Transplantation of pigmented and non-pigmented scales into the ocular and blind sides of the Japanese flounder *Paralichthys olivaceus*, suggesting the presence of ocular-side characteristic inducer in pigmented scales

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Abstract After metamorphosis of Japanese flounder *Paralichthys olivaceus*, both the eyes are located on its left side, and only the ocular side becomes pigmented. Staining, or hypermelanosis, occurs on the blind side at 2–3 months after metamorphosis, thereby lowering the market price of the fish. To understand the pigmentation expansion, we performed scale transplantation between the blind and ocular sides of an individual. About 40% of transplanted scales were successfully engrafted, regardless of donor or recipient site. When blind-side scales were transplanted to the ocular side, they became pigmented after 2 weeks, while no change was observed when the scales were transplanted to the blind side. Ocular-side scales did not lose pigment, regardless of the recipient site. However, after removal of transplanted ocular-side scales, pigmented scales regenerated after 3 weeks, even at blind-side sites. Identical results were obtained when the stained area on the blind side was used as the recipient location. When an ocular-side scale with skin tissue was inserted under blind-side scales, the scales immediately above the transplanted area became pigmented, whereas ocular-side scales stripped of tissue did not induce pigmentation. The results strongly suggest the presence of an ocular-side characteristic inducer in pigmented scale tissues.

Keywords sand substrate, asymmetric coloration, ocular-side induction, one-way differentiation, Japanese flounder, staining, scale transplantation

Introduction

The Japanese flounder *Paralichthys olivaceus* is one of the most important flatfishes in Japanese fisheries, and their juveniles are successfully produced on an industrial scale in many hatcheries [1]. The larvae are symmetric before metamorphosis; however, during metamorphosis into asymmetric juveniles, the flatfish experiences significant morphological changes, such as eye migration [2]. Body coloration is another distinctive character of the juvenile's asymmetry: the blind side of the fish remains white, while the ocular side becomes pigmented as a result of the presence of melanophores and xanthophores in the ocular-side scales [3]. One of the possible explanations for this color asymmetry is related to the inhibited development of blind-side skin into adult type. As discussed in Yoshikawa et al [4], there are several lines of evidence suggesting the larval nature of blind-side skin, as well as the adult nature of ocular-side skin.

In fishery production of flatfish juveniles, abnormal pigmentation, widely known as staining-type hypermelanosis, frequently occurs in areas on the blind side of the fish after metamorphosis [2]. This color anomaly is a major problem for flatfish hatcheries because it decreases the market price of the adult fish [5]. We have suggested in our previous report that staining is a change in status of body surface conditions into those of the ocular side, and that staining of these areas is irreversible [6]. Though staining is effectively prevented by use of a sand substrate [6-10], an alternative method to prevent staining is needed because sand creates cleaning difficulties for hatcheries. Further, hatchery stocks experience another color anomaly called pseudoalbinism, in which a significant portion of the ocular side lacks pigmentation (due to absence of melanophores and xanthophores), though this has been largely overcome by improvements in nutrition [11-13]. We previously confirmed that most of the non-pigmented areas on the ocular side of pseudoalbino individuals become darkened several months after metamorphosis [14]. Consequently, it is very likely that the non-pigmented (blind-side type) skin can become pigmented (ocular-side-type) skin, while pigmented skin cannot become non-pigmented.

From these speculations, one-way differentiation seems probable. However, there is no study demonstrating the one-way differentiation of blind-side type pigmentation into ocular side type in flatfishes. Therefore, the current study aimed to investigate differentiation of skin pigmentation of Japanese flounder by using transplantation of scales: a method mainly utilized in immunological studies

[15-21].

Materials and Methods

Subjects

Eight juvenile flounders (divided into 2 tanks) were used in this experiment. Juveniles at 73 days post-hatch (DPH) were transported from the Chiba Prefectural Fisheries Research Center, Chiba, Japan. They were reared with artificial diet (Nagisa K1 [0.8–1.2 mm in diameter, 74–103 DPH] and Nagisa K2 [1.2–2.8 mm in diameter, 103–123 DPH], Oriental Yeast Co. Ltd., Japan; Hirame EP-F2 [1.9–2.3 mm in diameter, after 123 DPH], Marubeni Nissin Feed, Tokyo, Japan) at 25 °C with a filtering system in a 60-L transparent acrylic tank filled with simulated seawater (New Marin Merit, Matsuda Co. Ltd, Japan) and without a sandy substrate. At 200 DPH, after the fish reached a terminal state of hypermelanosis (with complete staining and dark coloration at the base of dorsal and anal fins, which is typical for juvenile Japanese flounders in captivity), a sandy substrate was introduced to the tank. At the beginning of the experiment, average body length was 19.0 ± 0.9 cm.

First experiment: simple transplantation between ocular and blind side

At 224 DPH, 4 individuals (referred as #1 - #4) were used for scale transplantation. More than 20 scales were randomly extracted from ocular side and white area of the blind side of each individual, and washed in phosphate-buffered saline (PBS) solution (0.01 M, pH 7.2, 0.9% NaCl). Because all scales extracted from the ocular side were black and those from blind side were white, scales from ocular side and blind side were labeled “black” and “white” respectively. In order to distinguish the transplanted scales from native or regenerated scales, scales were stained with alizarin red solution (0.5 g alizarin red S, 5 ml acetic acid, 10 ml glycerol, 60 ml 1% chloral hydrate) [6] diluted 50 times with PBS for 5 minutes. After staining, the scales were washed in PBS and transplanted to either the ocular or blind side of the individuals from which the scales were obtained.

The naming convention for the transplantation patterns was donor scale type/recipient area. In

the first experiment, transplantation was carried out for the following 4 patterns: Black/Ocular, Black/Blind, White/Ocular, and White/Blind. Native scales around transplanted scales were also removed in order to examine the newly regenerated scales in the area. Two weeks after the transplantation, the number and color of successfully engrafted scales were recorded.

After analyzing the results from the first 2 weeks of our study, we speculated that the tissue under the removed black scales has the potential to induce the development of dark pigmentation (an ocular-side characteristic) on the transplanted white scales. To test for the possible presence of the ocular-side characteristics inducer (OCI) in black scales, as well as for the possible transfer of the OCI to the tissues under the transplanted black scales, successfully engrafted black and white scales on the blind side were removed, and the color of the newly regenerated scales was observed at 3 and 4 weeks after removal.

All observations and operations were conducted after anesthetizing the target individuals in 0.02% 2-phenoxyethanol (Nacalai Tesque Inc., Kyoto, Japan). For the final sampling at 4 weeks after transplantation, transplanted scales and neighboring areas were examined and photographed in situ with a digital camera (DV-Vi1-L2, Nikon, Japan) equipped with a microscope (SMZ800, Nikon, Japan). The individuals were sacrificed with a lethal dose of 2-phenoxyethanol (0.1%) and fixed in 10% neutralized formalin (Nacalai Tesque Inc.).

Second experiment: transplantation in relation to the reproducibility, staining area, and components of scale

For the second experiment, the 4 other individuals (#5 - #8) housed in the second tank were used, at 245 DPH. Using scales from individual #5, an identical transplantation pattern to the first experiment was conducted to confirm reproducibility. In individual #6, because the stained area on the blind side was considered to be similar to the ocular side for chromatophores [6, 14], the black scales from the stained area were extracted, and transplanted to either the stained or normal white area of the blind side.

Two additional experimental transplantations were conducted in order to examine the possible presence of an OCI. In individual #7, the skin tissue above the scale plates was carefully removed from the black scales (Fig. 1) with tweezers, and used for transplantation to the normal (white) area of the blind

side. In individual #8, a small piece of scale plate, which contained black skin tissue, was cut out from the black scales, and inserted under white (normal) scales of the blind side, in order to reduce the possible contamination of skin tissue of black scales to the white scales of recipient area.

Two weeks after transplantation, the number and color of successfully engrafted scales were recorded. Successfully engrafted scales on the blind side (of individuals #5 and #6), stripped scales with regenerated skin tissue on the scale plates (of individual #7), and native blind-side scales above the piece of scale with black skin (of individual #8) were removed, and the color of the newly regenerated scales was observed 3 weeks after removal. After observation, individuals were sacrificed as described above.

Statistics

Statistical analyses were performed with online tools provided by the Osaka University (http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom). Chi-square test was used to compare the number of grafted scales in the 4 patterns of the first experiment. P value < 0.05 was considered significant.

Result

Transplantation between ocular and blind side

The colors of transplanted scales after 2 weeks, and the rate of successfully engrafted scales, are summarized in Table 1. In all individuals in the first experiment, all engrafted white scales darkened when transplanted to the ocular side after 2 weeks, while remaining white after transplantation to the blind side. The color of black scales did not change; they maintained their original dark color regardless of the recipient area. These results were reproducible, as demonstrated with individual #5 of the second experiment. The typical appearance of a scale from each transplantation pattern is indicated in Figure 2. For chromatophores, the presence of melanophores and the yellowish hue of xanthophores were confirmed in the scales of Black/Ocular, Black/Blind, and White/Ocular transplantations. In White/Blind transplantation, neither melanophores nor the yellowish hue of xanthophores was observed. Similar coloration was found both in newly generated skin tissue (located more peripherally than the red portion

of the transplanted scale plate) and in the original skin tissue on scale plate (located on the red portion). We could not detect a statistically significant difference among the grafting rates of the transplanted patterns (Table 1, $P > 0.05$).

Though it is not clear from the magnified photo in Figure 3, the presence of newly regenerated scales was confirmed 2 weeks after the transplantation around the transplanted scales, where native scales were removed prior to transplantation. On the blind side, regardless of the donor scale site (the red scale in Fig. 3), there were no melanophores in the newly regenerated scales, as would normally be found in intact blind-side scales (Fig. 3). Similarly, in transplantation to the ocular side, the presence of newly regenerated scales was confirmed, and these regenerated scales displayed dark pigmentation, as found in intact ocular-side scales (Fig. 4). Identical results were obtained in all 5 individuals.

The successfully engrafted scales on the blind side were removed, and regenerated scales were examined after 3 weeks (Fig. 5). The color of regenerated scales at Black/Blind transplantation was black for all of the 19 scales, while those at the White/Blind transplantation sites were white, like normal blind-side scales, in each of the 18 scales. In the magnified photograph taken at the end of the process (4 weeks after the removal), the presence of melanophores and yellowish hue of xanthophores was confirmed on the newly regenerated scales (Fig. 6). We have confirmed similar color changes and regenerations in all the individuals, #1 - #5.

Transplantation between the stained area and normal areas of the blind side

Even in individual #6, in which the stained area of the blind side was used instead of the normal ocular side, the color of successfully grafted scales after 2 weeks was almost identical to the scales in first experiment, except for one scale of the Black/Blind set, which stayed white (Table 2). In general, normal blind-side scales transplanted to stained areas expressed melanophores (Fig. 7). The color of all regenerated scales (more than 20 for each treatment) in the normal and stained areas of the blind sides were white and black, respectively, regardless of the donor scale site. In addition, all 3 regenerated scales on the normal blind side, at the point where stained scales had been removed for transplantation, were black.

Transplantation of the stripped scale plate and insertion of a small scale piece under native scale

When 10 stripped scales from the ocular side were transplanted into the blind side of individual #7, the regenerated skin tissue above the stripped scale displayed white (normal) coloration in all of the 3 successfully engrafted scales (Fig. 8a). Conversely, in individual #8, 10 scale pieces with black skin tissue induced dark coloration on the native blind-side scales above each piece, in all of the 4 successfully engrafted pieces. As shown in Figure 9, 2 populations of melanophores were distinguished in the transplanted area. Indistinct melanophores were observed both immediately after transplantation, and at 2 weeks later, suggesting the presence of melanophores beneath the scale plate (Fig. 9). Clearer melanophores were only observed after 2 weeks of the transplantation, suggesting the presence above the scale plate, as found in Figure 9B. Identical results were confirmed for all 4 successfully grafted pieces.

Two weeks after transplantation, stripped scales with regenerated skin tissue (individual #7), and transplanted scale pieces, along with superior melanophore-expressing native scales (individual #8), were removed. Regenerated scales were examined after 3 weeks. Normal blind-side scales were regenerated in individual #7 (Fig. 8b). On the other hand, melanophores and the yellowish hue of xanthophores were found in the regenerated scales in individual #8 (Fig. 10).

Discussion

In this study, we demonstrated the expression of ocular-side pigmentation on blind-side scales when transplanted to the ocular side of flounder, suggesting a process of one-way differentiation from blind-side to ocular-side characteristics. In addition, since the regenerated scales showed ocular-side coloration at the places where black scales were once located (regardless of whether they were native, stained or regenerated), the presence of OCI on the black scales, and/or in the tissue beneath the black scales, was proposed.

Suitability of the experimental protocol

In the present study, all the scales for transplantation were stained in advance with alizarin red before

transplantation. As indicated in Figure 2, transplanted scales in all donor/recipient combinations exhibited an alizarin red-unstained portion peripheral to the pigmented site, suggesting the growth of scale plate after the transplantation. Therefore, it is clear that the transplanted scales grew and that the skin tissue above the plate likely survived in all the engrafted scales.

In order to exclude the possible occurrence of staining at transplantation sites, experiments were conducted using individuals in which staining progression has completely ceased. In addition, a sand substrate was introduced to tanks, to further prevent the staining [6-10] and to minimize the occurrence of injury-induced darkening on the blind side (Echigo H and Tagawa M, unpubl. data, 2013). Therefore, it is probable that the occurrence of new black scales in the white area of the blind side is attributed to the transplantation and removal of black scales.

One-way differentiation of the scales from blind-side type into ocular-side type

When blind-side scales were transplanted into the ocular side of the flounder, melanophores and xanthophores, which normally exist only on ocular side, were expressed on the surface of transplanted scales. This result was demonstrated in the 4 individuals in the first experiment and in individual #5 of the second experiment. Because melanophores were found not only on newly generating tissue, but also on the native tissue of transplanted scales, it is highly possible that native tissue originally placed on the blind-side scale acquired other ocular-side characteristics, though it is not clear from our result whether the melanophore newly generated in transplanted blind-side scales, or moved from peripheral native ocular-side scales to transplanted blind-side scale as previously suggested in scale transplantation of goldfish [15]. However, although ocular-side scales successfully engrafted and grew on the blind side, chromatophores on the transplanted scales did not disappear. As a result, we suggest that there is “one-way differentiation” from blind-side to ocular-side characteristics, at least on the body surface of the juvenile flounder.

In normal development, it has been previously suggested that, in flatfishes, various characteristics of blind-side scales (chromatophore populations, scale types, mucus cells, etc.) remain similar to that of symmetrical larvae, and only the scales of the ocular-side progress to the type found on the ocular side of adults during and after metamorphoses [4]. This development-associated shift of skin characteristics is a

good example of one-way differentiation from blind-side to ocular side characteristics in flatfishes.

Another example is the irreversibility of staining and reversibility of pseudoalbinism. Staining is a type of abnormal coloration where darkening occurs on the blind side [2]; it is a serious problem for flatfish hatchery because the “dirty” appearance decreases the market price of affected fish [5]. One of the difficulties related to staining is the irreversibility: it never restores, once it has occurred on the blind side [14]. Also in the present study with individual #6, in which stained scales of the blind side were used instead of normal ocular scales, stained scales never turned “white” as found in ocular-side scales. On the other hand, pseudoalbinism (total or partial), is another color anomaly expressing non-pigmented (white) areas on the ocular side, similar to scales on the normal blind side [11, 22, 23]. This pseudoalbinism can restore, and scales will obtain relatively normal coloration after 2–3 months of juvenile growth [11, 14, 24].

Possible presence and origin of the ocular-side character inducer (OCI)

As we have shown, an ocular-side transplantation site induced ocular-side pigmentation in transplanted white scales, while a blind-side site did not induce blind-side coloration in black scales. These observations suggest the presence of an ocular-side characteristics inducer (OCI) and the absence of an inducer for blind-side characteristics. Furthermore, after removing the successfully engrafted black scales from the blind side, scales exhibiting melanophores and xanthophores regenerated at the site where transplanted ocular-side scales had been located. This result can be explained by assuming the transfer of OCI as follows: 1) ocular-side scales themselves possess OCI, 2) blind-side skin obtains ocular-side characteristics by the OCI transferred from ocular-side scales during transplantation, and 3) scales with ocular-side characteristics were regenerated.

In the transplantation experiment with individual #7, stripped scale plates from the ocular side were transplanted to the blind side. Interestingly, the color of the skin tissue regenerated on the stripped scale was white. In addition, in the transplantation experiment with individual #8, pieces of ocular-side scales with skin tissue were inserted under blind-side scales. After transplantation, the native white scales superior to the inserted pieces turned black. In this case, it is possible that the skin tissue on the native scales, superior to the inserted scale piece, did not contain transplanted tissues, suggesting that direct

contact of black skin tissue was not needed for the change of scale color. Furthermore, in individual #8, when the darkened native scale and inserted piece were removed, black scales continued to regenerate at the site. Although we cannot exclude the possible presence and contribution of stem cell of chromatophor that has been determined to differentiate spontaneously into adult type melanophores to those results, these findings imply the presence of some diffusible and transferrable substance(s) in the skin tissue above the scale plate of pigmented scales that induces ocular-side color on blind-side scales.

In this study, the appearance of spines of ctenoid scale was not examined because the duration after the transplantation was not long enough. This point would be important when speculating the function of OCI in the next step, because ctenoid is another specific character of ocular-side scale and appears on the stained area of blind side [6]. If ctenoid spines newly appear on the transplanted and/or regenerated scales, it is possible that the OCI is a common inducer of melanophores of adult type and ctenoid, and therefore the real determinant of ocular-side character. For further confirmation of the presence of OCI, together with its function, it is necessary to establish an in vitro culture system of scales, and examine the possible differentiation of white cycloid scales into black ctenoid scales when extracts of ocular-side skin is added.

Possible mechanism for staining progression and for stasis by sandy substrate

Identical results were obtained between staining area of blind side and native ocular side, both as donor and recipient, suggesting a similar contribution of OCI to staining. It is noteworthy that expansion of staining is restricted to the neighboring area [14]. Though the mechanism of first occurrence of staining is unknown, our model of OCI suggests a possible process of staining expansion as follows: the OCI is released from the stained area to the normal neighboring area; the neighboring area develops ocular-side characteristics; and the site, in turn, releases the OCI. However, the occurrence of staining differs between sites on the blind side, and staining expansion stops at a certain period within a certain area individually [14]. Therefore, some mechanism(s) to inhibit the effect or diffusion of OCI may exist locally in normal blind-side tissues.

The results of our study suggest the presence of an OCI in the tissues of the Japanese flounder. The

299 postulated OCI seems to be a powerful determinant in development of the characteristics of the ocular
300 side: when black scales were transplanted, they induced dark pigmentation, even in non-pigmented sites
301 of individuals reared in a tank with a sandy substrate. Therefore, future studies aimed at identification and
302 characterization of OCI may greatly help our understanding of the asymmetrical skin formation of
303 flatfishes, as well as abnormal coloration, including hypermelanosis and pseudoalbinism. Revealing the
304 manner and mechanism of OCI transfer, as well as any inhibition mechanisms of the transfer, may be of
305 help in developing practical and efficient methods for preventing hypermelanosis without the use of sand
306 substrates.
307
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Table

Table 1 Result of scale transplantation between ocular and blind side

(footnote) Ten scales were used in each treatment. E = number of successfully grafted scale; B = number of black (pigmented) scales; W = number of white (non-pigmented) scales; Engrafted ratio = number of successfully grafted scales/number of all transplanted scales in the first transplantation. No statistical difference in the grafted ratio was observed among the 4 transplantation patterns.

Table 2 Result of scale transplantation between stained and normal area of blind side

(footnote) Ten scales were used in each treatment. E = number of successfully engrafted scale; B = number of black (pigmented) scales; W = number of white (non-pigmented) scales.

Figure captions

Fig. 1 Typical appearance of a stripped scale plate of an ocular-side scale used for the transplantation of individual #7 in the second experiment. Scale bar indicates 1 mm

Fig. 2 Successfully engrafted scales 2 weeks after the transplantation between ocular and blind sides in the first experiment. a) Black scale on ocular side; b) Black scale on blind side; c) White scale on ocular side; d) White scale on blind side. Scale bar indicates 0.5 mm

Fig. 3 Regenerated scales in the neighboring area of transplanted scales on the blind side in the first experiment (individual #2). Scales with red markings were transplanted. a) Black scales were transplanted from ocular side; and b) White scales were transplanted from blind side. Most of the native scales in the transplanted area were removed 2 weeks prior. Though it is not clear in the figure, the presence of newly regenerated scales was confirmed. Scale bar indicates 1 mm

Fig. 4 Regenerated scales in the neighboring area of transplanted scales on the ocular side in the first

experiment (individual #2). The scale in the white circle with a red marking was transplanted from ocular side. Most of the native scales in the transplanted area had been removed 2 weeks prior. Though it is not clear in the figure, the presence of newly regenerated scales was confirmed. Scale bar indicates 1 mm

Fig. 5 Regenerated scales after removing the transplanted scales in the first experiment (individual #2). Black arrow heads indicate lateral line of the fish. a) Red scales superior and inferior to the lateral line were successfully engrafted black and white scales, respectively, 2 weeks after the transplantation. b) Black scales and white scales were regenerated 3 weeks after removing the transplanted black and white scales, respectively, at the donation site. Scale bar indicates 1 cm

Fig. 6 Regenerated black scale on the blind side, 4 weeks after removing the successfully engrafted black scale in the first experiment (individual #2). Scale bar indicates 0.5 mm

Fig. 7 Transplantated normal blind-side scale into stained area in the second experiment (individual #6). Two weeks after the transplantation. Scale bar indicates 0.5 mm

Fig. 8 Transplantation of stripped scale plate of ocular-side scale into blind side in the second experiment (individual #7). a) Stripped-scale plate of black scales, 2 weeks after the transplantation. Three red-stained scales (black circle) were successfully engrafted, and 2 red-stained scales (green circle with broken line) failed to engrafted, judging by the presence or absence of skin tissue above the scale. b) White scales (black circle) were regenerated 3 weeks after removing the transplanted scales at the donation site. Although it is not clear from the figure, the presence of newly regenerated scales was confirmed. Scale bar indicates 1 cm

Fig. 9 Insertion of small piece of ocular-side scale under native blind-side scales in the second experiment (individual #8). a) Indeterminate image of melanophores on the inserted scale piece were observed just after the insertion. b) Both indeterminate (inserted scale piece) and clear images (newly expressed on the native blind-side scale) of melanophores were observed, 2 weeks after insertion. Scale bar indicates 1 mm

460 Fig. 10 Regenerated scale after removing the inserted piece of Fig. 9 and the melanophore-expressing
461 native blind-side scale in the second experiment (individual #8). Scale bar indicates 0.5 mm

Fig. 1

Fig. 2

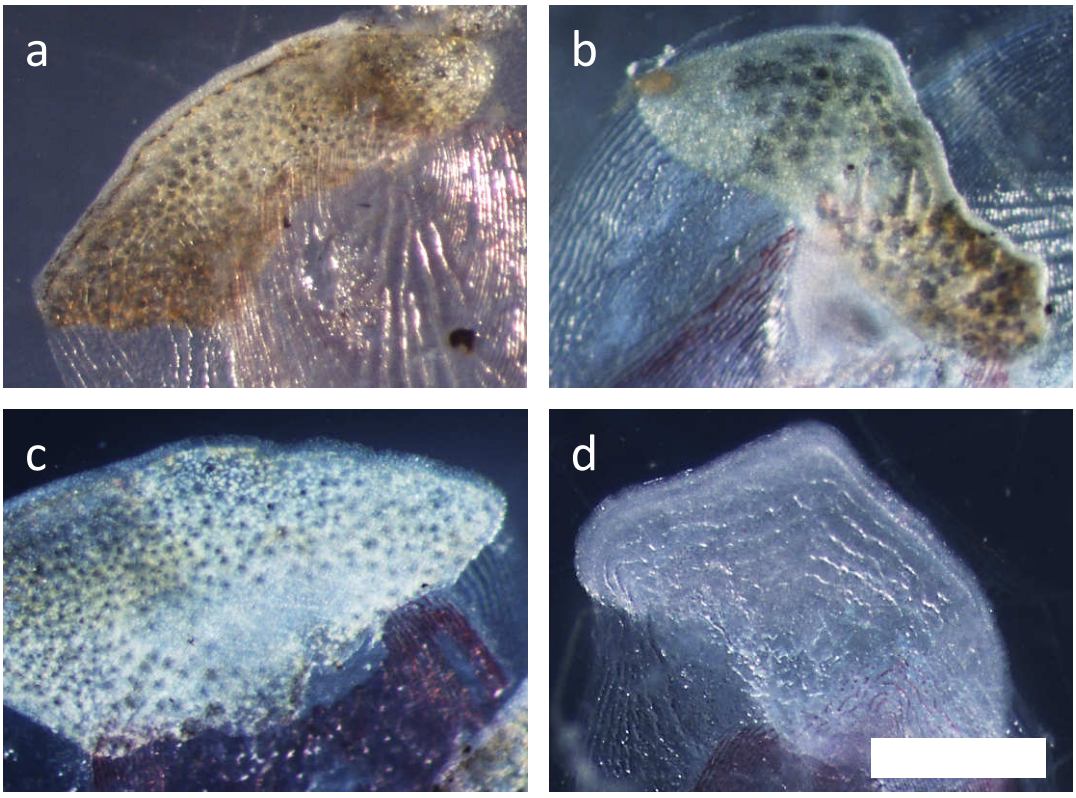

Fig. 3

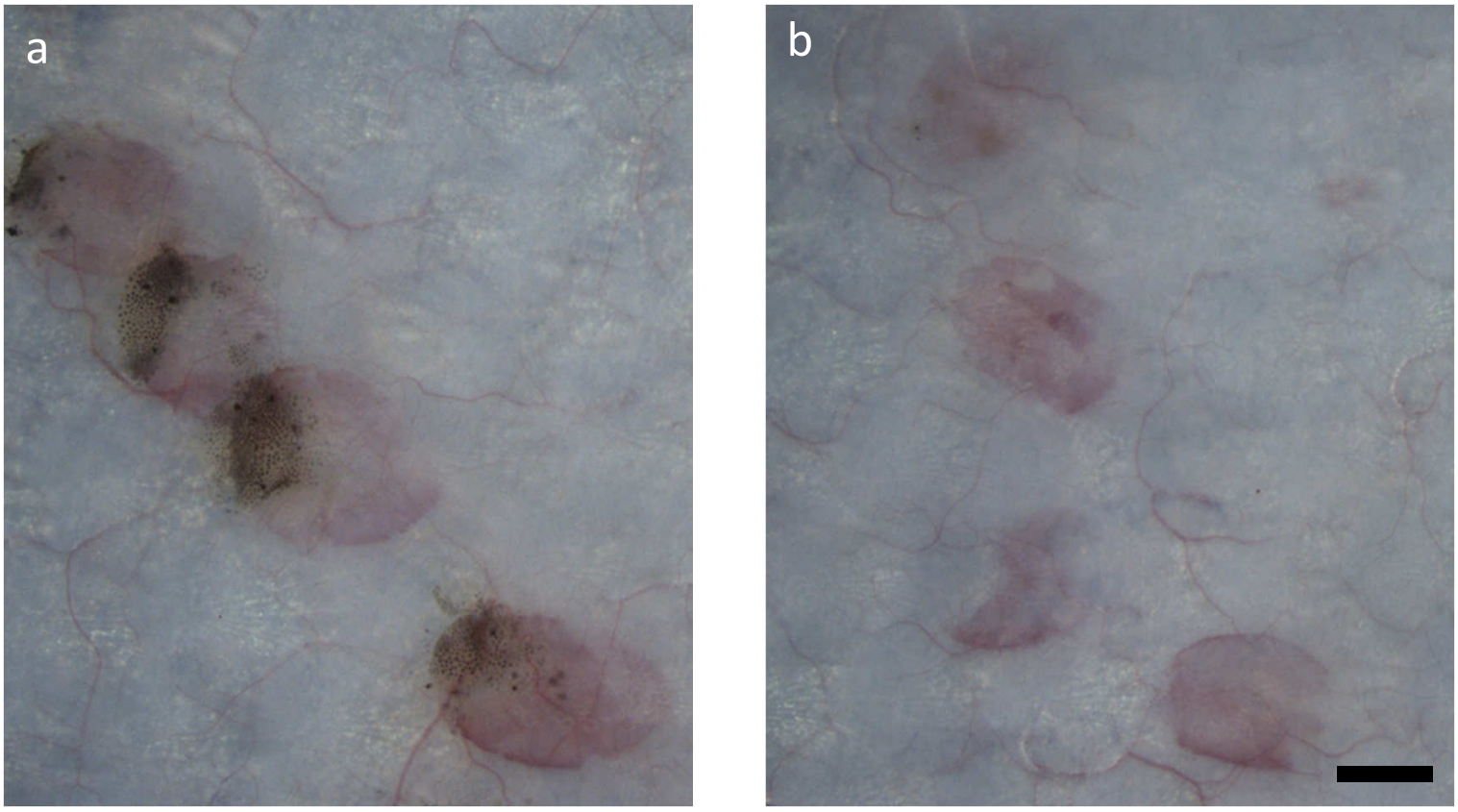

Fig. 4

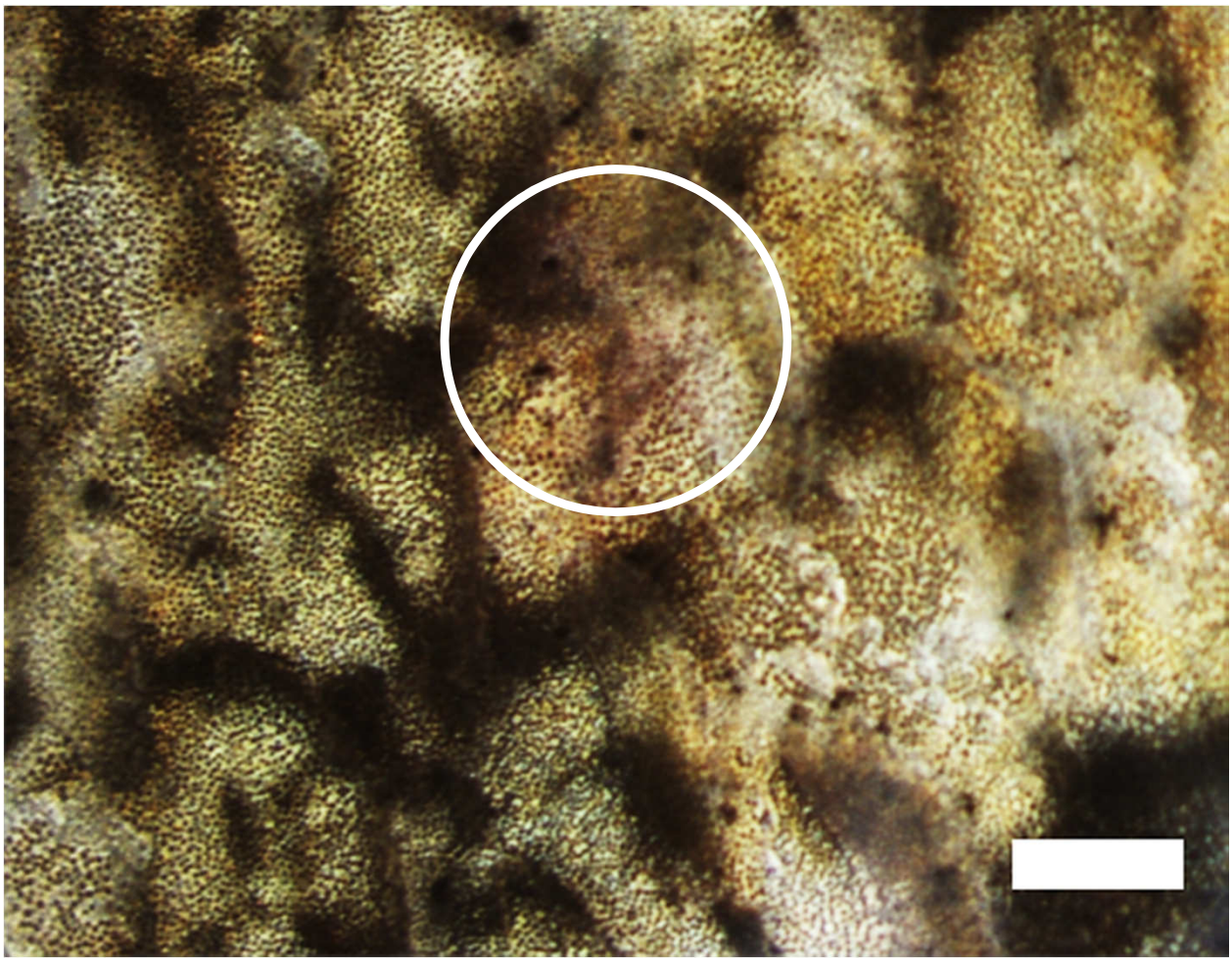

Fig. 5

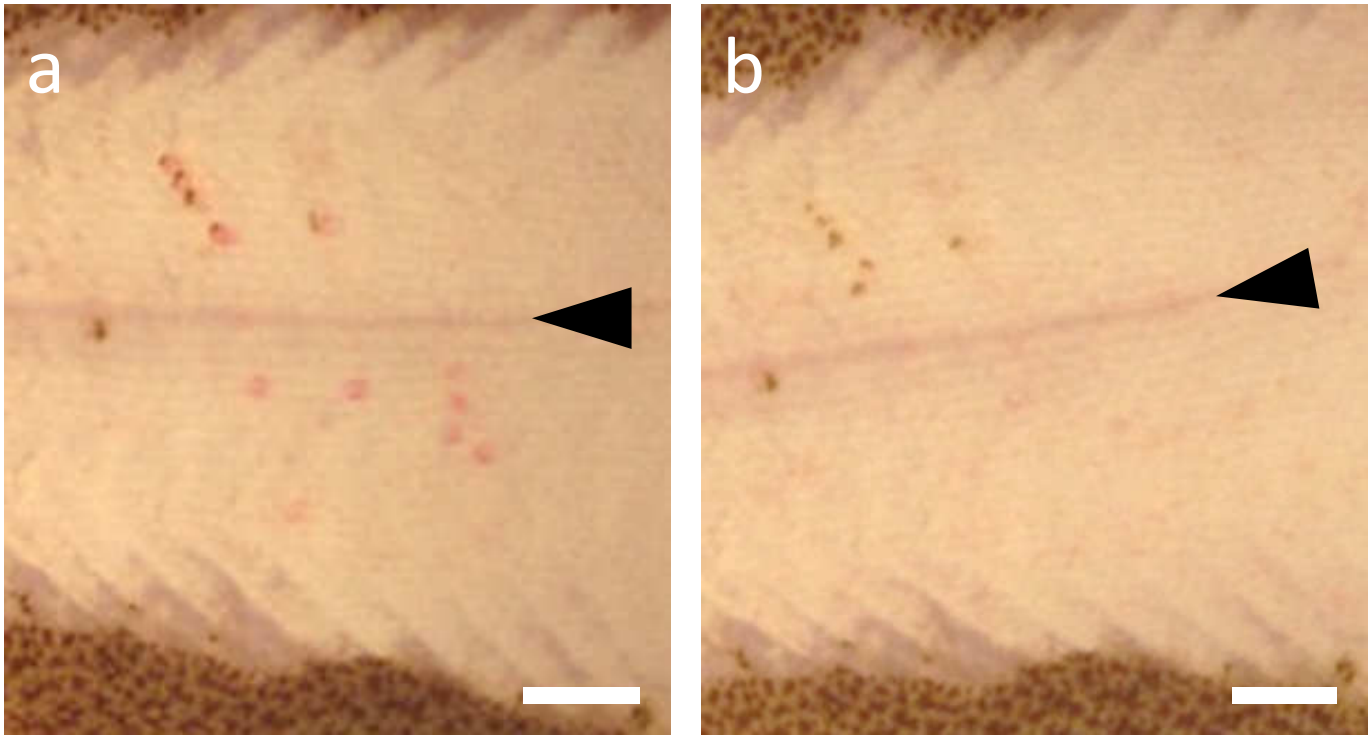

Fig. 6

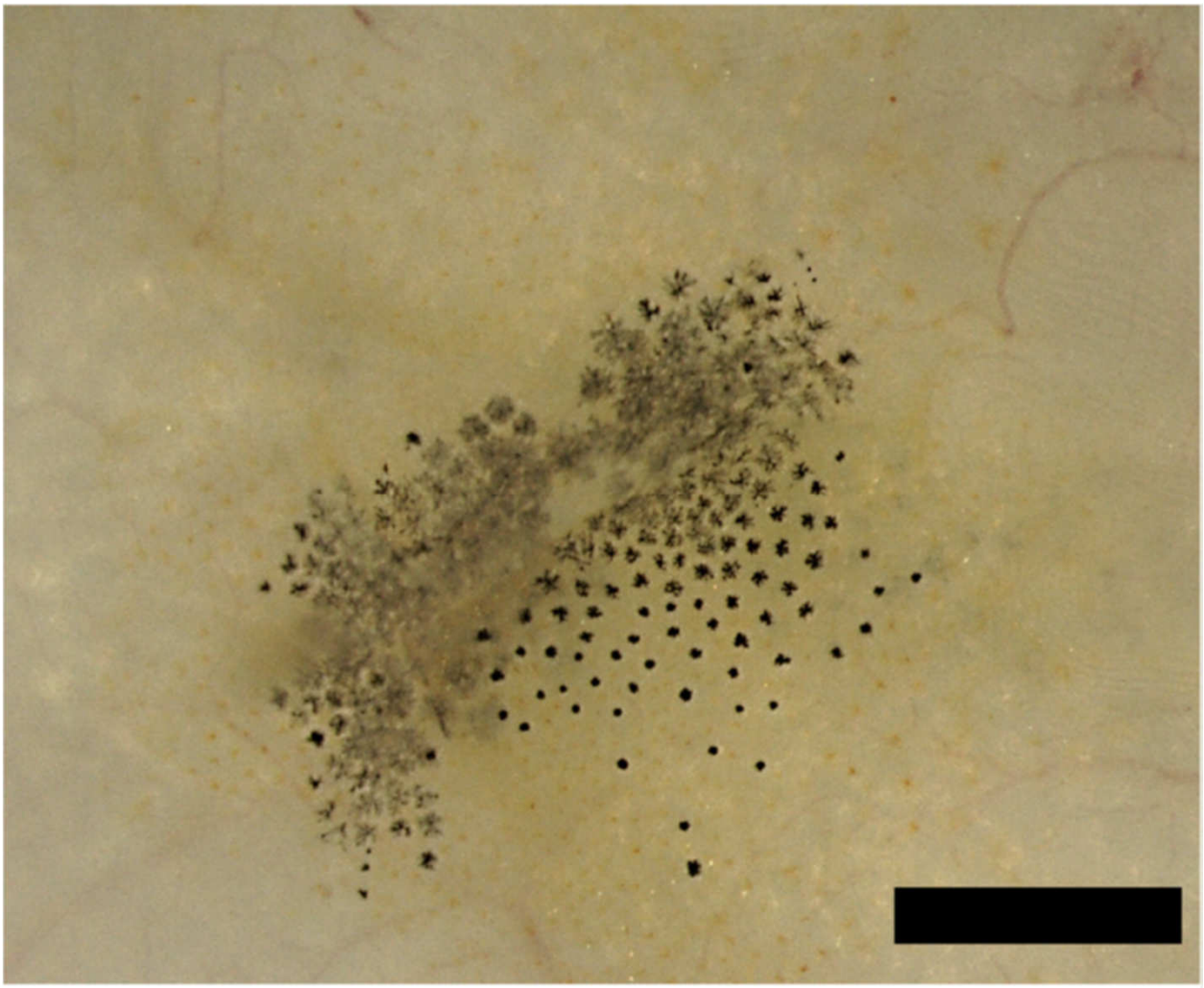

Fig. 7

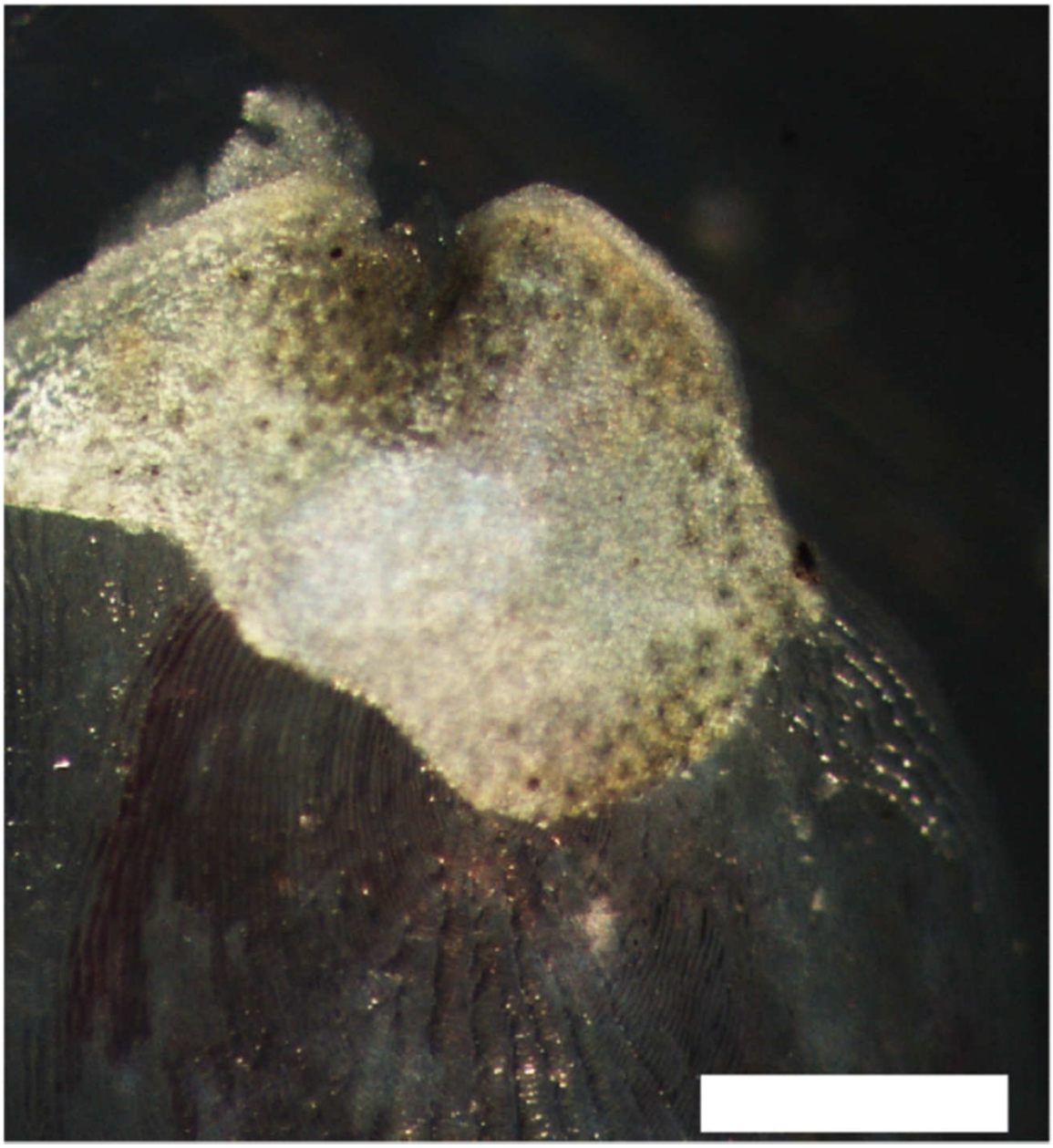

Fig. 8

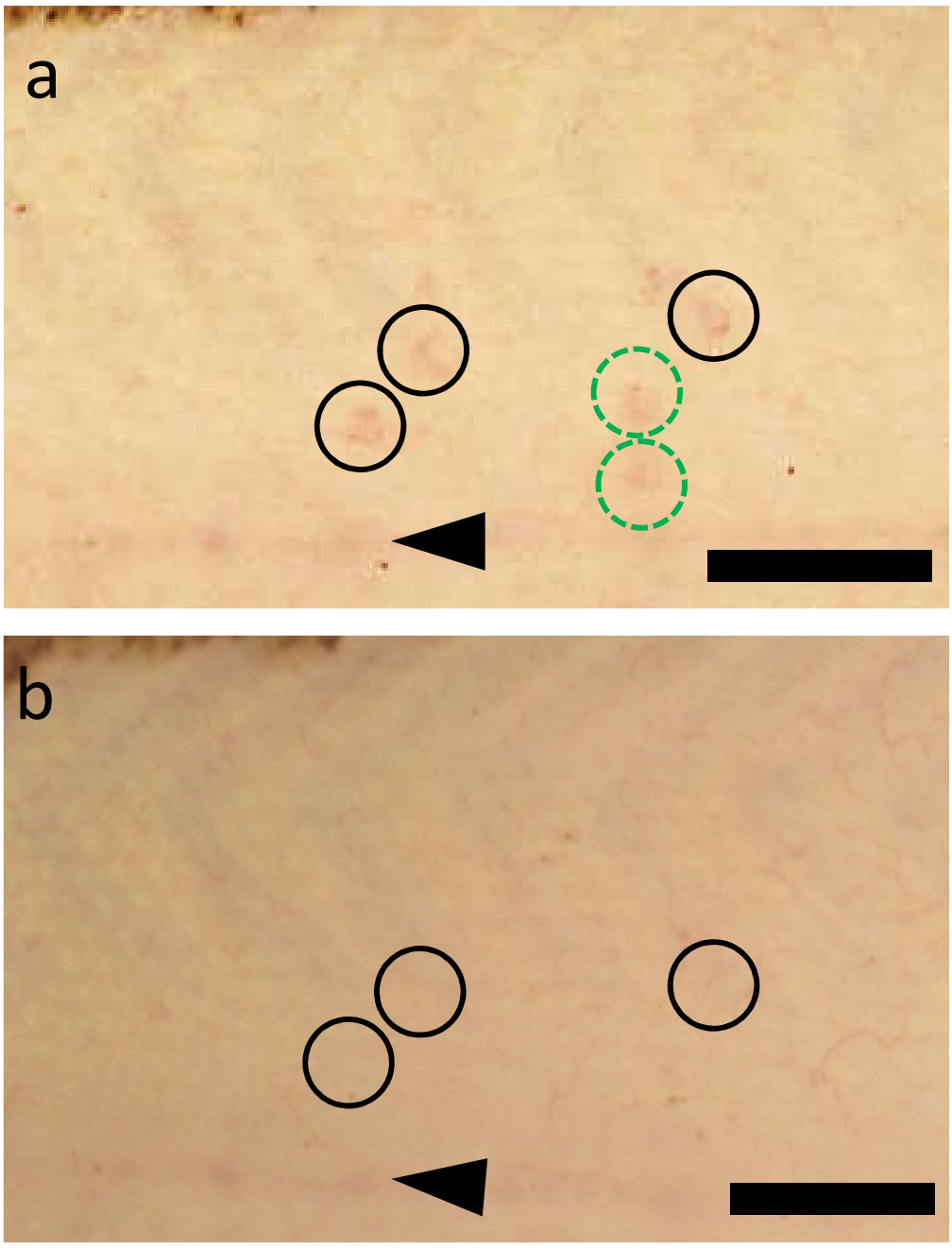

Fig. 9

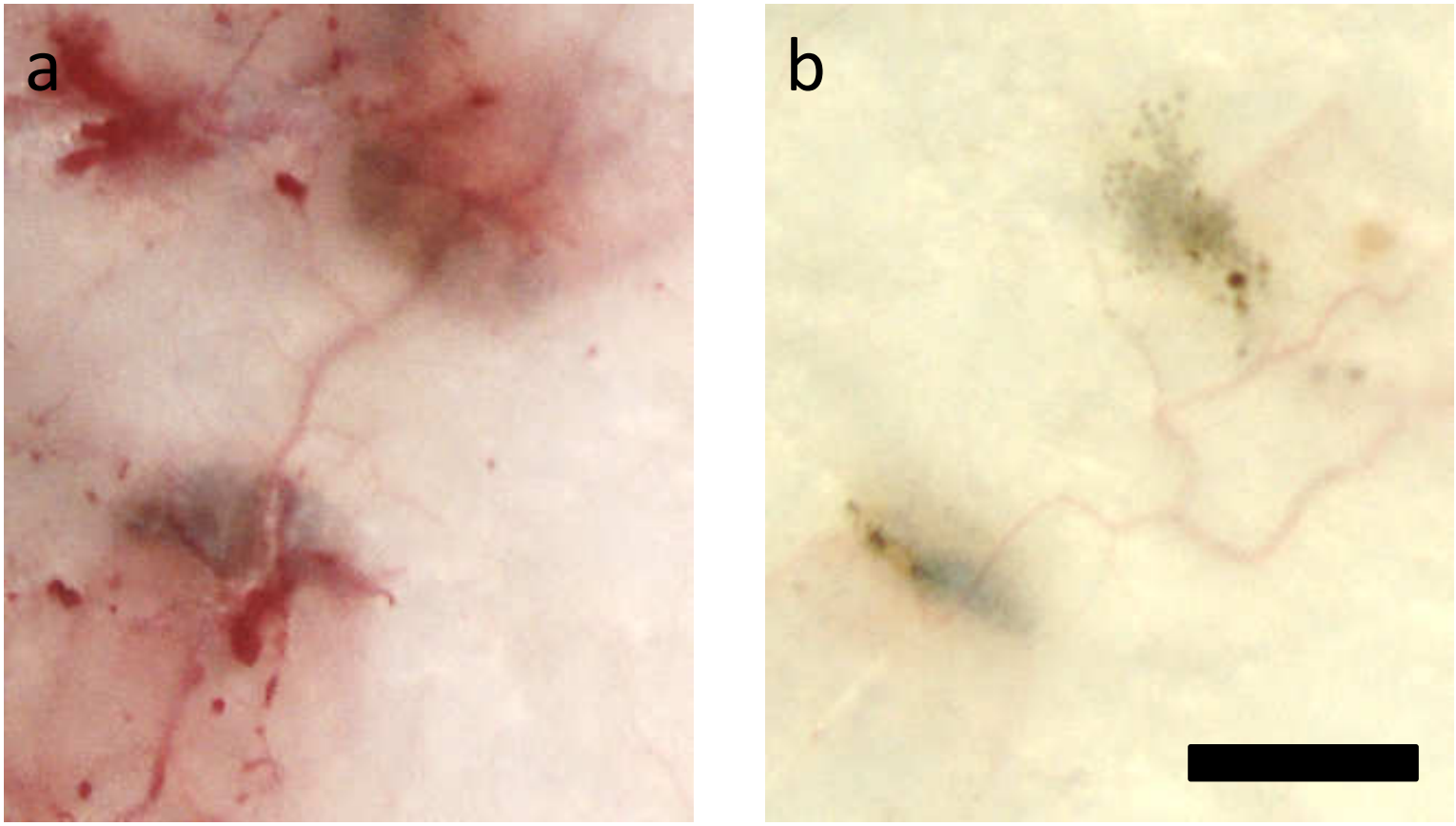

Fig. 10

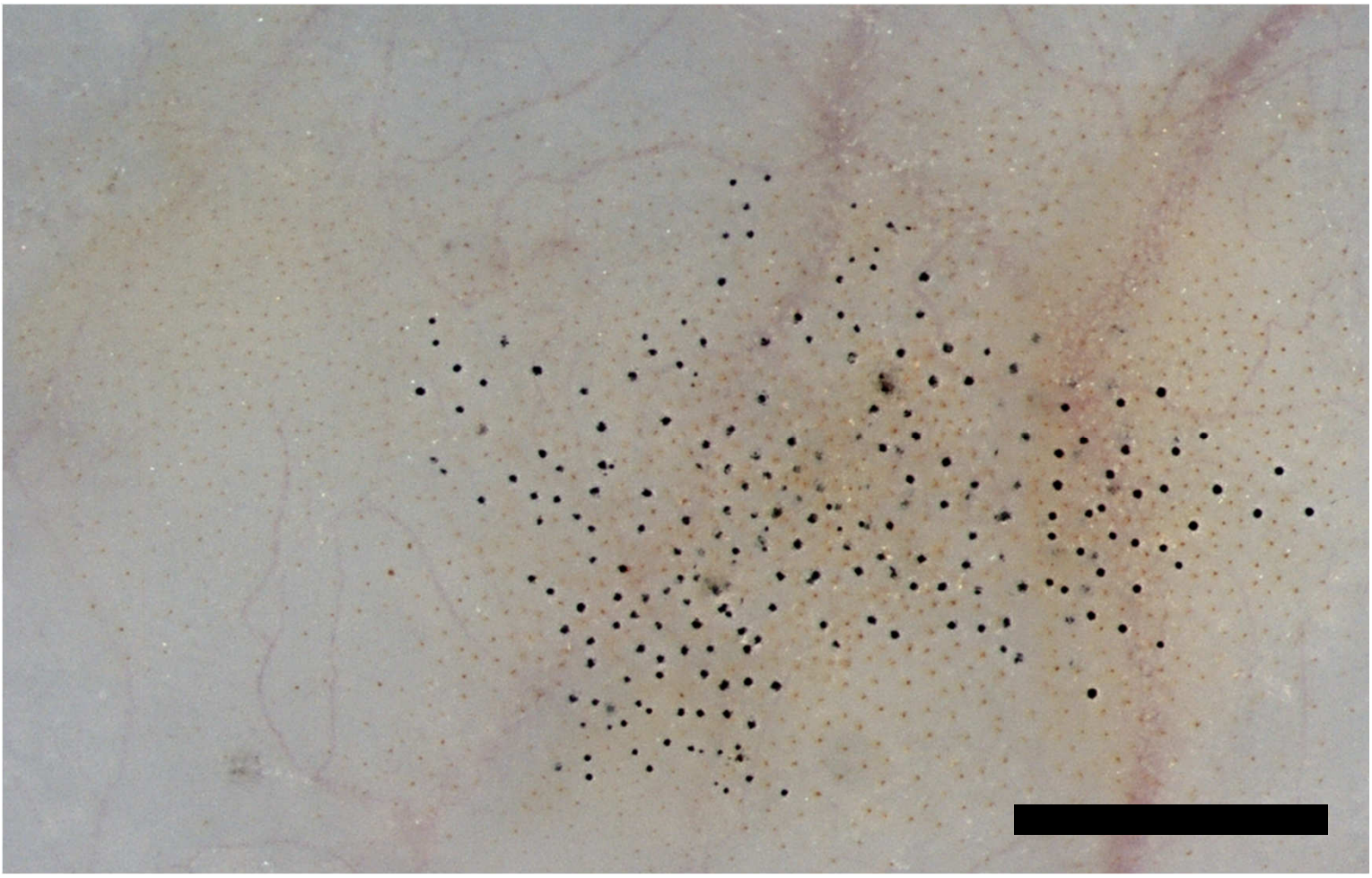

Table 1

Table 1 Result of scale transplantation between ocular and blind side

	Treatment(Scale type / recipient area)											
	Black/Ocular			Black/Blind			White/Ocular			White/Blind		
Individuals	E	B	W	E	B	W	E	B	W	E	B	W
# 1	6	6	0	2	2	0	7	7	0	2	0	2
# 2	5	5	0	5	5	0	5	5	0	3	0	3
# 3	5	5	0	2	2	0	6	6	0	6	0	6
# 4	5	5	0	5	5	0	5	5	0	2	0	2
Engrafted rate	21/40			14/40			23/40			13/40		
# 5	1	1	0	5	5	0	2	2	0	5	0	5

Ten scales were used in each treatment. E = number of successfully grafted scale; B = number of black (pigmented) scales; W = number of white (non-pigmented) scales; Engrafted ratio = number of successfully grafted scales/number of all transplanted scales in the first transplantation. No statistical difference in the grafted ratio was observed among the 4 transplantation patterns

Table 2

Table 2 Result of scale transplantation between stained and normal area of blind side

	Treatment(Scale type / recipient area)											
	Black/Stained			Black/Blind			White/Stained			White/Blind		
Individuals	E	B	W	E	B	W	E	B	W	E	B	W
# 6	5	5	0	3	3	0	5	4	1	4	0	4

Ten scales were used in each treatment. E = number of successfully engrafted scale; B = number of black (pigmented) scales; W = number of white (non-pigmented) scales