Effects of time and duration of rearing with bottom sand on the occurrence and expansion of staining-type hypermelanosis in the Japanese flounder *Paralichthys olivaceus*

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Abstract We previously reported that the progression of staining-type hypermelanosis spontaneously ceased at a specific time and area in Japanese flounder *Paralichthys olivaceus*. To examine whether time is a limiting factor in the spontaneous cessation of staining, we experimentally controlled the initiation and duration of staining by manipulating the bottom substrate condition in the fish tanks. At 151 days post hatching (DPH; 11 weeks), spontaneous cessation of staining was observed in fish reared in tanks without sandy substrate. However, staining resumed (or was initiated) in tanks where sand was removed form 11 weeks, indicating a strong but temporary effect of bottom sand and the absence of time limitation in the staining progression by 151 DPH. Extended duration of the inhibitory period of hypermelanosis expansion (9 weeks or more) aided in only 20% reduction of the final staining area because of the increased rate of staining expansion, The bottom sandy substrate decreased the visibility of the staining area in individuals, but this was observed only before the completion of the staining expansion. These findings were discussed in relation to possible presence of area limitation of future staining, as well as the fundamental nature of staining.

Keywords bottom sand, color anomaly, hatchery production, hypermelanosis, individual identification, Japanese flounder, staining, time limitation

Introduction

Juveniles of the Japanese flounder *Paralichthys olivaceus* are successfully produced on an industrial scale in hatcheries [1] but occurrence of staining-type hypermelanosis is a major problem of hatchery-reared individuals. Staining is a color anomaly that occurs after the completion of metamorphosis, which expresses darkened areas on the blind side of fish [2]. This color anomaly is a serious problem in the production of fish juveniles because it decreases their market price [3]. Recently, information suggesting a morphological similarity between staining area and the normal ocular side has been reported [4-11]. Similar to Seikai's notion [12] of "true ambicoloration," a different type of darkening that is also distinguishable immediately following the completion of metamorphosis, we have described the fundamental nature of staining as a "status change in the body surface condition from the blind to the ocular side" [11].

For the prevention of staining, a number of factors could be beneficial for industrial use, namely, background color, density, light intensity, feeding, and bottom sand [10, 11, 13-18]. Among these, bottom sand appears to have the strongest effect and the highest reproducibility [11, 13, 14, 17, 19]. Applicability for barfin flounder *Verasper moseri*[20] and importance of burrowing in sand [21]are also reported. Therefore, bottom sand is the most promising method for staining prevention, at present. However, it is not clear if the preventive effect of bottom sand is permanent or temporary; in other words, it is unknown if staining progression will remain suppressed even after the removal of bottom sand.

Such information would be useful for industrial purposes and could help to further elucidate the underlying mechanisms involved in staining.

We have indicated that the progression of staining spontaneously ceases at approximately five months of age, to a certain extent, even in the absence of bottom sand in the Japanese flounder [22]. Therefore, it is expected that area or time limitations are present for staining expansion. In the other words, the possible staining areas are individually prefixed before the onset of staining (i.e., area limitation) or the possible period for the expansion of staining is prefixed (i.e., time limitation). If an area limitation is present, then studies investigating the determinant factor for a prefixed area are required. However, if there is a time limitation, staining may be irreversibly prevented by rearing fish in the presence of bottom sand for a period of time that is longer than the, as yet to be determined, time limitation. Therefore, in the current study, we examined the possible presence of a time limitation using bottom sand to suppress staining expansion for a set period.

The results suggest that bottom sand has a strong, but temporary, suppressing effect. The absence of a time limitation against staining progression, in turn, suggests the presence of an area limitation for the spontaneous stasis of staining expansion.

Materials and Methods

Rearing before experiments

Fertilized eggs of the Japanese flounder were obtained by natural spawning from mature adults maintained at Chiba Prefectural Fisheries Research Center, Chiba, Japan. Metamorphosis began at 25 days post hatching (DPH) and was completed at 32 DPH. At 72 DPH, about one hundred juveniles with small darkened areas on the blind side (e.g., see Fig. 1), with a body length of 5-6 cm, were selected, since flounders of this condition easily express significant staining [22]. They were transported to Kyoto University by commercial parcel transport with oxygen at ambient temperature. They arrived the next day, and 60 flounders were marked with three colors (red, blue, and green) using a Visible Implant Elastomer (Northwest Marine Technology, Inc., USA) at four points on the blind side to enable identification of these individuals.

Experimental design

At 74 DPH, juveniles were randomly distributed into 6 experimental tanks. As the controls, Tanks 1 (sandless, positive control; no sand on the bottom for the duration of the experiment) and 2 (sandy, negative control; sand on the bottom for the duration of the experiment) were employed.

The rearing experiment was divided into three periods (i.e., A, B, and C). All three were defined by the progression of the darkened area in Tank 1 (sandless). Period A began with the start of the experiment and ended with the onset of rapid darkening. Since rapid darkening occurred just after the ratio of darkening (see below) exceeded 0.1 [22], we considered the onset of the rapid darkening period to occur when the ratio exceeded 0.1 in 7/10 individuals in Tank 1. Period B was defined as the period from the end of Period A to the stasis of rapid darkening. The beginning of stasis was defined by an increase in the ratio of darkening (see below) that was < 1.1 when compared to that of the last measurement. Period C began with the end of Period B and continued to the end of the experiment.

To examine the presence of a time limitation for the initiation of rapid darkening period, two tanks were designed. The bottom sand that was initially present was removed at the beginning of period B in Tank 3 (i.e., 2-week delay) and of period C in Tank 4 (11-week delay). In addition, to examine the inhibitory effect of bottom sand against once-initiated rapid darkening, bottom sand was introduced at the beginning of period B in Tank 5 (suspend); however, the similarly-introduced bottom sand was removed at the beginning of period C in Tank 6 (suspend-restart). The presence or absence of bottom sand for each tank is shown in Table 1 for each period.

Rearing procedure and final sampling

Ten juveniles of each tank were stocked in a 60-l transparent acrylic tank filled with artificial seawater

(New Marin Merit, Matsuda Co. Ltd, Japan) at 25 °C with a filtering system equipped with chiller. Circulation ratio was about 8 time / hour. Salinity and pH were constantly at 34 - 36 and 7.9 - 8.1, respectively, during the rearing period. Juveniles were fed four times a day with artificial diets of suitable size (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 EP2 [1.9–2.3 mm in diameter, after 123 DPH], Marubeni Nissin Feed, Tokyo, Japan). Time of exposure to light irradiation was extended to 17 h/d in the current experiment, and the density of juveniles was not adjusted in response to their growth. However, as follows, individuals in Tanks 1, 4 and 6 were further divided into 2 tanks at 18th week in order to secure enough space for their growth.

By week 18 (200 DPH), the expansion of darkening in Tanks 1 (sandless) and 3 (2-week delay) ceased, as well as in Tanks 2 (sandy) and 5 (suspend). Rearing experiment of Tanks 2, 3, and 5 was terminated at week 18. Since individuals in Tank 1 were further used for examining the effect of bottom sand on completed darkening, they were reared about 3 more weeks, after divided into 2 tanks. However, since darkening expansion continued in Tanks 4 (11-week delay) and 6 (suspend-restart), rearing of these two tanks was continued until week 24 (242 DPH). At week 18, the fish from these two tanks were divided into 4 tanks (5 individuals per tanks), in order to secure a larger area for future growth. At the end of the rearing experiment of each tank, all juveniles were anesthetized in 0.1% 2-phenoxyethanol (Nacalai Tesque Inc.) and fixed in 10% neutralized formalin (Nacalai Tesque Inc.).

Measurement of darkened areas

The blind side of each flounder was photographed once a week or every 2 weeks without anesthesia from the underside of a transparent tank. The measurement of the darkened area and calculations of the ratio of darkening was conducted according to Isojima et al. [22]. In brief, the darkened area was measured by NIH Image J (http://rsbweb.nih.gov/ij/; National Institute of Health, USA) by using digital images. The ratio of darkening on the blind side was calculated by dividing the size of the darkened area by the size of the blind side, excluding the fins [22]. The ratio of darkening on the blind side at the end of experiment was regarded as the maximum ratio of darkening.

In Tanks 1 (sandless), 3 (2-week delay), and 4 (11-week delay), steady expansion of the darkened area was observed, and the darkening speed was calculated over time (i.e., from the beginning of the experiment [Tank 1, sandless] or from the removal of bottom sand [Tanks 3, 2-week delay, and 4, 11-week delay] to the darkening of 90% of the maximum ratio). The darkening speed in Tank 6 was not determined. In this tank, significant area darkened at the removal of bottom sand at week 11 depending on individuals, therefore the meaning of darkening speed may be different from other 3 tanks. The increase in the ratio of darkening over time (i.e., per week) during this period was calculated as the darkening speed as follows:

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

Detailed observations on the putative recovery from darkening

On the basis of the photographs taken for the whole blind side of live fish in Tanks 5 (suspend) and 6 (suspend-restart), a significant portion of the darkened area observed at the end of period A was no longer visible during period B following the introduction of bottom sand. These putative recovery areas were further examined using a fixed sample from Tank 5 (suspend) at the end of the experiment (week 18, 200 DPH) using a microscope (SMZ800, Nikon, Japan) equipped with a digital camera system (DV-Vi1-L2, Nikon, Japan).

Effect of bottom sand on completed darkening

In order to examine whether the recovery also occurred after complete expansion of the darkened areas, bottom sand was introduced into Tank 1 (sandless) starting at week 18. After 17 d, the darkened areas on the blind side of the juveniles were compared to those at week 18.

Statistical analysis

For statistical analyses, online tools provided by the Osaka University (http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom) were used. Student’s *t*-test was used to compare two means, and the Tukey–Kramer method was used for comparing the means of more than three groups. A P value <0.05 was considered significant.

Results

Body length and daily growth rate in relation to the bottom sand

The average initial body length was about 5.4 cm for all tanks, and there was no significant difference among the tanks ($P > 0.05$) (Table 2). During the experimental period, daily growth rate in body length/day fluctuated from 0.2 to 2.1 mm/day and was not correlated with the presence or absence of bottom sand for all tanks (Fig. 2). At week 18, the average body length was about 19 cm in all tanks (not statistically significant; $P > 0.05$) (Table 2). At week 24, the average body length was about 25 cm in extended rearing tanks (Tanks 4 and 6; not statistically significant at $P > 0.05$).

Changes in the ratio of darkening in relation to bottom sand

In the positive control, Tank 1 (sandless), rapid darkening and completion of darkening expansion (see Materials and Methods) were observed from weeks 2–11 and at week 18, respectively (Fig. 3). Therefore, periods A, B, and C were defined as 0–2, 2–11, and 11–18 weeks, respectively. Almost no expansion of the darkened area was observed in the negative control (i.e., Tank 2, sandy) (Fig. 3). When the ratio of darkening was compared between the beginning (week 0, 0.036 ± 0.003) and end (week 18, 0.045 ± 0.006) of the experiment, there was no significant difference ($P > 0.05$).

In tanks where initially-present bottom sand was removed, quick darkening was observed almost immediately following the removal of the bottom sand [i.e., at the beginning of periods B and C in Tanks 3 (2-week delay) and 4 (11-week delay), respectively] (Fig. 4). The completion of darkening expansion was observed at week 22 in Tank 4 (11-week delay).

In tanks where bottom sand was introduced after the onset of the quick expansion of darkening (i.e., Tanks 5 and 6), darkening expansion ceased immediately and the ratio of darkening significantly decreased from 0.23 ± 0.03 (at the end of period A) to 0.11 ± 0.02 (a level equivalent to the initial value at the end of period B) (Fig. 5). A similar ratio of darkening was maintained until the end of the experiment or until the removal of bottom sand in Tanks 5 (suspend) or 6 (suspend-restart), respectively. When the bottom sand was removed, quick darkening was observed in period C for Tank 6 (suspend-restart), and completion of darkening expansion occurred at week 22 (Fig. 5).

No significant differences were observed between the ratios of darkening calculated for the end of period A (week 2) among all the sandless tanks (i.e., Tanks 1 [sandless], 5 [suspend], and 6 [suspend-restart]). Further, the values for the darkening ratio at the end of period A for Tanks 1, 5, and 6 were significantly higher than those for Tanks 2 (sandy), 3 (2-week delay), and 4 (11-week delay) ($P < 0.05$, Fig. 6a). A comparison of the maximum ratio of darkening among Tanks 1 (sandless), 3 (2-week delay), 4 (11-week delay), and 6 (suspend-restart) showed that the former 2 tanks (0.59 ± 0.03 and 0.61 ± 0.02, respectively) were significantly larger than those for the latter 2 tanks (0.47 ± 0.03 and 0.48 ± 0.02, respectively, $P < 0.05$) (Fig. 6b).

Observations of putative recovery areas

As quantitatively shown in Fig. 5, a significant portion of the darkened area became pale in coloration (therefore, not recognizable as a darkened area) after introducing bottom sand to Tanks 5 (suspend) and 6 (suspend-restart). Figure 7 shows the typical location for darkened areas in Tank 5 (suspend) one week before the end of period A (Fig. 7a) and at the end of periods A (Fig. 7b) and C (Fig. 7c). From a comparison of Figure 7b and c, it is clear that the darkened area at the base of the dorsal and anal fins was diminished. The remaining darkened area in Figure 7c was similar to the earlier-darkened area shown in Figure 7a

After completion of the rearing experiment, the putative recovery area was examined under a microscope using formalin-fixed samples from Tank 5 (suspend). There were no melanophores in normal blind side (Fig. 8a). As shown in Figure 8b, melanophores of uniform size (81.6 ± 5.4 mm in diameter, $n = 30$) were present at a low density of about 30 cells/mm^2 in the putative recovery area. Since the melanophores were no longer present on the fish after removing the scale (data not shown), they were, therefore, present on the scale. Similarly, melanophores of significantly smaller size (70.3 ± 4.6 mm in diameter, $n = 30$, $P<0.05$) were present on the scale in areas that remained darkened (Fig. 8c) and the density (about 260 cells/mm^2) was about 10 times higher than that in the putative recovery areas.

Figure 9 shows the re-darkening process following the removal of bottom sand in Tank 6 (suspend-restart). A significant area became darkened in the absence of bottom sand (Fig. 9a), and a large darkened area at the base of the dorsal and anal fins diminished with the addition of bottom sand (Fig. 9b). One week after the removal of bottom sand, the darkened area began to expand, starting with the putative recovery area (Fig. 9c), resulting in an overall darkened area similar to that observed prior to the addition of bottom sand (Fig. 9a).

Effect of bottom sand on the completed darkening

In an additional experiment, to examine the possible recovery from darkening, bottom sand was introduced to the fish that had experienced complete darkening in Tank 1 (sandless) starting from week 18. However, these darkened areas remained, even at 17 d after the introduction of bottom sand (Fig. 10). The ratio of darkening at 17 d after the addition of bottom sand (0.59 ± 0.03) was not statistically different from that at week 18 without bottom sand (0.58 ± 0.03, $P > 0.05$).

Relationship between maximum ratio of darkening and darkening speed

For individuals in Tanks 1 (sandless), 3 (2-week delay), and 4 (11-week delay), there was a strong linear relationship between the maximum ratio of darkening and the darkening speed (R^2 was calculated at 0.80, 0.76, and 0.79, respectively). Since the darkening speed was in proportion to the maximum ratio of darkening and, was, therefore, necessary to avoid differences in the maximum ratio of darkening among the three tanks, a comparison of darkening speed was conducted using the slope of the three regression lines. As shown in Figure 11, although the slopes of the regression lines for Tanks 1 and 3 were similar, that of Tank 4 (11-week delay) was approximately two times higher than the former two tanks, indicating a faster darkening speed for Tank 4 (11-week delay). This was mainly due to the significantly shorter darkening period of Tank 4 (11-week delay; 7.0 ± 0.5 weeks) than that of Tanks 1 (sandless; 11.9 ± 0.5 weeks) and 3 (2-week delay; 11.5 ± 0.2, $P < 0.05$), as shown in

Figures 3 and 4.

Discussion

In the preceding studies, the onset or progression of staining was investigated in constant conditions (e.g., rearing with or without bottom sand). However, in the present study, the timing for the onset of staining and the progression or stasis of staining were artificially controlled by adding or removing bottom sand. In addition, the effect of staining stasis and timing of staining initiation was successfully clarified on the final extent and expansion speed of staining.

Suitability of rearing and bottom sand as a means of suspending darkening

As shown in Table 2 and Figure 2, the daily growth rates of individuals in all experimental tanks were within the normal range for this species in rearing conditions, without exhibiting statistical differences among tanks. A similar increase in daily growth rate was observed after week 8 for all tanks, probably due to a change in diet from K2 (sinking type) into EP2 (floating type) at 123 DPH (about week 7). Thereafter, the daily growth rate gradually decreased in all tanks in a similar manor. Since the pH was constant at 7.9-8.1 in all tanks throughout the experimental period, the decrease in daily growth rate may be caused not by the decrease of water quality, but probably by the increase in density due to growth, irrespective of the presence or absence of bottom sand. We have no explanation for the increase in the daily growth rate after week 22. Anyway, it is clear that the presence or absence of bottom sand did not affect the growth of individuals. Therefore, when differences in staining are detected among tanks in the current study, the differences are caused by direct effect of bottom sand, not indirect effect mediated by growth and body size differences, for example.

In the current experiment, almost all darkening on the blind side occurred after the completion of metamorphosis. Therefore, they are regarded as "staining", not "true ambicoloration", areas following Norman's definition (cited in Seikai [2]). As previously reported [11, 13, 14, 17, 19], the staining-preventive effect of bottom sand was strongly confirmed with constant conditions (Fig. 3). Moreover, the addition and/or removal of bottom sand during rearing (Figs. 4 and 5) indicated that the effect of bottom sand was only temporary; thus, the inhibition of staining only occurred in the presence of bottom sand. Although the darkening ratio decreased with the addition of bottom sand in Tanks 5 (suspend) and 6 (suspend-restart), these data do not indicate recovery of a once-darkened area as described later in detail. From these results, it appears that the manipulation of bottom sand may be an excellent method for inducing the expression or temporal stasis of staining.

Effect of bottom sand on rapidly-darkened area and completed-darkened area

A significant portion of the rapidly darkened areas was observed to diminish in fish after the addition of bottom sand in Tanks 5 (suspend) and 6 (suspend-restart) (Figs. 5, 7, and 9), while a similar change was not observed following complete staining for fish in Tank 1 (sandless) (Fig. 10). From the results indicating that newly- and early-darkened areas did and did not diminish (Fig. 7), respectively, and the fact that melanophore density in the putative recovery areas was remarkably low (Fig. 8), it is highly possible that the staining process had not progressed to completion in the newly-darkened areas and was only arrested by the addition of bottom sand. However, bottom sand does not lead to a complete recovery in staining but changes the appearance of the area into the normal blind side, as evidenced by the presence of a small number of melanophores that were equivalent in size to those in the undisappeared darkened area of blind side in Tank 5 (suspend). In addition, staining resumed at a faster speed than that in other areas after the bottom sand was removed for the second time in Tank 6 (suspend-restart). However, from the present study, it is not clear whether the quick darkening following the removal of bottom sand was the result of simple increase in visibility due to expansion of melanophores or of the rapid progress of normal darkening process.

Possible absence of time limitation for staining expression

The quick expansion of staining in fish began in Tanks 4 (11-week delay, Fig. 4) and 6 (suspend-restart, Fig. 5) after the removal of bottom sand at week 11, and the stasis of rapid-staining expansion was observed at week 11 in Tank 1 (sandless). In addition, slow expansion continued in Tanks 4 and 6 even after complete stasis of staining expansion in Tank 1 (i.e., week 18 and thereafter up to week 22). Consequently, time limitation seems absent on the onset of quick expansion or the progression of staining before weeks 11 (151 DPH) and 22 (228 DPH), respectively.

Possible presence of an “underlying” process of staining in the white area on the blind side

In order to compare the darkening speed, it is necessary to consider a strong positive correlation with the maximum ratio of darkening [22]. As shown in Figure 11, the darkening speed in Tank 4 (11-week delay) was approximately two times faster than that in Tank 1 (sandless) at a similar maximum ratio of darkening, which may be due, in part, to the significantly shorter darkening period of the former. This difference was not observed between Tanks 1 (sandless) and 3 (2-week delay), possibly due to the shorter suspension period by bottom sand in Tank 3, which could have caused the suspension effect to be negligible. These results, indicating a faster staining speed in Tanks 4 suggest that staining after the removal of bottom sand leads to the progression of darkening that begins with the midpoint of progression (rather than restarting the process from the beginning). Thus, with the addition of bottom sand, the staining process could still be present at an underlying level, even in the “white” area of the blind side, especially at neighboring area to completely-darkened staining area before the addition of bottom sand.

As shown in Figure 6b, the maximum ratio of darkening in Tanks 4 (11-week delay) and 6 (suspend-restart) was significantly lower than that in Tank 1 (sandless). This indicates that the prolonged suspension of staining (i.e., >9 weeks) decreased the maximum ratio of darkening. This phenomenon may also be explained by assuming that: 1) the progression of the underlying process was suspended with the presence of bottom sand, and 2) a time limitation was imposed for expansion of the underlying process. Thus, it is understandable that an area affected by underlying mechanisms is quickly darkened after the removal of bottom sand, but the final darkened area itself is often smaller because the total time to completion is shorter than the time to completion for the fish in Tank 1 (sandless). At present, the age (or body size) of the time limitation for the underlying process of staining, as well as its cellular and molecular bases, are still unknown.

On this point, we previously suggested that staining is “a status change in the body surface conditions from the blind side to that on the ocular side,” at least for pigment cells and scale types [11]. During the normal development of flatfish, asymmetric characteristics in pigmentation and scale formation are caused by the additional appearance of adult-type melanophores, xanthophores, and ctenoid scales only on the ocular side after metamorphosis [2, 4, 8, 9, 23, 24]. In other words, at least for pigmentation and scale formation, differentiation on the ocular side is considered as addition of new characters to the skin on the blind side of the fish. From these reasons, the notion that the blind-side skin is the larval type (transient phase) and the ocular is the adult type (terminal phase) was recently proposed [25]. This notion could also be true for staining. Pigmentation and ctenoid formation normally occur only on the ocular side and only for a short period soon after metamorphosis [26]. However, in the case of staining, a similar process may also occur on the differentiated blind side even long after metamorphosis. Thus, the essential nature of staining could be “delayed and mislocated” initiation of procedure for ocular side construction on blind side.

At present, there is no idea for the delayed occurrence of ocular side formation, but for the location, there is suggestive information. By performing Dopa assay on the normal blind side, an increased amount of chromoblasts was found at the edge of the trunk and at the base of the pectoral fin [13]; these are specific areas where darkening frequently occurs [5, 10, 11, 13, 15, 16, 22]. If future areas of staining could be detected in advance by assessing the density of chromoblasts, then the time limitation for the onset of the darkening process may correspond to the time limitation for the proliferation of chromoblasts. In addition, it is clear that the time (with regard to age or body size) was not the direct reason for the stasis of staining expansion observed at week 11 in Tank 1. In turn, it may be more plausible that the maximum area of staining is prefixed, on an individual basis, during development. At any rate, further detailed examination of the morphological and physiological changes is required, especially for the possible staining area on the blind side after metamorphosis.

Effectiveness of bottom sand against staining

It is well known that bottom sand strongly inhibits staining [11, 13, 14, 17, 19]. We discovered that bottom sand also stopped staining already in progress, as shown in Tanks 5 (suspend) and 6 (suspend-restart). Unfortunately, a time limitation after which staining no longer occurred was not observed, at least before 151 DPH (11 weeks in the current experiment). Therefore, bottom sand has a temporary effect only (i.e., not a permanent or irreversible effect) on the suppression of staining. While the staining area became smaller in Tanks 4 and 6; even after a long period of stasis, this remission only occurred in about 20% of the stained area, which is probably not enough to improve the market price of Japanese flounders [3]. From these results, bottom sand may be an effective but temporary inhibitor against staining. For stock enhancement, since juvenile flounders are released to the sea where bottom sand is present. Thus, reducing the risk of the onset staining, and the prevention of staining is only required during the artificial rearing period. Consequently, rearing with bottom sand until release should be an effective strategy. However, for juveniles intended for use as seedlings in aquaculture, alternative and more permanent methods for the prevention of staining need to be developed.

Acknowledgments

We express our gratitude toward the members of the laboratory of marine stock-enhancement biology, Graduate School of Agriculture, Kyoto University, for their useful discussions and encouragement throughout the course of the study. This study was supported in part by the Kyoto University Research Funds to Back-up Scientists at Core Stage FY2011 and JSPS KAKENHI Grant Number 24580266 to M.T.

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Table

Table 1 presence (+) or absence (-) of bottom sand in tanks during each period

Table 2 Comparison of the initial and final body lengths (cm) among the six tanks

(footnote) Mean ± standard error (SE), *n* = 10; no statistical differences were observed between tanks with similar measurement timings. Data on week 24 are lacking for Tanks 1, 2, 3, and 5, because experiments were terminatedat week 18 or 22 in those tanks

Figure captions

Fig. 1 Typical appearance of the blind side at the beginning of the experiment. An individual from Tank 1 (sandless) is used (body length, 5.0 cm). Although there are strong white areas on abdomen and around eye due to light reflection, the absence of darkened area had been confirmed. Juveniles were marked with three colors (red, blue, and green) using a Visible Implant Elastomer at four points on the blind side to enable individual identification. The black bar indicates 1 cm

Fig. 2 Changes in the daily growth rate (mm/day) during the experimental period. Closed circles, open circles, closed triangles, open triangles, closed squares, and open squares indicate Tanks 1 (sandless), 2 (sandy), 3 (2 weeks delay), 4 (11 weeks delay), 5 (suspend), and 6 (suspend-restart), respectively. *A*, *B*, and *C* indicate the periods A (before week 2), B (week 2–11), and C (after week 11), respectively. Mean ± standard error (SE), *n* = 10

Fig. 3 Changes in the individual ratio of darkening on the blind side in Tanks 1 (sandless, upper) and 2 (sandy, lower). Shaded pattern indicate the period where bottom sand was added to the tanks. Open circles in the upper panel (Tank 1) indicate the end of the rapid-darkening period. The broken line in the upper panel corresponds to the individual shown in Figure 10

Fig. 4 Changes in the individual ratio of darkening on the blind side in Tanks 3 (2-week delay, upper) and 4 (11-week delay, lower). The shaded pattern indicated the period where bottom sand was added to the tanks. Open circles indicate the end of the rapid-darkening period

Fig. 5 Changes in the individual ratio of darkening on the blind side in Tanks 5 (suspend, upper) and 6 (suspend-restart, lower). The shaded pattern indicates the period where bottom sand was added to the tanks. The broken lines in the upper (Tank 5) and lower panel (Tank 6) correspond to the individuals in

Figures 7 and 9, respectively. The thick line in the upper panel (Tank 5) corresponds to the individual in Figure 8

Fig. 6 Comparison of darkened area at (a) end of period A and (b) end of the experiment among tanks. Mean ± standard error (SE), $n = 10$. Experiments (1) sandless, (2) sandy, (3) 2-week delay, (4) 11-week delay, (5) suspend, and (6) suspend-restart. The end of the experiment was week 24 for (4) 11-week delay and (6) suspend-restart, and week 18 for other tanks. Different characters indicate statistical difference ($P < 0.05$)

Fig. 7 Typical pattern of darkening and disappearance of the darkened area in an individual in Tank 5 (suspend, the individual shown with broken line in the upper panel of Fig. 5) by adding bottom sand. a, 1 week before the end of period A (week 1, ratio of darkening = 0.09, body length = 5.8 cm); b, end of period A (week 2, 0.27, 6.0 cm); and c, end of period C (week 18, 0.11, 17.2 cm)

Fig. 8 Photographs of normal blind side (a), putative recovery (b) and visible (c) darkened areas. An individual in Tank 5 (suspend, indicated by thick line in the upper panel of Fig. 5). Body length = 18.0 cm. White bars indicate 1 mm

Fig. 9 Typical pattern of the re-darkening process by removing bottom sand for an individual in Tank 6 (suspend-restart indicated by the broken line in the lower panel in Fig. 5). a, 2 weeks after the beginning of the experiment (week 2, ratio of darkening = 0.20, body length = 6.7 cm); b, 4 weeks after adding bottom sand (week 6, 0.07, 8.7 cm); and c, 1 week after the removal of bottom sand (week 12, 0.21, 15.9 cm)

Fig. 10 Absence of the putative recovery effect of bottom sand against an individual experienced completion of darkening (Tank 1, indicated by broken line in the upper panel of Fig. 3). a, before the addition of bottom sand (week 18 [day 126 after the beginning of the experiment], ratio of darkening = 0.59, body length = 16.9 cm); and b, 17 days after the addition of bottom sand (day 143, 0.58, 17.8 cm)

Fig. 11 Relationship between the maximum ratio of darkening and darkening speed (increase in the ratio of darkening per week) for individuals in Tanks 1 (sandless), 3 (2-week delay), and 4 (11-week delay); closed square, triangle, and circle indicate individuals in Tanks 1, 3, and 4, respectively

Table 1

Table 1 presence (+) or absence (-) of bottom sand in tanks during each period

	Period		
Tank	A (week 0-2)	B (week 2-11)	C (week 11-18)
(1) Sandless	**-**	**-**	**-**
(2) Sandy	**+**	**+**	**+**
(3) 2-week delay	**+**	**-**	**-**
(4)11-week delay	**+**	**+**	**-**
(5) Suspend	**-**	**+**	**+**
(6) Suspend-restart	**-**	**+**	**-**

Table 2

Table 2 Comparison of the initial and final body lengths (cm) among the six tanks

Tank	0 week (cm)	18 week (cm)	24 week (cm)
(1) Sandless	5.35±0.07	18.77±0.90	
(2) Sandy	5.38±0.05	18.83±0.34	
(3) 2-week delay	5.28±0.08	18.66±0.43	
(4)11-week delay	5.32±0.05	18.99±0.42	24.12±0.45
(5) Suspend	5.40±0.06	18.54±0.73	
(6) Suspend-restart	5.44±0.05	19.80±0.38	25.19±0.44

*Mean ± standard error (SE), n = 10; no statistical differences were observed between tanks with similar measurement timings. Data on week 24 are lacking for Tanks 1, 2, 3, and 5, because experiments were terminated at week 18 or 22 in those tanks

Fig. 1

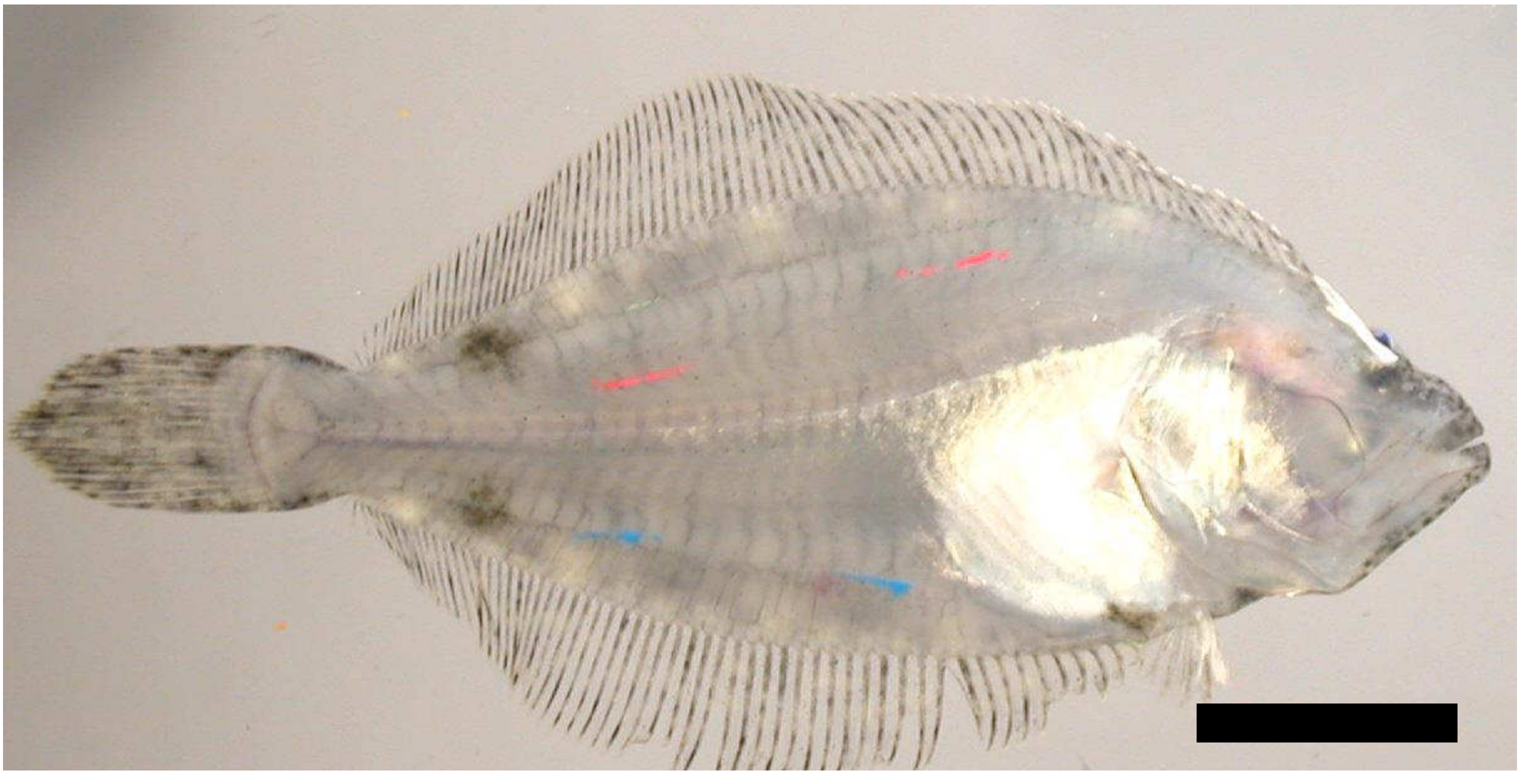
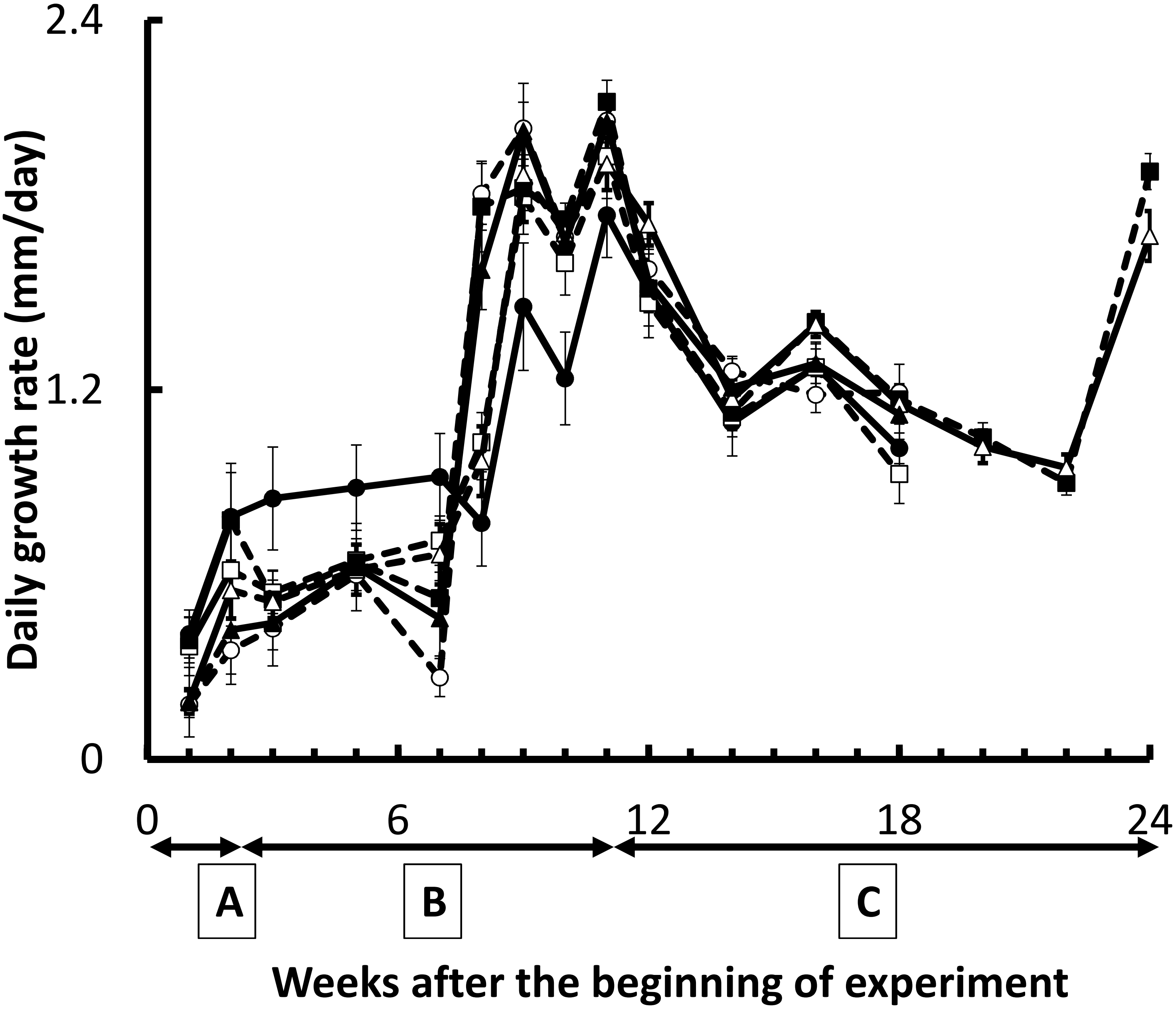


Fig. 2



Daily growth rate (mm/day)
2.4
1.2
0
0
6
12
18
24
A
B
C
Weeks after the beginning of experiment

Fig. 3

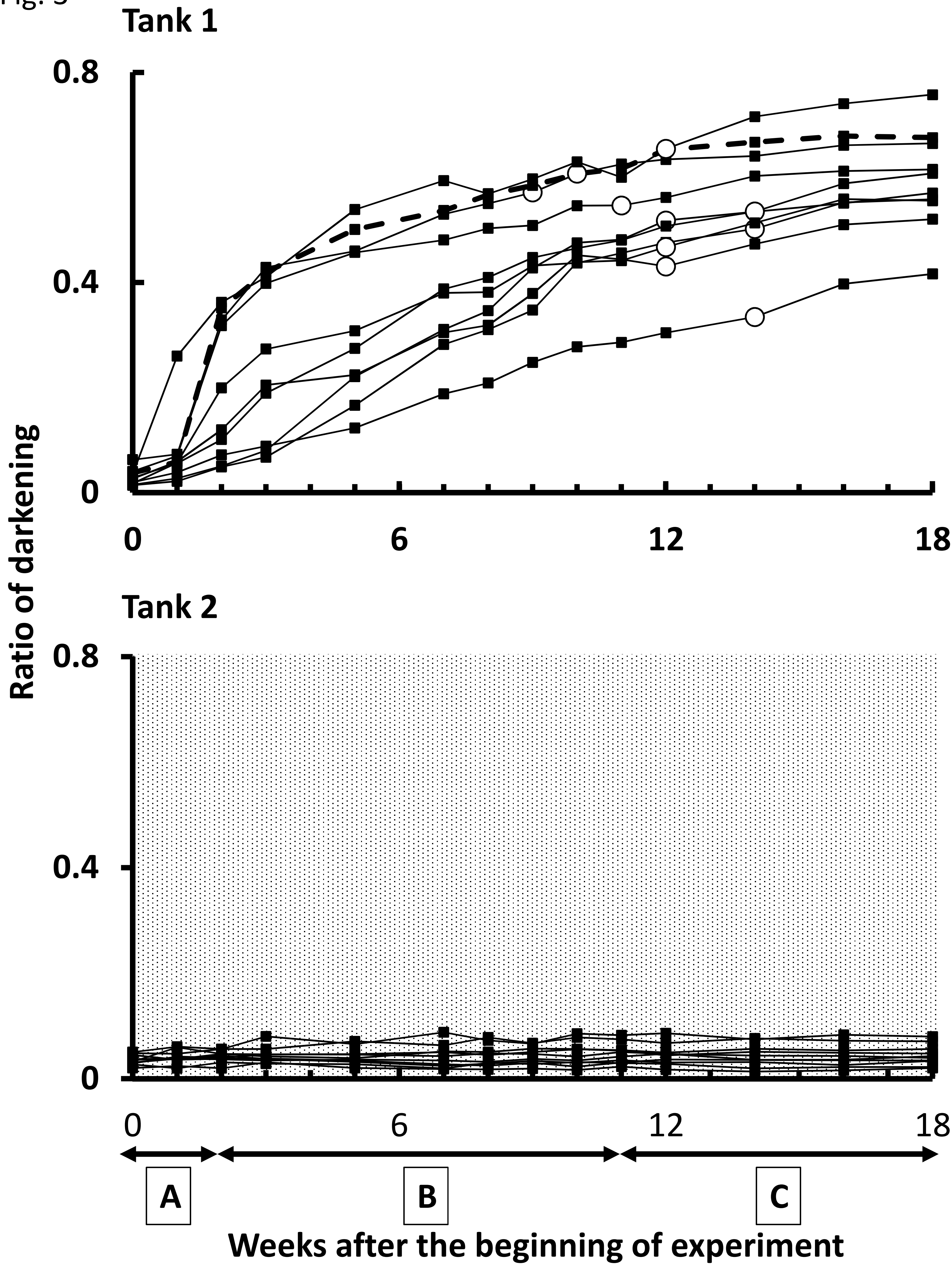

Tank 1
0.8
0.4
0
0
6
12
18
Ratio of darkening
Tank 2
0.8
0.4
0
0
6
12
18
A
B
C
Weeks after the beginning of experiment

Fig. 4

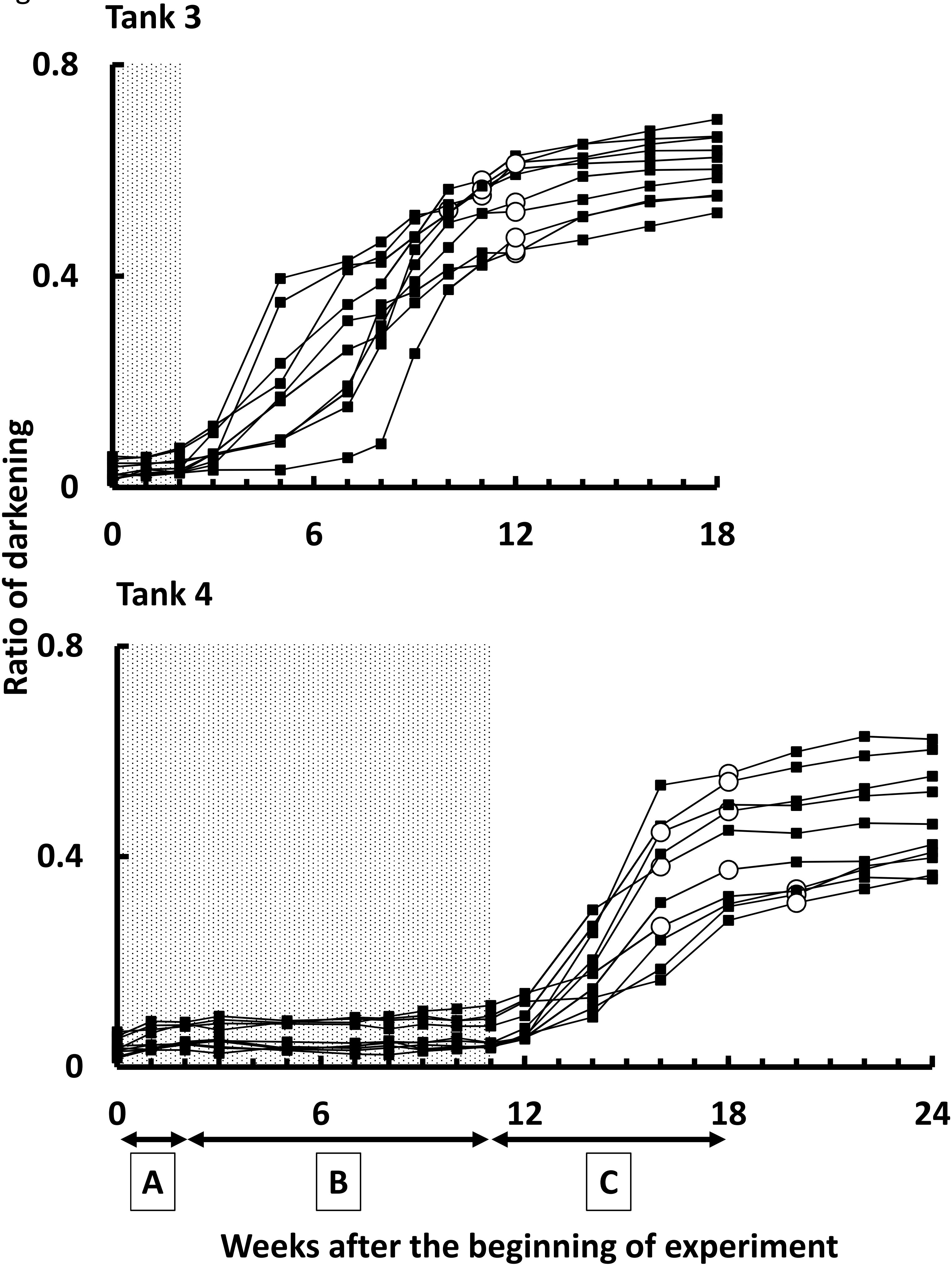

Tank 3
0.8
0.4
0
0
6
12
18
Ratio of darkening
Tank 4
0.8
0.4
0
0
6
12
18
24
A
B
C
Weeks after the beginning of experiment

Fig. 5

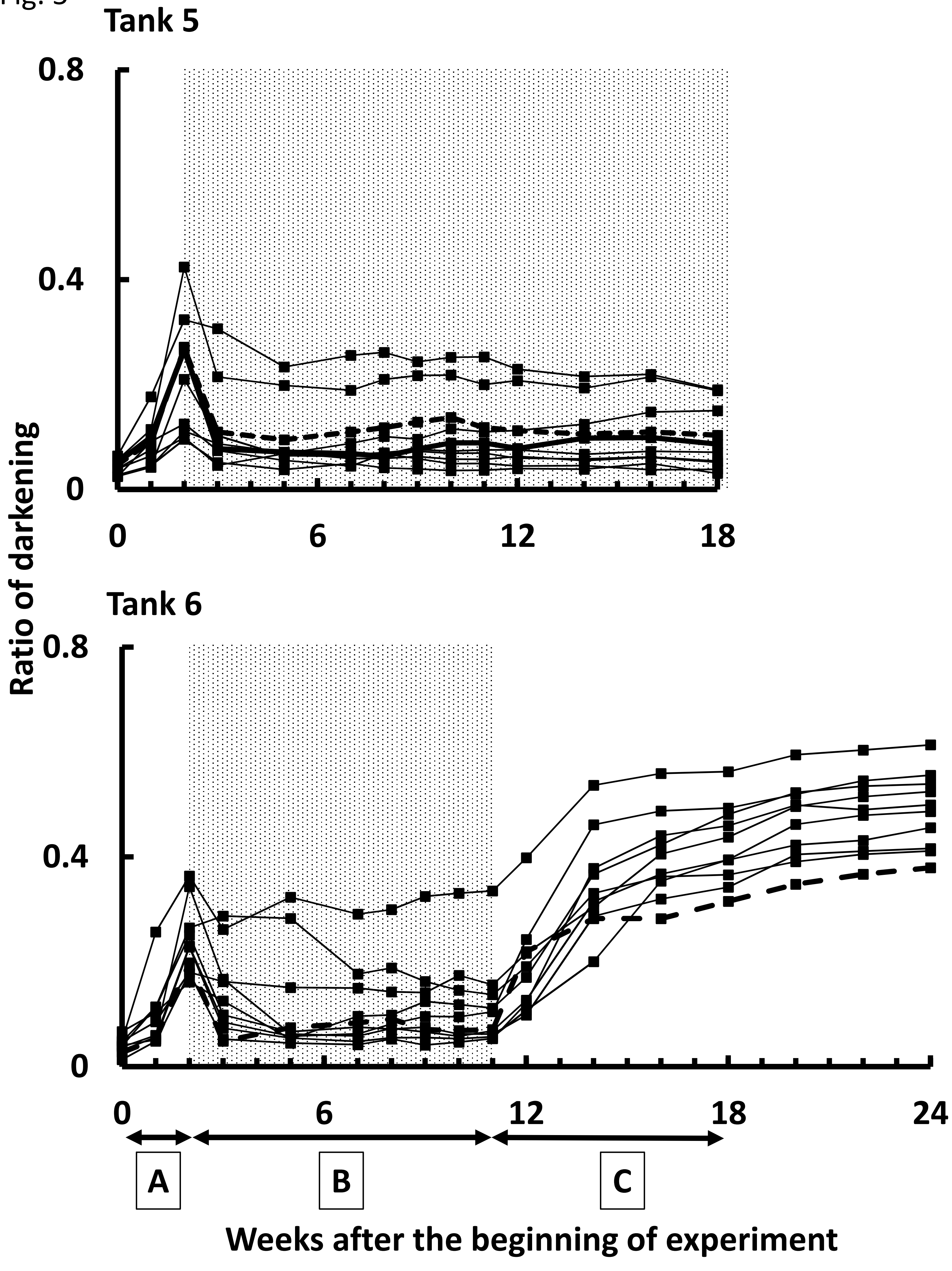

Tank 5
0.8
0.4
0
0
6
12
18
Ratio of darkening
Tank 6
0.8
0.4
0
0
6
12
18
24
A
B
C
Weeks after the beginning of experiment

Fig. 6

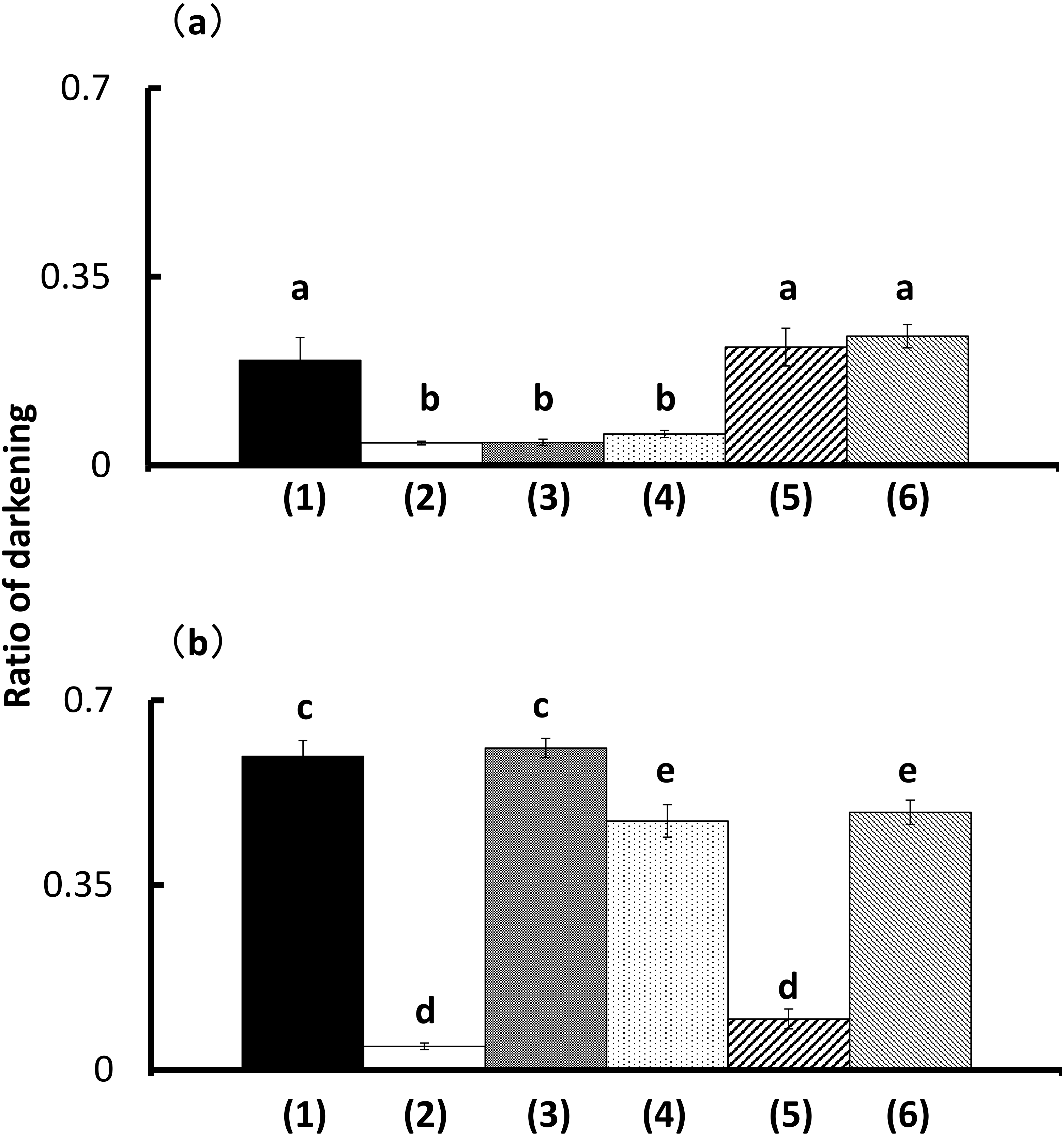

(a)
0.7
0.35
0
a
b
b
b
a
a
(1)
(2)
(3)
(4)
(5)
(6)
Ratio of darkening
(b)
0.7
0.35
0
c
d
c
e
d
e
(1)
(2)
(3)
(4)
(5)
(6)

Fig. 7

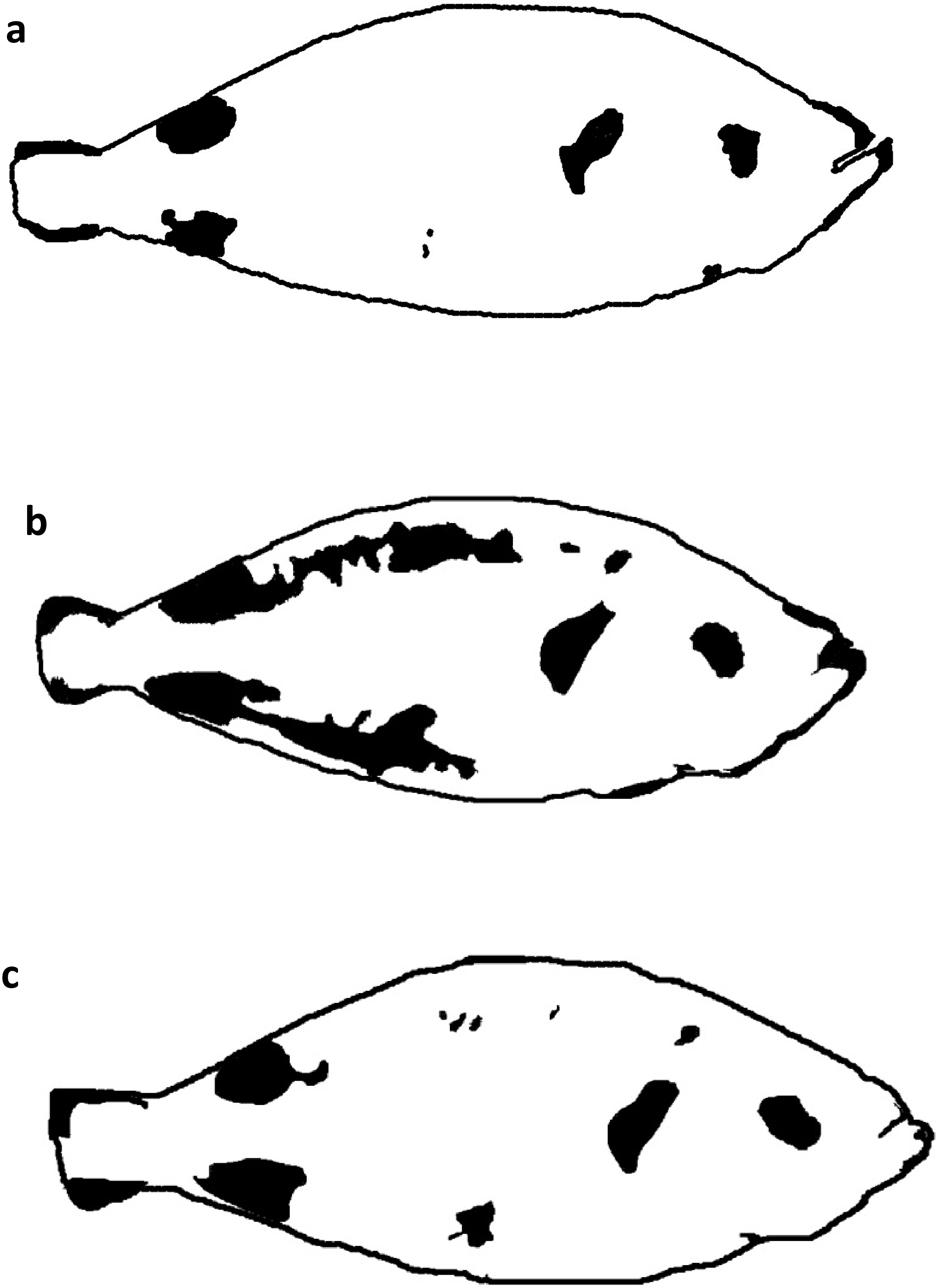


Fig. 8

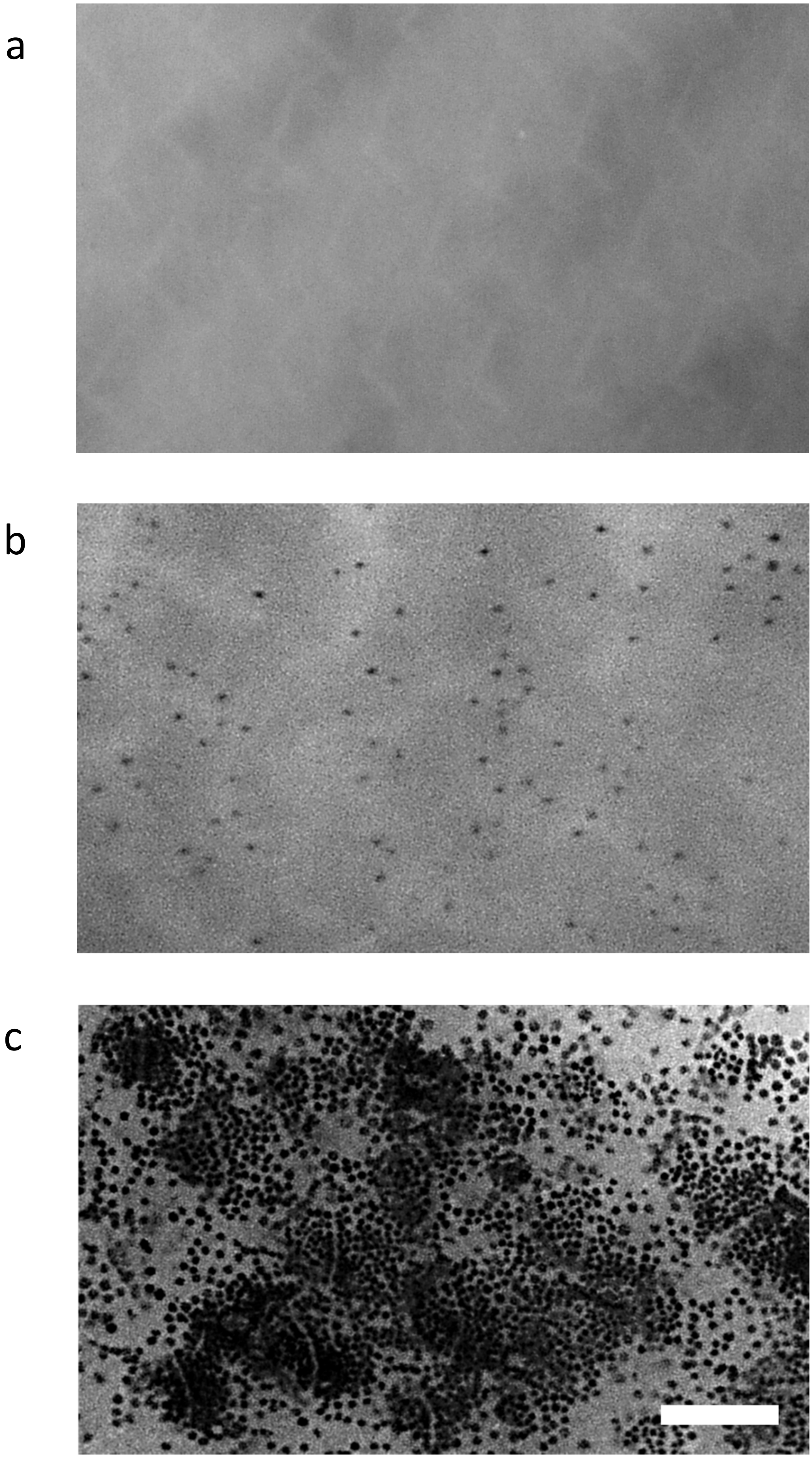


Fig. 9

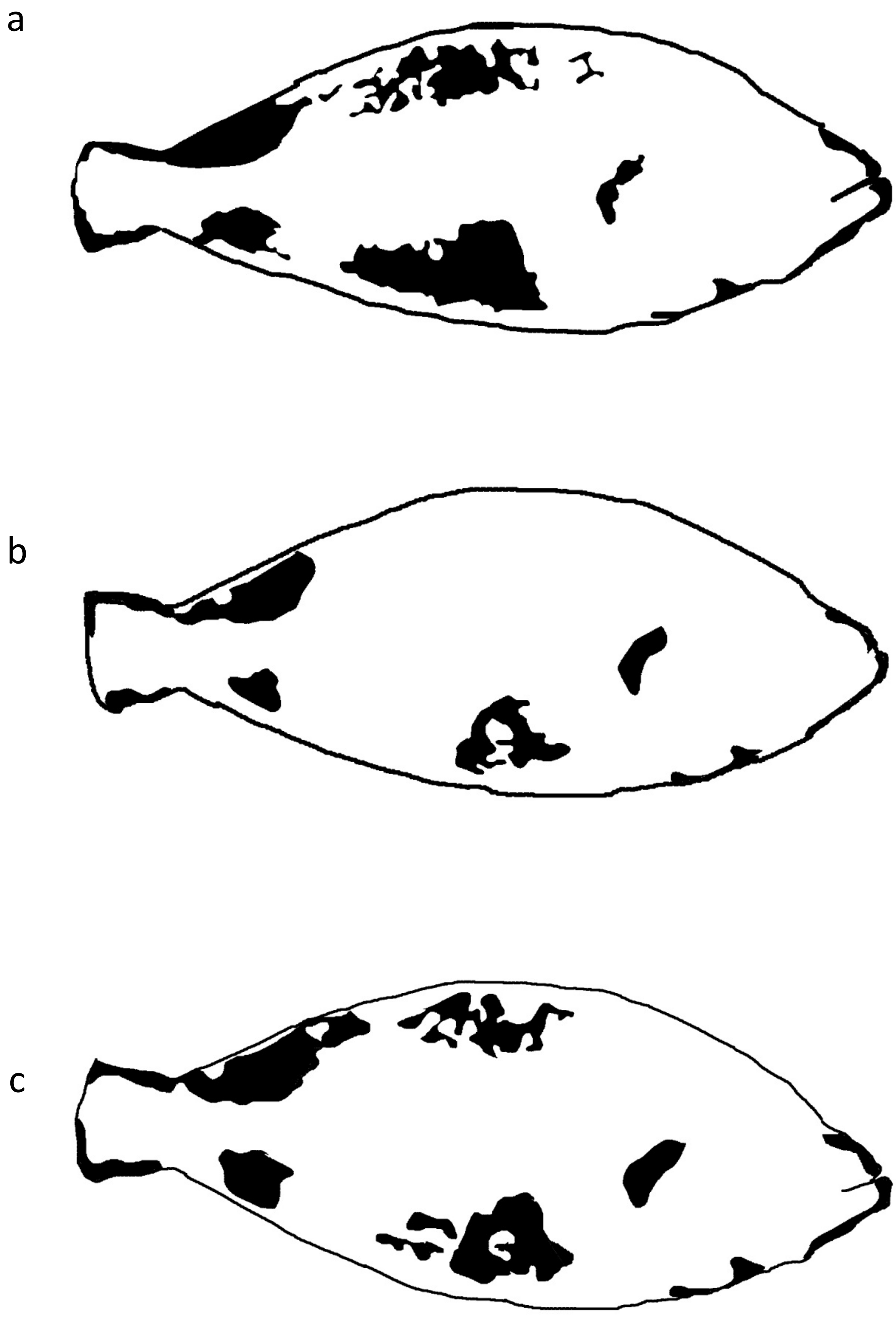


Fig. 10

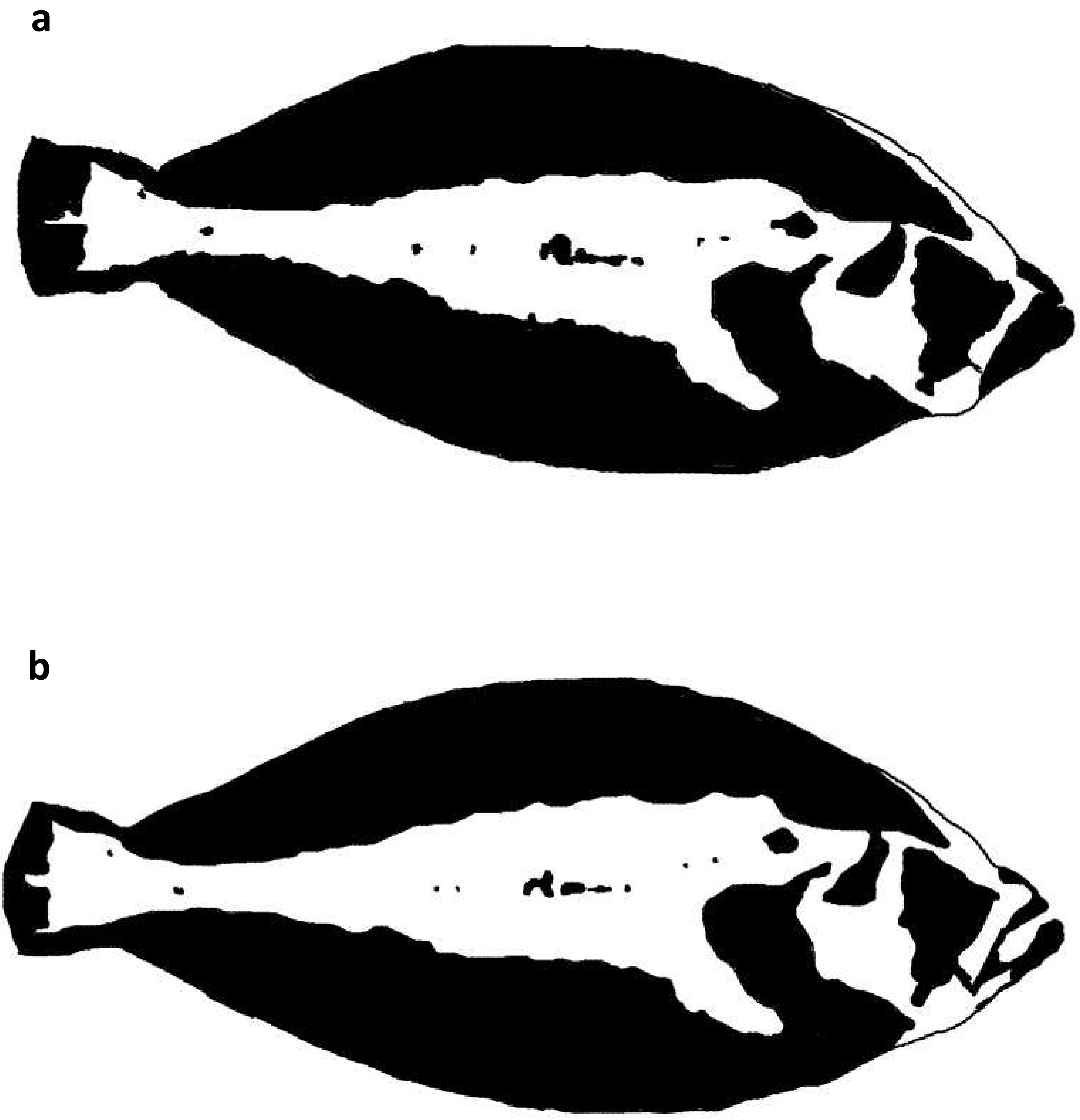
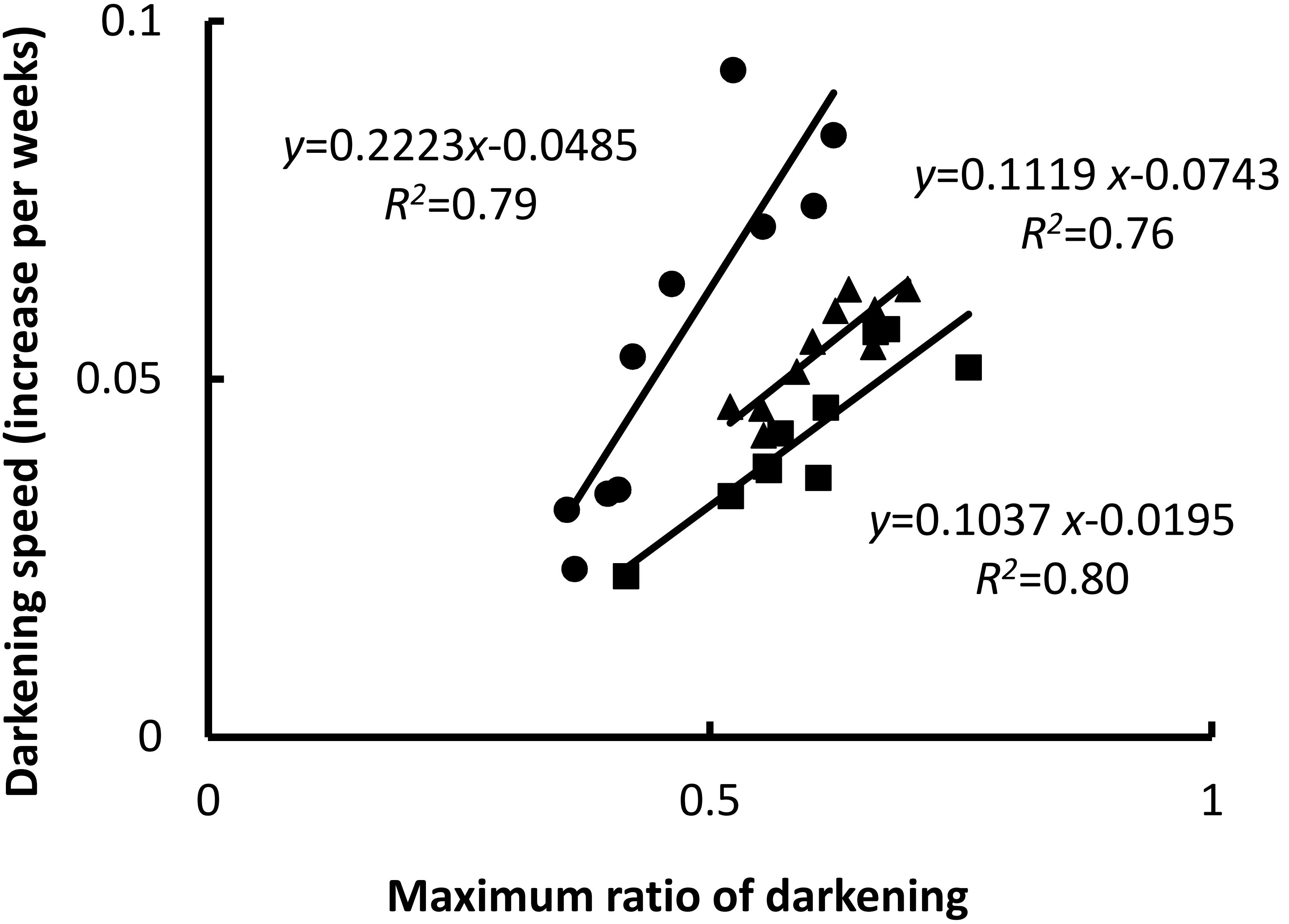


Fig. 11



0.1
0.05
0
0
0.5
1
Darkening speed (increase per weeks)
Maximum ratio of darkening
y=0.2223x-0.0485
R^2=0.79
y=0.1119 x-0.0743
R^2=0.76
y=0.1037 x-0.0195
R^2=0.80