side, except for the fins, were measured on the digital photographs using NIH Image J (available on
183 line, http://rsbweb.nih.gov/ij/; National Institute of Health, USA). The ratio of stained areas on the blind
184 side (ratio of stained area) and the ratio of areas covered by the ctenoid scales in the stained area (ratio of
185 ctenoid scales in stained area) were calculated.
186
187 Observation of scale morphology using the replica method
188
189 For detailed examination of the scale morphology without altering the skin color by the use of alizarin red,
190 we made replicas of the scales. For this, specimens at 120 DAH from sandless tanks were used.
191 After immersion in 70% ethanol for more than 1 night, the specimens were air-dried. A piece
192 of paper with a round hole (5 mm diameter) was placed on the target area of the skin. After capturing a
193 photograph, by viewing through the dissection microscope, the exposed skin surface together with the
194 edge of the paper was covered with a liquid adhesive (Ekiban, Taihei Kogyo Co, Ltd., Ibaraki, Japan).
195 After 15 min of drying, the liquid adhesive was carefully detached from the skin by gently pulling up the
196 paper. Photographs of the skin surface replica were taken under the same conditions using the
197 microscope. The scale types were identified based on photographs of two target areas.
198
199 Statistics

200

201 For statistical analyses, on-line tools provided by Osaka University (available from
202 http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom) were used. For comparing growth between
203 flounders in sandy and sandless tanks, two-way factorial ANOVA without replication was used. An
204 *F*-test was performed to compare the size distribution between deeper and shallower melanophores.
205 Student's *t*-tests followed by subsequent multiple comparisons using the Tukey–Kramer method were
206 used to compare the density of melanophores among various stained and normal areas.

207

208 Results

209

210 Pigment cells before fixation in the stained area

211

212 Because xanthophores and iridophores disappeared within 3 days of immersion in a 10% neutral formalin
213 solution (data not shown), observations on these chromatophores were performed under anesthesia. For
214 melanophores, detailed examination was conducted using fixed samples as described later.

215 The stained area on the blind side of the 120 DAH samples was very similar to the ocular side
216 of the fish (Fig. 1a, b). In addition to the size and density of melanophores, hue and reflection were
217 essentially the same to that observed on the ocular side. Near the boundaries of the stained area (Fig. 2),

larger melanophores and xanthophores were observed as faint images behind the clear images of scale rings, while smaller melanophores were not covered by the scale rings.

Classification of the degree of staining

Intensity of staining varied considerably among juveniles, even in a single tank. To evaluate and compare the intensity of staining among tanks, and to examine the time-course changes during the experiment, individual intensity of staining was classified as follows:

Level 0 (no staining): Pigmented area is absent on the blind side (Fig. 3a).

Level 1 (faint staining): Very little area is faintly pigmented (Fig. 3b).

Level 2 (tail-base staining): Pigmented areas are present only on the base of the posterior part of the dorsal and anal fins (Fig. 3c).

Level 3 (DA-base staining): Pigmented areas are spread anteriorly along the base of the dorsal and anal fins (Fig. 3d).

Level 4 (pectoral staining): The dorsal part of the pigmented area reaches the head, and the ventral part of the pigmented area reaches to along the operculum and the base of the pectoral fin (Fig. 3e).

Level 5 (head staining): Non-pigmented area only present near the lateral line (Fig. 3f).

Level 6 (whole staining): All the blind side is covered by the pigmented area (Fig. 3g).

236

237 Process of staining expansion in sandy and sandless tanks

238

239 At the beginning of the experiment using 50 DAH juveniles, and thereafter until 70 DAH, we did not
240 notice pigmentation on the blind side of juveniles. Significant staining was first observed at 80 DAH
241 and sampling began from this age. There was no significant difference between the growth of juveniles
242 in the sandy and sandless tanks (data not shown; $P > 0.05$).

243 At 80 DAH, more than 50% of juveniles were classified in the level 0 and level 1 categories for
244 staining intensity and 25-33% were classified as medium staining (level 2, 3, and 4) in the sandy and
245 sandless tanks. Although intensive staining (level 5 and 6) was only observed in juveniles in the
246 sandless tank, there was no obvious difference between the tanks (Fig. 4). Thereafter, the proportions of
247 medium and intensive stained juveniles did not change considerably in the sandy tank. In the sandless
248 tank, however, the proportion of normal juveniles decreased and the proportion of juveniles with medium
249 staining increased significantly. The proportion of juveniles with intensive staining did not increase in
250 the sandless tank, even at the end of the experiment (Fig. 4).

251

252 Classification of stained area, and qualitative similarity to normal ocular and normal blind side
253 melanophore

When scales from the stained areas were removed, 3 types of change were observed in the melanophores on the skin: (1) a large number of melanophores disappeared and a small number of them remained, (2) no melanophores disappeared, or (3) all melanophores disappeared. Although the inner surface (facing to the body) of removed scales was carefully examined, no melanophores were present. All the melanophores were present on the outer sides of the scales in (1) and (2). Tentatively in this results section, we named the stained areas within the 3 change categories as: (1) double-layered, (2) deeper-layered, and (3) shallower-layered staining, respectively. When the normal area of reared juveniles was examined, the ocular side was equivalent to double-layered and the blind side was equivalent to deeper-layered staining.

In wild fish, the ocular side was also judged as equivalent to double-layered staining. Both deeper-layered and shallower-layered stained areas tended to exist near the boundaries between double-layered stained areas and normal areas on the blind side of the juveniles.

Shallower melanophores were significantly smaller than deeper ones, although the size variation was large, especially in deeper melanophores (Fig. 5). In an individual of 110 DAH (TL 7.1 cm), the diameter of deeper melanophores (45.7 ± 1.8 μm, mean ± SE) was significantly greater than that of shallower melanophores (26.3 ± 0.5 μm; $P < 0.05$; $n = 100$]. We observed similar results in 2 more individuals at 110 DAH. In terms of appearance, deeper melanophores on the normal blind side of

juveniles were darker and more visible than the deeper melanophores of the stained area and the normal ocular side.

Changes in melanophore density with time

The density of shallower melanophores in the double-layered stained areas, and on the normal ocular side of juveniles, decreased in a similar way during the experiment, and reached a level of approximately 1000 cells/mm^2 after 90 DAH (Fig. 6). At 130 DAH, there was no significant difference between the density of shallower melanophores in the double-layered stained areas and the normal ocular side of the juveniles ($P > 0.05$, *t* -test).

Conversely, the density of deeper melanophores prior to 90 DAH differed significantly among the different categories (Fig. 7, $P < 0.05$), while the densities were approximately constant after 90 DAH in all cases, as found in shallower melanophores. The density of deeper melanophores was approximately 200 cells/mm^2 on the normal ocular side of juveniles, 140 cells/mm^2 in double-layered and deeper-layered stained areas, and 5 cells/mm^2 on the normal blind side of juveniles.

On the ocular side of the 3 wild fish, the density of shallower melanophores was 649 ± 15, 628 ± 26, and 780 ± 59 cells/mm^2 in individuals A, B, and C, respectively, while the density of deeper melanophores was 190, 187,and 322 cells/mm^2, respectively.

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291 Distribution of ctenoid scales on the blind side of juveniles

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293 Figure 8 indicates the ratios of white-cycloid (normal), white-ctenoid, black-cycloid, and black-ctenoid
294 scales in individuals at 100 DAH and 120 DAH. On the blind side of all examined individuals, the
295 presence of ctenoid scales was confirmed almost exclusively within stained areas, together with cycloid
296 scales.

297 During the experiment, the proportion of ctenoid scales in the stained areas was not
298 significantly different between 100 DAH and 120 DAH juveniles (*t*-test, $P > 0.05$), possibly due to the
299 large variance within the sampling dates. When considering the relationship between the "ratio of
300 staining" and "ratio of ctenoid scales in stained areas" of each individual, there was a strong linear
301 relationship ($R^2 = 0.72$; Fig. 9).

302

303 Number of spines in ctenoid scales from the stained area

304

305 Three types of scales were observed on the stained areas in addition to cycloid scales (normal scale on
306 blind side); normal cycloid (Fig. 10a), ctenoid with single spine (Fig. 10b), ctenoid with 3 spines (Fig.
307 10c), and ctenoid with more than 5 spines (Fig. 10d). As shown in Fig. 11, ctenoid scales with fewer

spines were located near the boundaries, and those with more than 5 spines were located in the central part of the pigmented area.

Discussion

Occurrence of staining in sandy and sandless tanks

For the criterion of staining degree, there have been a criterion proposed by Fisheries Agency [19], which classifies the staining level by the quantity of the pigmented area for individual body parts (body trunk, head, tail, and fin). This criterion is precise, but too complicated to show the time course result of staining in one figure. Later, Fukunaga (2004) proposed simplified criterion based on the pigmentation ratio mainly on the trunk. Our criterion is somewhat similar to this criterion. But we defined the level by the location of staining, and as the result, we further divided the type + of Fukunaga's into level 2, level 3 and level 4. By the use of staining level based on the location, it is expected from Fig. 3 and 4 that the staining starts from the tail base, expands anterior along dorsal and anal fin base up to the pectoral fin base.

At 80 DAH, there was no clear difference in the composition of staining levels between juveniles in the sandy and sandless tanks. Although the number of juveniles with different levels of staining fluctuated, probably due to the small sample size (40 individuals per age), the composition of the

different levels of staining categories in the sandy tanks did not show a clear shift towards any staining category at 120 DAH, suggesting that staining only occurred in a small number of individuals after 80 DAH. In contrast, in the sandless tank from 80 DAH to 120 DAH, the proportion of juveniles exhibiting no or slight staining (levels 0 and 1) decreased to less than 10%, and juveniles with medium level staining (levels 2 to 4) increased to more than 80%. Consequently, it is clear that bottom sand suppresses the occurrence of staining in juveniles of the Japanese flounder as previously reported. [7-9]

Interestingly, the proportion of juveniles observed with severe staining (levels 5 and 6) did not increase in either substrate of the tanks. Therefore, it is possible that staining progresses to medium level staining but does not advance beyond this to severe level staining as suggested previously [9]. To confirm this possibility, it is necessary to verify the stasis of staining progression in juveniles after 120 DAH. In addition, the change in stained area needs to be examined individually without sacrificing the target individuals at the time of measurement.

Unexpectedly, the proportion of juveniles with severe level staining did not differ significantly between the two tanks. Although we did not notice the presence of these individuals from the beginning of experiment to 70 DAH, we cannot exclude the possibility that juveniles with severe staining were present by 80 DAH, due to the confusion with ocular side coloration. Thus, it is possible that all the severe level staining had occurred by 80 DAH, possibly at the time of completion of metamorphosis, and regardless of bottom sand.

It is clear that medium level staining in sandless tanks is definitely staining, not true-ambicoloration, because the difference between sandy and sandless tank appeared after metamorphosis and flounders having middle level staining specifically increased in sandless tanks. Therefore, in the later part of this study, samples with medium level staining taken from sandless tanks were used to characterize the morphology of the stained area.

Comparison of chromatophores in the stained area with those on the normal ocular and blind side

In our previous study, we reported 3 types of chromatophores (i. e., adult type melanophore, xanthophore, and iridophore) on the ocular side of 50-80 mm TL Japanese flounder juveniles [12], which were equivalent body size to 120 DAH individuals in this study. As shown in Fig. 1, although the density was not examined, the presence of xanthophores in the stained area was obvious by the yellowish hue, as well as the presence of iridophores by the white reflection. Since the color and reflection in the stained area is similar to the ocular side of the fish, these two chromatophores are expected to exist at a level similar to that observed on the normal ocular side. Zhu et al. reported the presence of three types of chromatophores on the pigmented area of blind side [16, 17]. However, it is not clear whether they observed staining or true-ambicoloration. For the effects of sandy bottom, the responsiveness to bottom sand is a characteristic of staining as suggested by previous papers [7-9]. So, by the presence of this

result, the idea of "what we observed was staining" gets stronger persuasiveness. Therefore, as far as we know, this is the first report confirming the similarity of chromatophores between stained area and normal ocular side.

When the boundaries of the stained area were examined (Fig. 2), large melanophores were observed behind the image of the scale rings, at a density much higher than that observed on the normal blind side of the fish, suggesting that the depth of the melanophore is deeper than the scale plate. Therefore in this study, the melanophore densities were examined by distinguishing shallower and deeper melanophores relative to scales. The presence of size differences between deeper and shallower melanophores (Fig. 5) on the blind side of juveniles strongly suggests a qualitative difference between them. By using the scale removal method, the density of deeper melanophores was first measured in this study, which would be impossible by ordinary histological observation.

From the results of melanophore depth, double-layered presence of melanophores was unexpectedly observed on the normal ocular side of the juveniles. In order to confirm whether wild fish also has dense melanophores under scales on ocular side, the presence and density of deeper layered melanophores was confirmed in wild juveniles. As described in the results section, deeper melanophores were also present on the ocular side of 3 wild fish at a density equivalent to those in reared fish. This is the first report on the presence of deeper melanophores on the ocular side of juveniles at a much higher density than on the normal blind side.

The stained area that showed similarity to the normal ocular side of juveniles was the double-layered stained area. For shallower melanophores, density and appearance were very similar to that observed on the ocular side. Zhu et al. also reported the similarity of melanophore density between ocular side and pigmented area (either staining or true-ambicoloration) on blind side, without paying attention to the vertical location [16, 17]. In addition, density of deeper melanophores was also comparable at a difference of less than double. From these results, it is possible that the double-layered stained area is characterized by a change in melanophore conditions from that of the blind side to the ocular side, both above and under the scales.

The deeper and shallower stained areas were similar to the deeper and shallower layers of the double-layered stained area, respectively. The density of deeper melanophores (Fig. 7) was similar between deeper- and double-layered stained areas, suggesting that the change in melanophores that reflected the ocular side condition occurred only underneath the scales. Although the density was not examined in shallower stained areas due to the very limited area, melanophore appearance resembled that of the double-layered stained area, suggesting the change in melanophores to reflect the ocular side conditions occurred only above the scales. Consequently, it is possible that deeper- and shallower-layered stained areas are transient phases to double-layered stained areas. It is also speculated that changes in melanophore condition are regulated independently above and underneath the scale, at least in part, because shallower- and deeper-layered stained areas were simultaneously observed at the

398 organism level.

399 Unexpectedly, the densities of all types of melanophores did not increase with time. This
400 observation suggests that melanophores in specific small areas appeared simultaneously, and did not
401 appear gradually. The expression of melanophores on the blind side of juveniles may be controlled at a
402 level of unit area.

403 All melanophores in normal area of blind side (open circle in Fig. 7) must be of larval type as
404 previously reported [11, 12]. And those in shallower layer (both double-layered and normal ocular side
405 in Fig. 6) may be of adult type, because adult type melanophores are present shallower than larval type
406 [12, 20]. On the ocular side of various flatfishes, presence of two types in melanophores and differential
407 location of the cells at two depths were reported [21]. Although we have newly found a population of
408 melanophores in deeper layer (in deeper stained area, double-layered stained area, and normal ocular
409 side), it is impossible to distinguish newly found melanophores from larval type, from the size and depth.
410 However, the ratio of newly found melanophore and larval type can be roughly estimated as 100: 3-6,
411 based on the assumption that the larval type melanophores did not increase after metamorphosis and did
412 distribute equally on ocular and blind side [11, 12].

413 In ambicolored (possibly true-ambicoloration) juveniles of Japanese flounder, induced by
414 retinoic-acid immersion, the presence of two types of melanophores was reported in the stained area on
415 the blind side [22]. Although the authors of the paper considered the larger melanophores as a larval

type, we speculate that the melanophores are equivalent to our deeper melanophores, because the density of the cells should be much higher than the larval type from the photograph [22].

From these considerations, it is highly likely that staining is caused by the body-surface change towards conditions typically observed on the ocular side of fish, at least for melanophores. From the normal blind side, the process to reconstruct the ocular side skin starts as deeper- or shallower- layered staining, independently, and finishes as double-layered staining.

In this study, we could not examine the distribution and density of xanthophores in detail because observation on xanthophores is impossible using fixed samples, although more information on xanthophores gives stronger support to the similarity between stained area of blind side and normal ocular side.

Ctenoid scale formation in the stained area

In all specimens at 100 and 120 DAH, significant parts of the stained area were covered with ctenoid scales (Fig. 8). This indicates that body-surface change to that observed on the ocular side of fish also occurred in scale shape, in addition to melanophores, because it is the ocular side that is normally covered by ctenoid scales. Although there were also stained areas covered with cycloid scales, as normally found on the blind side, there was almost no white (not darkened) area covered with ctenoid scales.

Therefore, it is speculated that the melanophore change to ocular side condition is required as a precondition for the changes in scale shape. Especially near the edge of the stained areas, there were ctenoid scales with fewer spines (Fig. 11), as previously reported [15, 16]. The distribution pattern of cycloids, less-spine ctenoids, and ctenoids suggests that an increase in melanophore density occurs in certain areas as the first step, and then a change to ctenoid scales occurs as the second step, by developing spines on the original cycloid scale. Our finding on this point is supported by previous research in which the factors for the change to ctenoid scales were thought to exert the effect only on hypermelanized areas [13]. Therefore, the possible process of staining is as follows; a change to ocular side condition first occurs in the melanophores and then in the scales.

No significant difference was observed in the "ratio of ctenoid scales in stained area" between specimens at 100 DAH and 120 DAH, thus, growth within 20 days may not have had any major effect on the change to ctenoid scales. In contrast, the presence of a common driving force is suggested to exist in increasing melanophore density and causing the change to ctenoid scales, because of the strong linear relationship between them regardless of the sampling age of 100 and 120 DAH (Fig. 9).

Staining as a body-surface change to ocular side condition

As described above, our study clarified the essential characteristics of staining. Although some of our

results were reconfirmations of previous reports, information of those reports was fragmental. What is lacking is the broad concept which can explain the total image of staining. Therefore, we reconfirmed those data by ourselves, for proposing a concept of staining.

Although detailed analyses were not carried out on xanthophores and iridophores, staining was shown to be a phenomenon, possibly regarded as the change to ocular side condition, which occurred in three types of pigment cells with shallower and deeper stained areas as transient phases, and double-layered stained areas as the terminal phase. In addition, some scales located on possibly earlier stained areas were ctenoid scales, suggesting that ocular side condition also appears on the scales. Staining, caused by melanophore increase, is only one aspect of the staining phenomenon.

Consequently, staining is regarded as a reconstruction process towards a change to the ocular side body surface, which occurs on chromatophores and scales on the blind side of juveniles. This idea suggests that the prevention approaches directly and exclusively on melanophore expression may not be essential, alternatively, approaches from the idea of preventing the local formation of ocular side skin are emphasized. In order to prevent the staining in hatcheries, identifications of the fundamental determinant factor for ocular side characteristics and the timing of this factor is indispensable, and will be of great help to develop effective methods and protocols in seed production.

Acknowledgments

We are grateful to Dr. Naoshi Makino, Chiba Prefectural Sea Farming Center, Chiba, Japan for providing us with fertilized eggs of Japanese flounder, and encouragement during the course of the study. We are also grateful to Dr. Masahiro Ueno and Dr. Yoshiaki Kai for providing us with samples of wild Japanese flounder. This study was supported in part by Grants-in-Aid for the Ministry of Education, Culture, Sports, Science and Technology to M.T.

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560 Figure Captions

561

562 Fig. 1 Photographs of pigment cells on the body surface of a 120 DAH juvenile Japanese flounder reared

563 in a sandless tank. (a) ocular side and (b) stained area. Scale bar indicates 2 mm

564

565 Fig. 2 Boundary between stained and normal area on the blind side of juvenile Japanese flounder in

566 sandless tank on 80 DAH. Scale bar indicates 0.1 mm

567

568 Fig. 3 Classification of degree of staining in 120 DAH juvenile Japanese flounder reared in sandless tanks.

569 (a) Level 0 (no staining, TL 81 mm), (b) Level 1 (faint staining, TL 92 mm), (c) Level 2 (tail-base

570 staining, TL 87 mm), (d) Level 3 (DA-base staining, TL 98 mm), (e) Level 4 (pectoral staining, TL 89

571 mm), (f) Level 5 (head staining, TL 89 mm), (g) Level 6 (whole staining, TL 101 mm)

572

573 Fig. 4 Changes in the staining-level composition in juvenile Japanese flounder during the experiment.

574 Open bar = no and slight staining (level 0 and 1); shaded bar = middle staining (level 2–4), black bar =

575 severe staining (level 5 and 6)

576

577 Fig. 5 Size distributions of shallower- and deeper- melanophores on the blind side of an individual

578 juvenile Japanese flounder. n = 100 each. Total length of the individual was 71mm

579

580 Fig. 6 Density changes in melanophores shallower than scales. Closed square = double-layered staining,

581 closed circle = ocular side. Mean ± SE, n = 6. The melanophore density on shallower stained areas

582 was not measured because of insufficient size. At 130 DAH, different characters indicate the presence

583 of a significant difference ($P < 0.05$)

584

585 Fig. 7 Density changes in melanophores deeper than scales. Closed square = double-layered staining,

586 closed circle = ocular side, open square = deeper-layered staining, open circle = normal blind side.

587 Mean ± SE, n = 6. At 130 DAH, different characters indicate the presence of a significant difference (P

588 < 0.05)

589

590 Fig. 8 Ratio of cycloid and ctenoid scales in stained and normal areas on the blind side of juvenile

591 Japanese flounder at 100 DAH and 120 DAH. The numbers 1–6 on the x-axis indicates the individual

592 identification number. Closed bar = stained area with ctenoid scale, opened bar = stained area with

593 cycloid scale, slashed bar (indicated by arrow) = normal (white) area with ctenoid scale

594

595 Fig. 9 Relationship between “ratio of staining” and “ratio of ctenoid scales in the stained area” in juvenile

596 Japanese flounder. Closed circles indicate individuals at 100 DAH and closed squares indicate
597 individuals at 120 DAH

598

599 Fig. 10 Scales in the stained area of juvenile Japanese flounder. (a) normal cycloid, (b) ctenoid with
600 single spine, (c) ctenoid with three spines, (d) ctenoid with more than 5 spines. Scale bars indicate 0.1
601 mm

602

603 Fig. 11 Scale types near the boundaries between stained and normal (white) areas on the blind side of
604 juvenile Japanese flounder. Scale bar indicates 1 mm. Open circle = cycloid scales, closed circle =
605 ctenoid scales with more than 5 spines, and slashed circles = ctenoid scales with 1–3 spines. Numbers in
606 slashed circles indicate the numbers of spines on the scale

Fig. 1

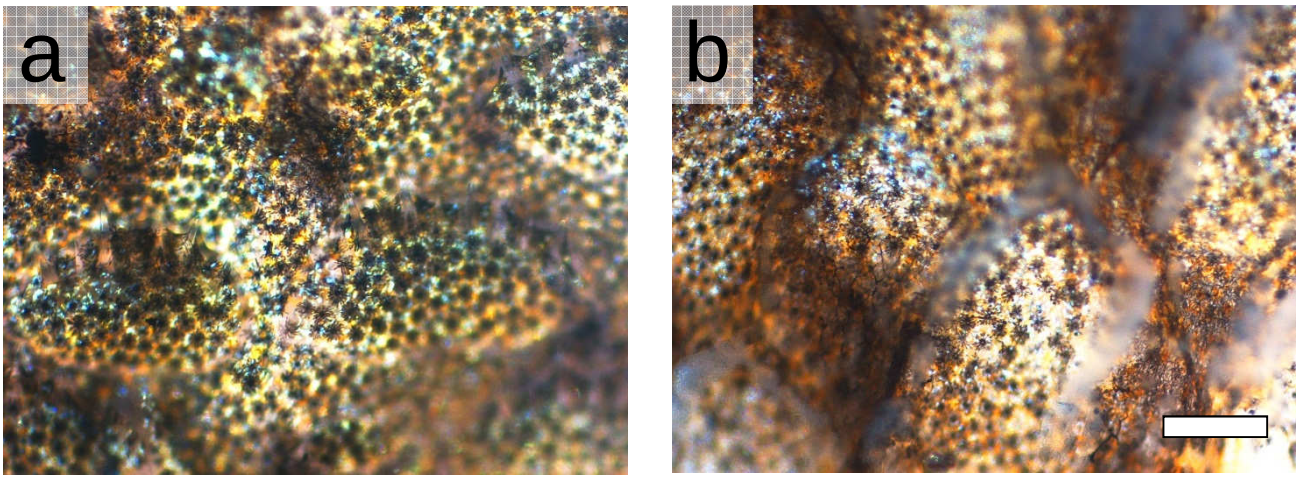


Fig. 2

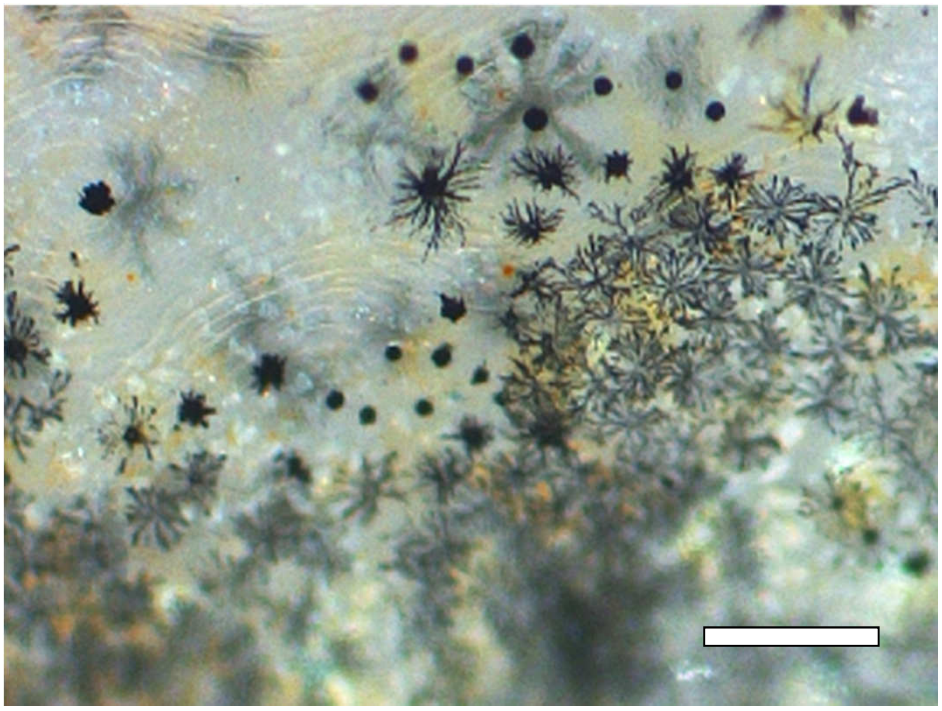


Fig. 3

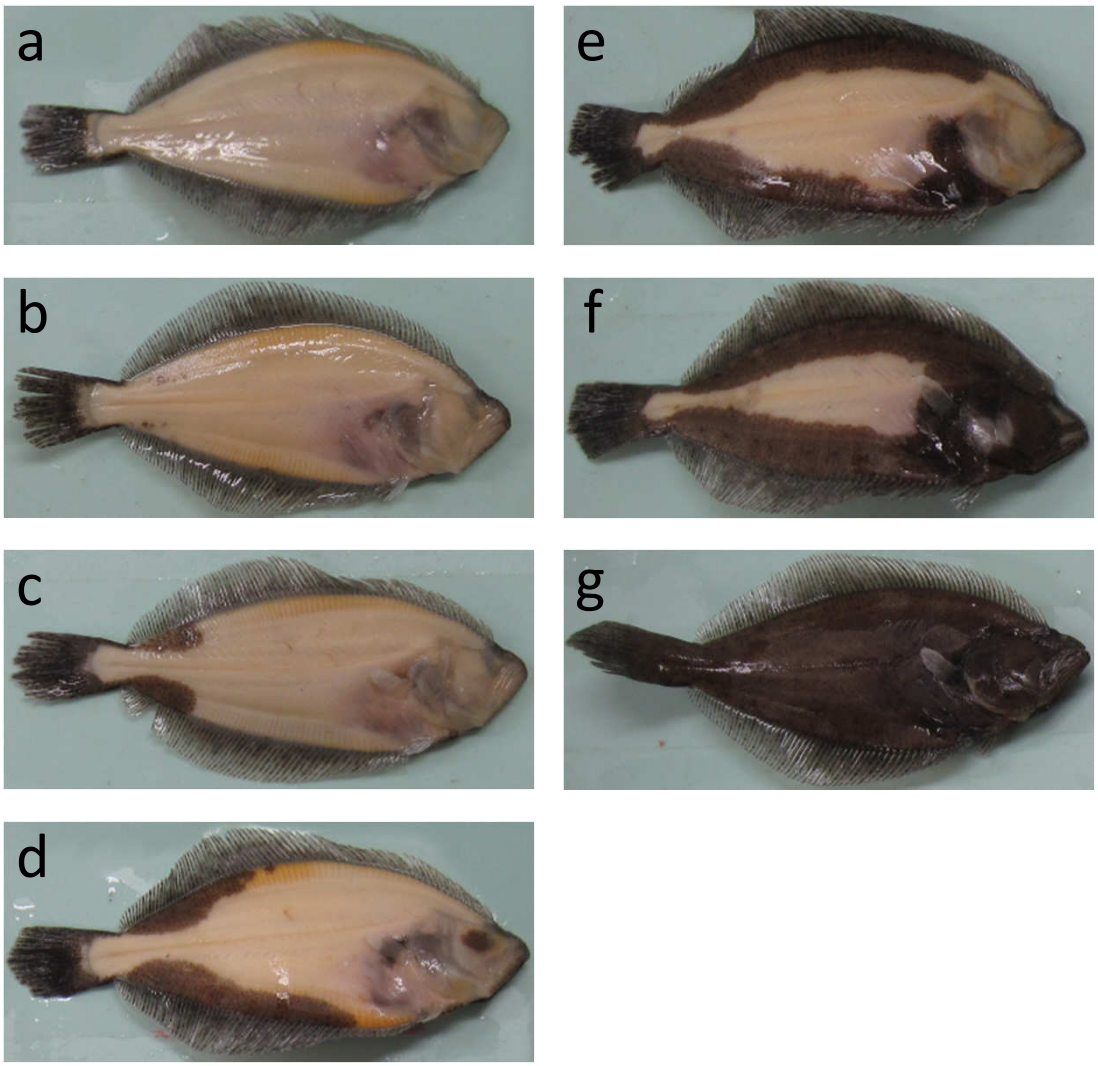


Fig. 4

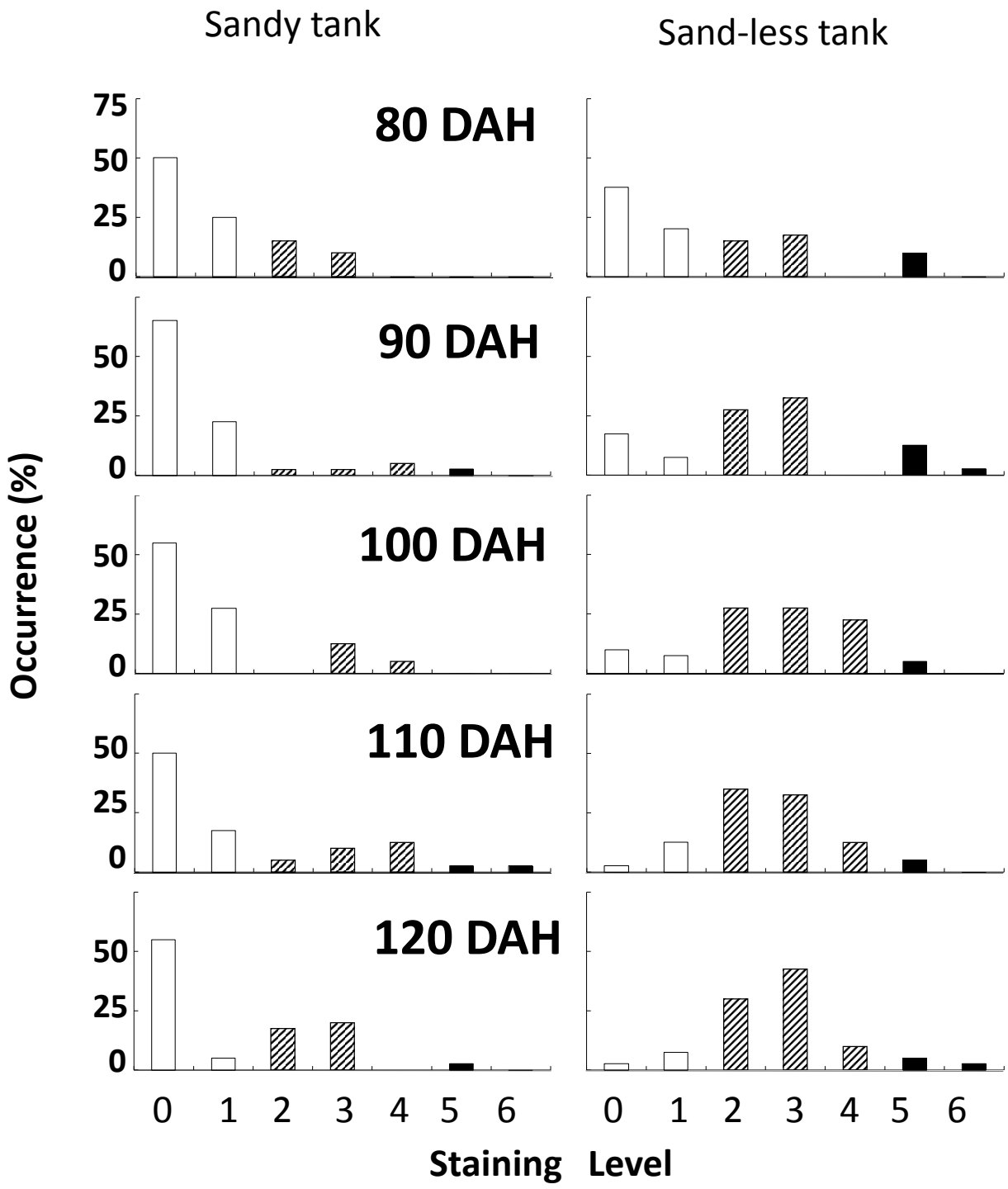

Sandy tank
Sand-less tank
80 DAH
90 DAH
100 DAH
110 DAH
120 DAH
Occurrence (%)
75
50
25
0
0 1 2 3 4 5 6
Staining Level

Fig. 5

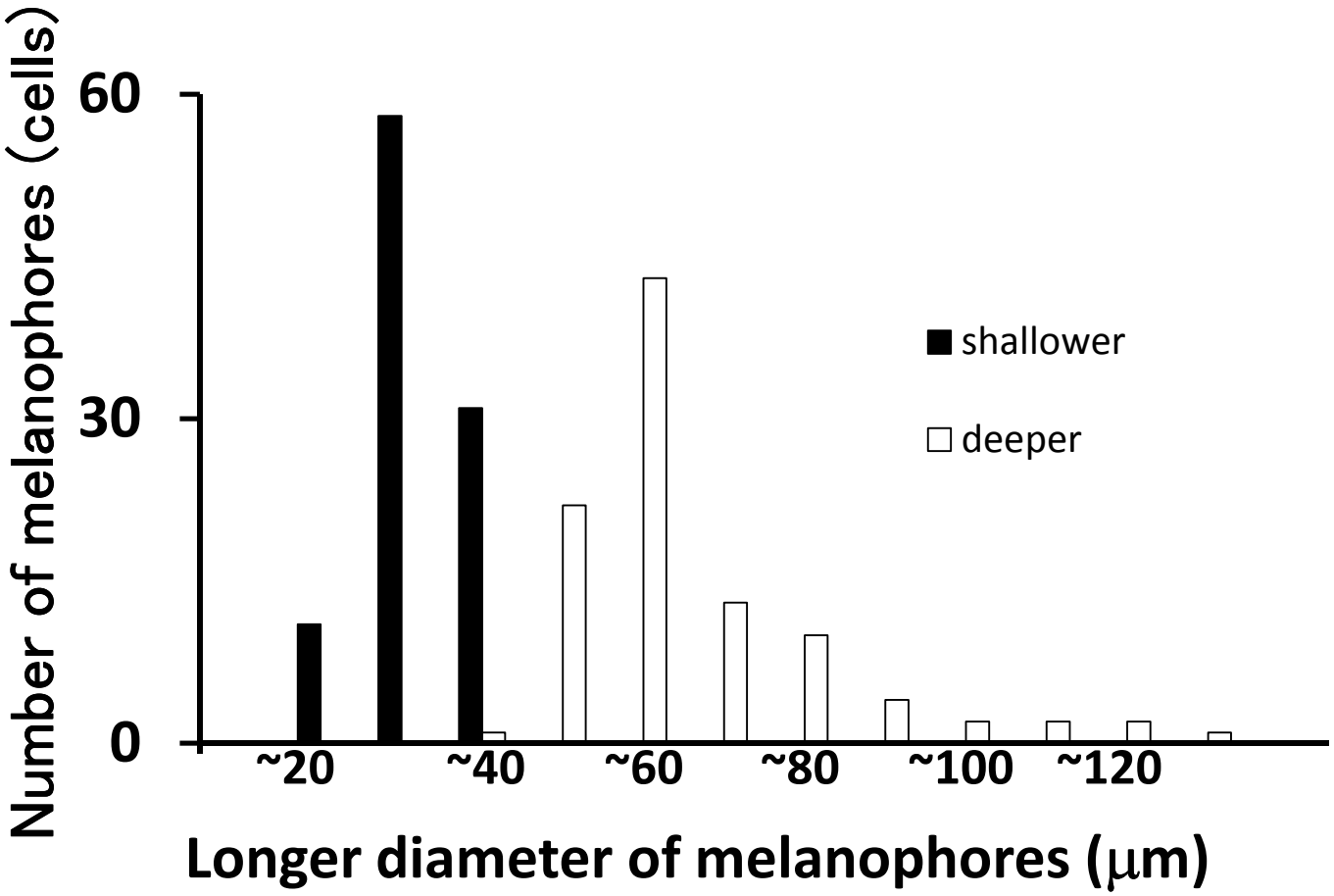

Number of melanophores (cells)
60
30
0
~20
~40
~60
~80
~100
~120
shallower
deeper
Longer diameter of melanophores (μm)

Fig. 6

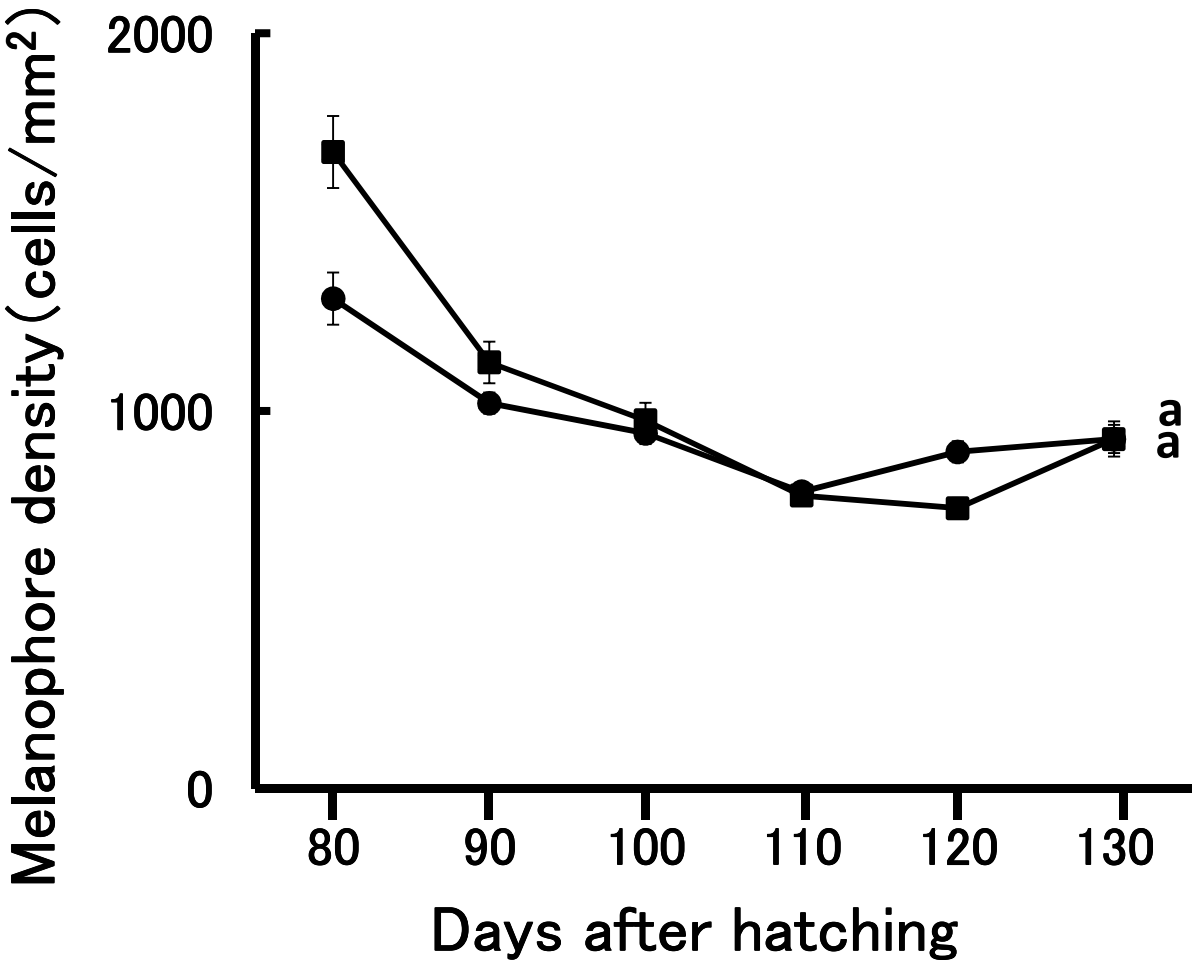

Melanophore density (cells/mm²)
2000
1000
0
80
90
100
110
120
130
Days after hatching
a
a

Fig. 7

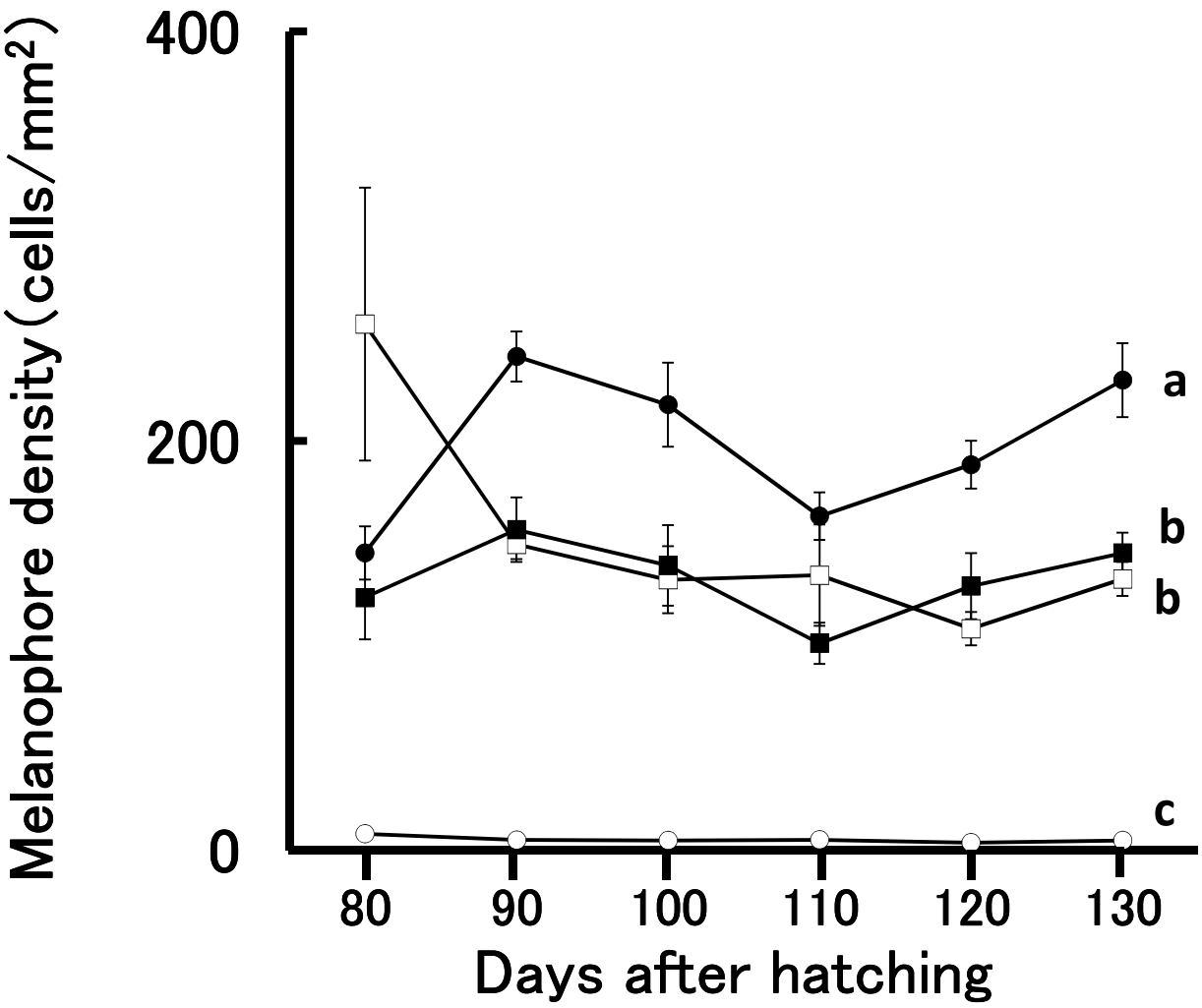

Melanophore density (cells/mm²)
400
200
0
80
90
100
110
120
130
Days after hatching
a
b
b
c

Fig. 8

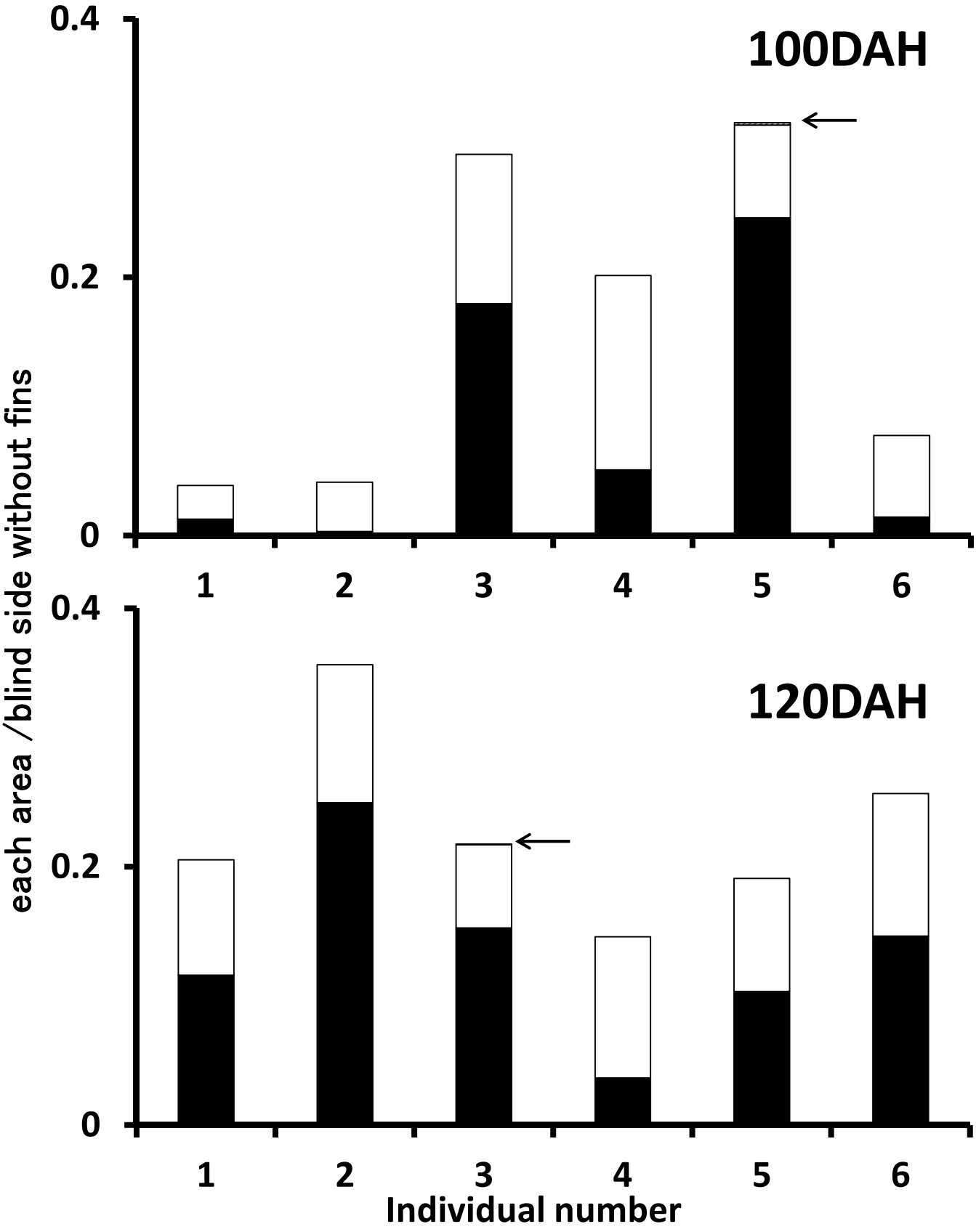

100DAH
120DAH
0.4
0.2
0
1
2
3
4
5
6
each area /blind side without fins
Individual number

Fig. 9

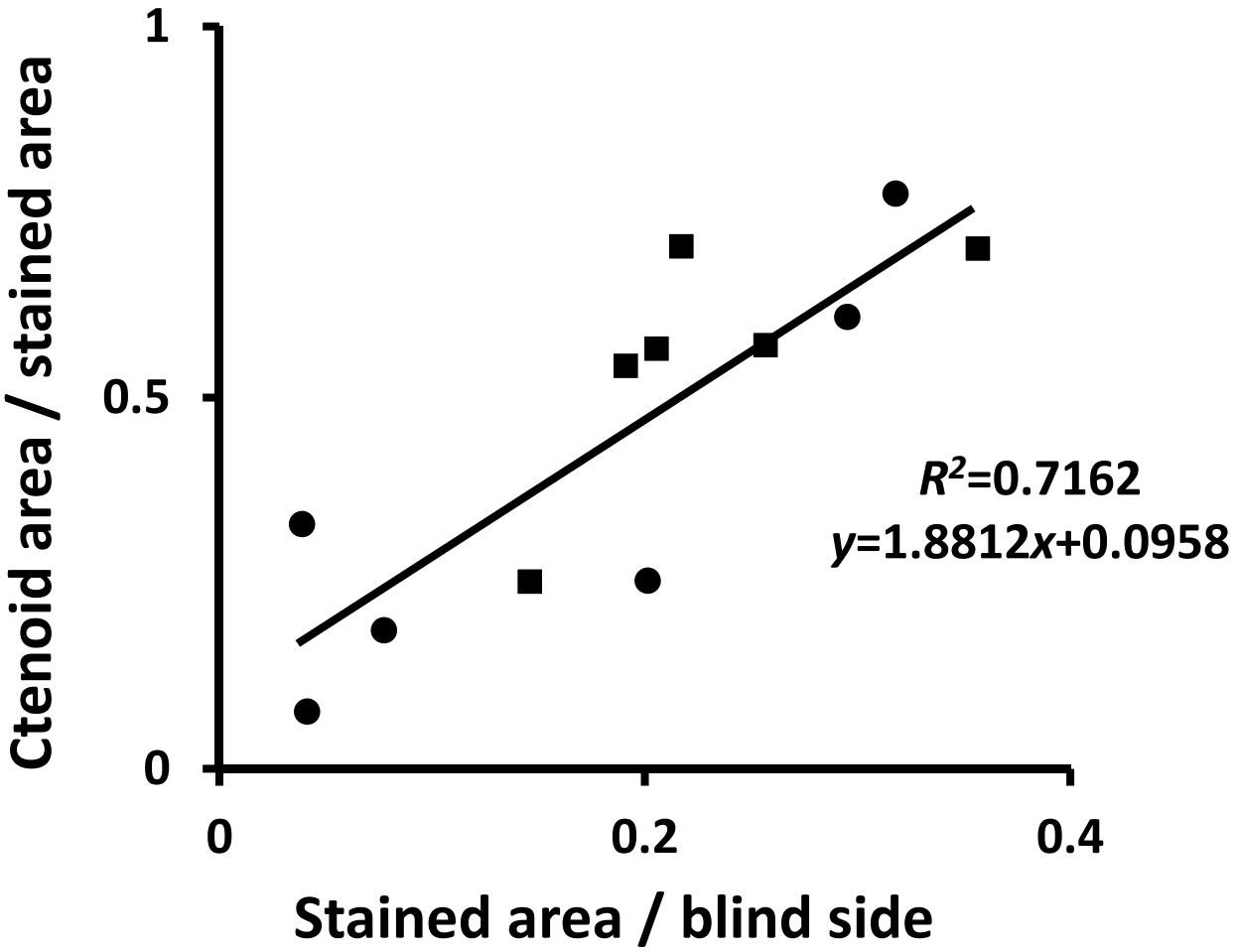

Ctenoid area / stained area
1
0.5
0
0
0.2
0.4
Stained area / blind side
R^2=0.7162
y=1.8812x+0.0958

Fig. 10

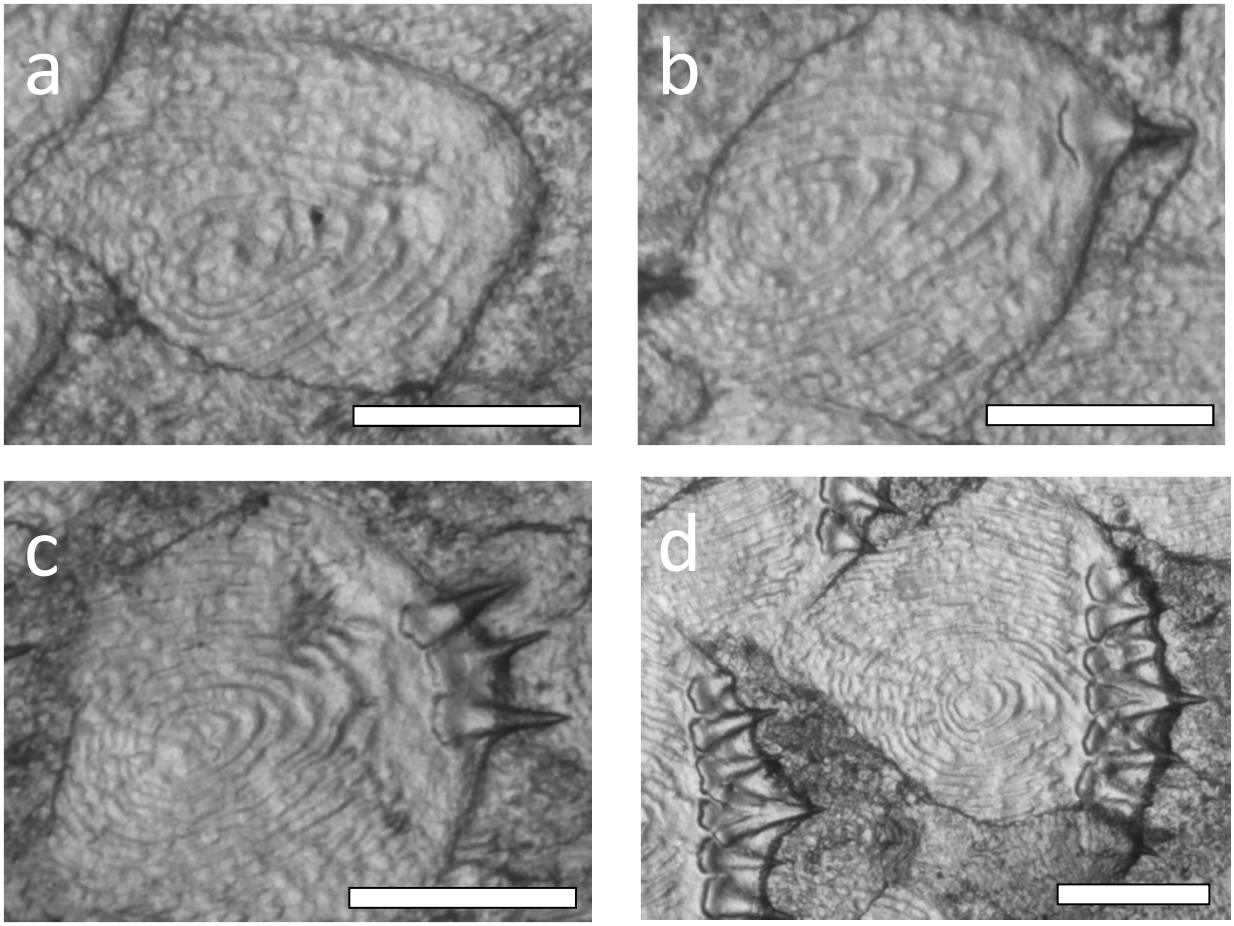
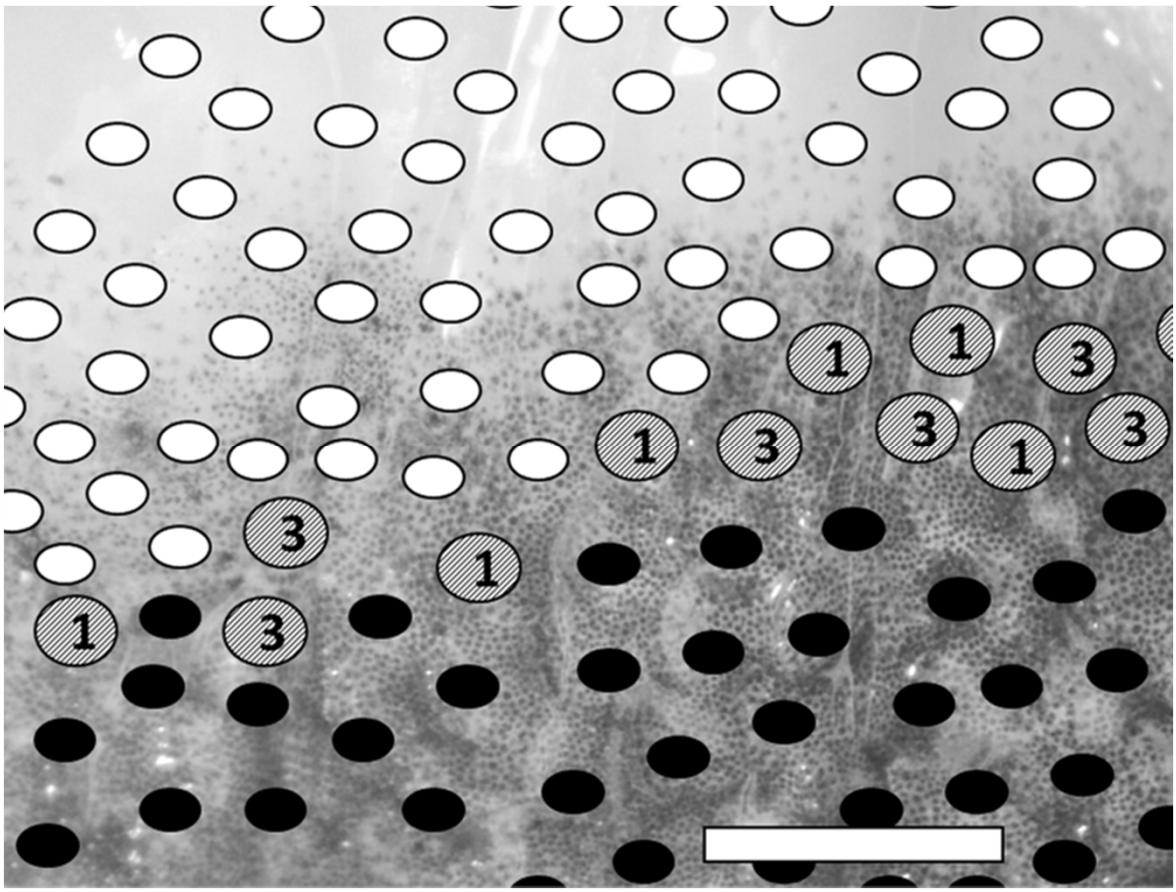


Fig. 11



ヒラメ無眼側着色型黒化における体表組織の特徴および進行過程の検討

礒島俊実，辻　寛人（京大院農），益田玲爾，田川正朋（京大フィールド研セ）

着色型黒化の生理・発生学的な特徴を理解するため、黒化の進行過程を詳細に観察した。観察期間の後半には激しい黒化個体の割合に増加が見られなくなり、着色型黒化は無眼側全面に至るまでに停止すると考えられた。また黒化部には、正常な有眼側と同様の各種色素胞及び櫛鱗が存在していた。特に黒色素胞は深部と浅部の２層に増殖が見られたが、これらは密度、深さ、形態でも正常な有眼側に極めて類似していた。以上より着色型黒化とは、単なる黒色素胞の増殖ではなく、無眼側に有眼側と同様の体表組織が形成される現象であると考えられた。