Prevention of hypermelanosis by rearing Japanese flounder *Paralichthys olivaceus* in net-lined tanks

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Abstract

In artificially reared flatfish, especially the Japanese flounder *Paralichthys olivaceus*, pigmented skin (hypermelanosis) frequently appears on the fish's blind side after normal metamorphosis. As no practical prevention method has yet been proposed, we examined the effectiveness of a loose net set inside the rearing tank to cover the bottom and walls like a pouch. When juveniles (standard length: 6 cm) were transferred to the net-lined tank (4-mm mesh) before the first appearance of hypermelanosis, the pigmented area after 2 months covered about 0.5% of the blind side; about 1/40th of the area covered in fish reared in an ordinary tank (20%). Although the initial appearance of pigmentation in the axilla area (the area covered by the pectoral fin) was not suppressed, utilization of a larger mesh size (12-mm mesh) decreased the expansion of pigmentation in this area. Juveniles reared in the net-lined tank were about 5-15% smaller (standard length) than those reared in the ordinary tank, but their body depth/length ratio was closer to that of wild-caught juveniles. From this study, net-lined rearing tanks with larger-sized mesh are proposed as a practical method of preventing hypermelanosis in Japanese flounder aquaculture.

Keywords: Abnormal skin color, Net-lined rearing tank, Flatfish aquaculture, Pleuronectiformes, Axilla darkening, Larger mesh size

Introduction

Most teleosts undergo remarkable bodily changes during their development from larva to juvenile (e.g., metamorphosis). The Japanese flounder *Paralichthys olivaceus*, a Pleuronectiformes, like other flatfish, has symmetrically located eyes in its larval form. During metamorphosis, the right eye relocates to the left side of the body; thus, the left and right sides of the body are referred to as the ocular and blind sides, respectively. Adult-type melanophores and xanthophores appear on the skin of the ocular side, which becomes dark brown, but are absent from the skin of the blind side, which remains white (Seikai et al. 1987; Nakamura et al. 2010).

Because the Japanese flounder is an important species for Japanese fisheries, extensive seed production for aquaculture and stock enhancement has been carried out. However, various color abnormalities occur during rearing in hatcheries. One of the most significant is hypermelanosis. In this color abnormality, pigmented skin appears and then gradually expands on the blind side of the flounder after the completion of normal metamorphosis. Flounders with hypermelanosis, both those reared to harvest size and those released into the sea for recapture by fishermen, were priced about 20-70% lower in fish markets (Kaji and Fukunaga 1999). Therefore, various studies have attempted to prevent hypermelanosis in Japanese flounders. Because lightly colored tank bottoms were found to decrease or delay hypermelanosis (Yamanome et al. 2005; Amiya et al. 2005; Kang and Kim 2013b), the contributions of melanophore-related hormones were investigated. These included the melanophore-stimulating hormone and its precursor, which were investigated as possible stimulators (Yamanome et al. 2007b; Kang and Kim 2012, 2015), and the melanin-concentrating hormone, investigated as a possible suppressor (Yamanome et al. 2005, 2007a; Kang and Kim 2012, 2013a). Gene expression studies of these hormones' receptors provide evidence to support their contribution to hypermelanosis (Matsuda et al. 2018a). A higher occurrence of hypermelanosis was empirically associated with stressful conditions, and cortisol, a stress-responsive hormone, was revealed to enhance hypermelanosis (Matsuda et al. 2018b). In addition, dietary studies suggest that vitamins A (Tarui et al. 2006) and D (Haga et al. 2004) have a stimulatory effect. Although those extensive and intensive efforts had been made, it is still difficult to prevent the hypermelanosis at industrial scale.

The effect of simulating the ocean environment with a sandy substrate has been examined by several groups independently, and hypermelanosis was consistently and very strongly suppressed in these studies (Seikai 1991; Iwata and Kikuchi 1998; Kang and Kim 2012, 2013; Isojima et al. 2013b, 2014). However, this method is not employed in large-scale tanks because the sandy bottom interferes with the satisfactory cleaning of the tank, making it difficult to wash out residual food and excrement. Our previous study, designed to find a sand substitute for juvenile Japanese flounders that already expressed a small degree of hypermelanosis, suggested that undulated tank bottoms were effective in suppressing hypermelanosis expansion (Nakata et al. 2017). By examining the pattern of contact between the blind side skin and the bottom surface of the tank, we proposed that contact stimulus from the bottom exerted a suppressive effect on hypermelanosis in the contact area.

As an easily installable undulated bottom, a loosely set net lining the inner surface of the rearing tank was tested, and its effectiveness in preventing the expansion of hypermelanosis was suggested (Nakata et al. 2017). However, its effectiveness in preventing the initial appearance of hypermelanosis was not tested. Therefore, the main purpose of this study is to examine the suppressive effects of a net-lined rearing tank on the initial appearance and subsequent expansion of hypermelanosis in juvenile flounders, starting the experiment before the appearance of any hypermelanosis. In addition, because the most difficult area in which to suppress darkening was the base of the pectoral fin (axilla area), further experiments were conducted to clarify the reason for this local specificity. The applicability of net-lined rearing tanks for industrial use is discussed in relation to growth and body proportion and directions for further research suggested.

Material and methods

Experiment 1: Effect of a net-lined rearing tank on juveniles before the appearance of hypermelanosis

This experiment was carried out at the Nagasaki Prefectural Institute of Fisheries. Juvenile Japanese flounders, hatched from eggs spawned by artificially-grown adults, were kindly provided by a private hatchery (Ootawa Shubyo, Saikai, Nagasaki, Japan). For the rearing experiment, two circular tanks (1,000 l, transparent polycarbonate) were used, one for control rearing (control tank) and one for net-lined rearing (net tank). In the control tank, the juveniles were reared directly in the tank. For the net tank, netting (wind-protection net, polyethylene, blue, Raschel knitted, mesh size 4 mm, Dio Chemicals Ltd, Tokyo, Japan) was fashioned into a large pouch about the same size as the tank, and loosely installed inside it (Fig. 1a). The juveniles were then reared inside the net pouch. Because the net pouch was simply inserted into the tank and only fixed along the surface, the net fabric undulated randomly. In order to match the color and pattern of the bottom in the net and control tanks, the same type of netting used in the lining was laid under the transparent bottom of the control tank, and both tanks were placed on a white Styrofoam sheet. To provide uniform color conditions, the vertical walls of both tanks were covered from the outside with a light blue plastic sheet (sky blue, SK Event Sheet, Tanaka Sanjiro, Fukuoka, Japan).

At the start of the experiment (December 19, 2016), juveniles were randomly selected from the stock tank, and 70 were assigned to each experimental tank. The remaining 73 juveniles were anaesthetized with 0.02% 2-phenoxyethanol and their blind sides were photographed with a digital camera (EOS kiss digital DS5041; lens: Canon EFS 18-55 mm, Canon, Tokyo, Japan). For taking photographs, a scale was placed near the fish, and all the lengths and areas were calculated using the scale as standard. The standard length (SL) of the initial sample was 60 ± 4 mm (mean ± SD), and 69 of the 73 individuals displayed no darkened areas on their blind sides. The total-darkened-area ratios (see 2.4 for definition, similar to the ratio of darkening in Isojima et al., 2013a) for the four individuals with darkened areas were 12.6%, 1.6%, 1.5%, and 0.7%.

The experimental tanks were installed indoors, and day length was controlled by ceiling fluorescent lights that were turned on at 0900 h and turned off at 1700 h. Sand-filtered (natural sand 0.612 mm and Anthracite 1.272 mm), ultraviolet-sterilized

(UV2500M-B, Ebara Corporation, Tokyo, Japan) natural seawater was supplied at a rate of two rotations per day. Inlet water was supplied from a single tube placed just below the water surface near the tank wall, and outlet water was discharged by overflow through a filter placed near the tank wall. Both tanks were equally aerated using an air stone for each tank placed at the center of the tank. For daily bottom cleaning, ordinary siphon tube was used. Because excrement and remnant food was visible and only accumulated on a few places on the bottom, it took about 5 minutes for both tanks. The lowest and highest water temperatures during the experiment were 9.1°C (February 10, 2017) and 18.0°C (April 16, 2017), respectively. Commercial pellets (floating type, Hirame EPF-1 and EPF-3, Nissin Marubeni Feed Co., Ltd., Tokyo, Japan) were provided in the morning and afternoon. At the end of experiment (after 122 days, April 20, 2017), all the juveniles from both tanks were photographed, as described above.

Experiment 2: Hypermelanosis suppression in the axilla area

Darkening around the axilla area was not suppressed by the net-lined rearing tank in Exp. 1, probably due to the interruption of contact between the net and the skin caused by the pectoral fin of the blind side. We therefore attempted to increase this contact by ablation of the pectoral fin and the use of a larger mesh size.

This experiment was carried out at Maizuru Fisheries Research Station, Kyoto University, using juvenile flounders produced at, and kindly provided by, the Futtsu Laboratory, Institute of Seed Production, Chiba Prefectural Fishery Research Center. Fertilized eggs were obtained from wild-collected adults and reared following the standard protocol of the center. On May 25 and 26 (69 and 70 days after hatching), 2017, juveniles of approximately 60 mm SL with a small darkened area on their blind side were selected. Based on our previous study, it could be assumed that similarly sized Japanese flounders produced at the Futtsu Laboratory which displayed a small darkened patch would, if left untreated, express significant hypermelanosis after 2 months (Isojima et al. 2013a). These fish were therefore suitable candidates for experiments on the suppression of hypermelanosis.

The juveniles were shipped to the Maizuru Fisheries Research Station on May 31, 2017 and arrived on June 1, 2017. Until the start of experiment (June 8, 2017), all juveniles were reared in a 500 l circular tank with a sandy bottom (1 cm thickness, Micros ceramic, MS-1, NORRA Co., Ltd., Ootsu, Japan). During the acclimation period, the juveniles were treated with antibiotics (ERUBAJYU for aquaculture, 50 ppm, Ueno Pharmaceutical Co., Ltd., Osaka, Japan).

Six experimental tanks (transparent 200 l circular tanks, polycarbonate, Tanaka

Sanjiro, Fukuoka, Japan) were set up with the following characteristics (Fig. 1b): (1: control) intact juveniles in a tank without a net lining, (2: finless) pectoral fin-ablated juveniles in a tank without a net lining, (3: net) intact juveniles in a tank with a standard net lining, (4: finless + net) pectoral fin-ablated juveniles in a tank with a standard net lining, (5: large mesh) intact juveniles in a tank with a net lining made from netting with a larger mesh size, and (6: sand) intact juveniles in a tank with a sandy bottom (Micros ceramic, MS - 1) about 1 cm thick.

Ablation of the pectoral fin on the blind side was performed with dissection scissors under anesthesia using 0.01% 2-phenoxyethanol. When the length of the regenerated pectoral fin exceeded the mesh size (4 mm), the fin was again ablated on the 21st day. Thereafter, the length of the regenerated pectoral fin did not exceed 4 mm.

For the net lined treatments, (3: net) and (4: finless + net), linings similar to those used in Exp. 1 were prepared using the same netting after adjusting the size to fit the 200 l tank. For the net lined treatment with the larger mesh size (5: larger mesh), a mesh size larger than the length of pectoral fin of blind side was used. This allowed the fin to protrude more easily through the mesh, giving the axilla area of the blind side a higher probability of making contact with the net. To estimate the pectoral fin length from the total length of the juveniles, the relationship between the total length and the length of the pectoral fin of the blindside was determined based on preserved specimens of juvenile flounders, and expressed as the following equation:

(pectoral fin length, mm) = 0.0815 x (total length, mm) + 1.1848 (N = 45, R^2=0.9701)

Consequently, considering the growth of the juveniles, a lining made from 12-mm mesh netting (AIDEA net, Raschel knitted, polyethylene, green, Dio chemical. Ltd., Tokyo, Japan) was used for the first 7 weeks of rearing, after which 25-mm mesh netting was used.

At the beginning of the experiment (June 8, 2017), juveniles without hypermelanosis on the axilla area were selected. Among these, 19 juveniles were randomly selected as the initial sample, anesthetized with 0.01% 2-phenoxyethanol, and their blind sides photographed with a digital camera (STYLUS TG-630 Tough, Olympus, Tokyo, Japan). For taking photographs, a scale was placed near the fish, and all the lengths and areas were calculated using the scale as standard. The SL of the initial sample was 62 ± 5 mm (mean ± SD). The median, 25 percentile, and 75 percentile of the total-darkened-area ratio were 1.15%, 0.74%, and 1.54%, respectively. Nineteen juveniles were randomly assigned into each experimental tank.

The bottom and vertical walls of the tanks were covered from the outside with light blue plastic sheets, as in Exp. 1. Sand-filtered (0.6 mm) and aerated natural seawater was supplied at a rate of 10 rotations per day. Inlet water was supplied from a single tube placed just above the center of water surface, and outlet water was discharged by overflow through a filter placed at the center of the tank. All tanks were equally aerated using an air stone for each tank placed at the center of the tank. For daily bottom cleaning of flat-bottom tanks (control and finless) and net-lined tanks (net, finless + net, and large mesh), ordinary siphon tube was used. Because excrement and remnant food was visible and only accumulated on a few places on the bottom, it took less than about 2 minutes for flat-bottom tanks and less than about 3 minutes for net-lined tanks. However, for daily bottom cleaning of sand tank, it took more than 15 minutes, because we had to take out most of the sand and wash strongly to clean. During the experiment, the water temperature was gradually increased from 19°C (at the start of experiment, June 8, 2017) to 27°C (at the end, August 4, 2017). The tanks were installed indoors without direct sunlight, and the day length was controlled by ceiling fluorescent lights that were turned on at 0600 h and turned off at 1800 h. Commercial pellets (sinking type, Otohime S2 and EP2, Nissin Marubeni Feed Co., Tokyo, Japan) were provided in the morning and afternoon.

Final sampling was performed 57 days after the start of rearing (August 4, 2017). All the juveniles were euthanized with 0.1% 2-phenoxyethanol and photographed as described above.

Collection of wild-caught juveniles

The body proportions of wild-caught Japanese flounder juveniles were measured for comparison. The wild-caught juveniles were caught at 5-10 m depths in western Wakasa Bay, Kyoto Prefecture, on July 13, 2016. The specimens were preserved in 99% ethanol until measurement.

Measurement and statistical analysis

In this experiment, all lengths and areas were measured from digital images using ImageJ (National Institute of Health, USA, http: //imagej.nih.gov/ij/). Because photographs were taken using "auto mode", pigmented areas were manually identified on the display. The total-darkened-area ratio was defined as the ratio of total pigmented areas to the entire blind side area without the fins. For more detail, the hypermelanosis was classified into the following four areas on the blind side (Fig. 2): along the base of the dorsal and anal fins (marginal area, Fig. 2a), around the base of the pectoral fin (axilla area, Fig. 2b), around the base of the pelvic fin (abdominal area, Fig. 2c), and the center of the head (head area, Fig. 2d). The axilla-darkened-area ratio was defined as the ratio of the pigmented area in the axilla region to the entire blind side area without fins. For the body depth, the largest body depth along the dorso-ventral axis without fins was measured.

For data values whose normality and equality of variance were not rejected by the Shapiro-Wilks test and Bartlett test, respectively, the statistical differences among the averages were parametrically examined by student *t*-tests (two groups). Parametrical tests for three groups or more were not needed in the present study. For other values, statistical analyses were non-parametrically examined by the Mann-Whitney *U*-test (two groups), or the Kruskal-Wallis test (three groups or more), followed by the Steel-Dwass post hoc test. For frequencies, statistical significance was tested by Fisher's exact test. The significance level was set at 0.05. All statistical analyses were performed using EZR (Kanda 2013), available at http://www.jichi.ac.jp/saitama-sct/, Saitama Medical Center, Jichi Medical University, Saitama, Japan, which is a graphical user interface for R (R Core Team, 2014).

Results

Experiment 1: Effect of a net-lined rearing tank on juveniles before the appearance of

hypermelanosis

Figure 3 shows the blind side photographs of 24 juveniles randomly selected from each tank. Juveniles with large darkened areas were abundant in the control tank, but very rare in the net tank. The total-darkened-area ratio was significantly lower in the juveniles in the net tank than in those in control tank (Fig. 4, Mann-Whitney *U*-test, $p < 0.05$). The median total-darkened-area ratio in the net tank (0.5%) was about 1/40th of that in the control tank (20%).

Table 1 shows the number of individuals in each tank that developed darkened skin in each blind side area and the total number of individuals with hypermelanosis at the end of the experiment. The net tank significantly suppressed the appearance of darkened areas in the marginal, abdominal, and head areas (Fisher exact test, $p < 0.05$), but not in the axilla area (Fisher exact test, $p > 0.05$). There was no statistical difference in survival rate between the control and net-lined tanks (Fisher exact test, $p > 0.05$).

At the end of Exp. 1, the median SL of the fish in the net tank was 154.8 mm, significantly smaller (by approximately 5%) than the corresponding value (163.4 mm) in the control tank (Fig. 5, Mann-Whitney *U*-test, $p < 0.05$). In addition, the body depth/SL ratio was slightly, but significantly, smaller in juveniles reared in the net tank (0.42 $\pm$ 0.02, mean $\pm$ SD) than in those reared in the control tank (0.04 $\pm$ 0.02) (*t*-test, $p < 0.05$).

Experiment 2: Hypermelanosis suppression in the axilla area

The total-darkened-area ratios at the end of Exp. 2 are shown in Figure 6. Regardless of the presence or absence of a net lining, ablation of the pectoral fin had no significant effect on the total-darkened-area ratio (Steel-Dwass test, $p > 0.05$). Furthermore, regardless of the presence or absence of the pectoral fin, experimental groups without a net lining showed significantly higher values for the total-darkened-area ratio than those with net linings or sand (Steel-Dwass test, $p < 0.05$). The total-darkened-area ratio of the sand group was significantly lower than all other experimental groups (Steel-Dwass test, $p < 0.05$).

At the end of Exp. 2, one juvenile died only in the large mesh treatment, and there was no statistical difference in the survival rate among treatments (Fisher's exact test, $p > 0.05$). The numbers of individuals with darkened axilla areas were 19/19 (control), 19/19 (finless), 19/19 (net), 18/19 (finless + net), 18/18 (large mesh), and 17/19 (sand), respectively; there was no statistical difference among the treatments (Fisher's exact test, $p > 0.05$). It is clear that the initial appearance of the darkened areas around the axilla was not suppressed by any treatment.

The axilla-darkened-area ratios are shown in Fig. 7. Regardless of the presence or absence of a net, the absence of the pectoral fin tended to suppress the size of the darkened areas around the axilla; however, the differences were not significant (Steel-Dwass test, $p > 0.05$). In addition, compared to the control tank, the axilla-darkened-area ratio was significantly lower in the finless + net, large mesh, and sand tanks (Steel-Dwass test, $p < 0.05$).

The SL and body depth/SL values at the end of Exp. 2 are shown in Fig. 8. The SL values were significantly larger in tanks without net linings (about 140 mm; control, finless, and sand treatments) than in those with net linings (about 117 mm; net, finless + net, and large mesh treatments) (Steel-Dwass test, $p < 0.05$). Although the body size of wild-caught Japanese flounder juveniles was much smaller (69 mm, Fig. 8a), we attempted a preliminary comparison between the body proportions of the experimental and wild-caught juveniles (Fig. 8b). The body depth/SL values of the wild-caught juveniles were more similar to those from tanks with net linings (about 0.39) than they were to those from tanks without net linings (about 0.42).

Discussion

In this study, we first showed that net-lined rearing tanks clearly suppress hypermelanosis in Japanese flounders; not only its expansion in juveniles with small darkened areas, but also its significant initial appearance in juveniles without darkened areas. Darkening in the axilla area, the most difficult area in which to suppress hypermelanosis, could also be suppressed by selecting netting with a larger mesh size. However, the juveniles reared in net-lined tanks experienced slower growth and had different body proportions to traditionally reared juvenile.

Effect of net-lined rearing tanks on hypermelanosis and growth in juvenile flounders

Nakata et al. (2017) demonstrated that an undulating bottom surface, such as that provided by a net lining, suppressed the "expansion" of hypermelanosis in juveniles that had already expressed a small degree of darkening. To examine whether net-lined rearing tanks also prevent the initial appearance of hypermelanosis, the juveniles were subjected to the treatments mostly before the appearance of any darkening. The initial appearance of hypermelanosis, especially in the marginal area, was almost entirely prevented (Table 1), and consequently, the total darkened area was remarkably reduced to about 1/40th of that of traditionally reared juveniles (Figs 3 and 4). This clearly indicates the effectiveness of the net lining in suppressing the initial appearance of hypermelanosis. For the expansion of hypermelanosis, the results of Exp. 2 were basically consistent with Nakata et al. (2017), in which juveniles reared in net-lined tank tended to show smaller areas of hypermelanosis compared to those reared in flat-bottom tank of the same bottom color. The total-darkened-area ratios were significantly smaller in all three tanks with net linings than they were in the two tanks without net linings (Fig. 6). Only a single tank was used for each treatment in the present study due to the limited equipment available, but the results were consistent between the tanks with and without net linings, indicating the strong reproducibility of the hypermelanosis-suppressing effect of net-lined rearing tanks.

Although rearing juveniles in sandy bottomed tanks strongly prevents hypermelanosis (Seikai 1991; Iwata and Kikuchi 1998; Kang and Kim 2012, 2013c; Isojima et al. 2013b, 2014), bottom sand requires more intense tank cleaning. A net lining can be an excellent substitute for bottom sand, and is a practical method for preventing hypermelanosis, after confirming the effectiveness in industrial-scale tanks holding much larger number of juveniles.

However, this study also revealed two drawbacks to the net-lined rearing tanks. The first is the slower growth. Nakata et al. (2017) also reported lower growth rates in net-lined rearing. Because they provided a diet of sinking-type pellets to the juveniles, a significant proportion of the food became inaccessible when it fell through and remained under the net. They thus speculated that the lower growth rate was caused by a lower amount of nutrients available to the net reared fish compared to the flat-bottom reared ones (control tank). This situation was identical to that in Exp. 2 of the present study. In Exp. 1, although a diet of floating-type pellets was provided, a significant number of them still escaped through the net and became inaccessible. It is possible that the lower growth rate was again caused by less nutrients being available in the net-lined rearing tanks. Before these net-lined rearing tanks can be applied at an industrial scale, methods of keeping the pellets inside the net are required to enable growth equivalent to that of traditionally reared flounders.

Prevention of axilla darkening

Another drawback of the net-lined rearing tanks is their ineffectiveness in suppressing axilla darkening. As shown in Table 1, the net-lined rearing tank significantly reduced the appearance of darkening in the marginal, abdominal, and head areas. Although the axilla area is much smaller than the marginal area, the appearance of darkening in this area was not suppressed. A possible reason for this is the presence of the pectoral fin between the blind side skin of the axilla area and the net. If contact with the bottom substrate suppresses darkening (Nakata et al. 2017), then the presence of a pectoral fin could interfere with this contact with the net, resulting in the appearance of darkening in this specific area. Therefore, the effect of pectoral fin ablation was examined.

The results of Exp. 2 show that the ablation of the pectoral fin did not affect the SL (Fig. 8a) or the total-darkened-area ratio (Fig. 6), regardless of the presence or absence of a net lining. These results suggest that fin ablation does not cause any major defects affecting growth. Also, it is speculated that fin ablation does not induce serious chronic stress, because stress-induced cortisol is known to increase hypermelanosis (Matsuda et al. 2018b).

Although the initial appearance of darkening in the axilla area was not suppressed by any of the treatments in Exp. 2, its expansion tended to be suppressed by pectoral fin ablation (Fig. 7). Therefore, the presence of the pectoral fin is a possible reason for the expansion of darkening in the axilla area.

The use of the net linings with larger mesh sizes resulted in a significantly smaller area of darkening in the axilla region compared to the control, and it was comparable to that in the sand tank (Fig. 7). We selected a mesh size larger than the pectoral fin of the blind side because we expected that pectoral fin protrusion through the mesh would provide better contact between the net and the skin of the axilla area. The resulting suppression of hypermelanosis further supports the idea that the presence of a pectoral fin induces local darkening in the axilla area by interfering with the hypermelanosis-suppressing effect of contact stimulation from the bottom substrate (Nakata et al. 2017). Net-lined rearing tanks using nets with larger mesh sizes can be an excellent solution for preventing hypermelanosis.

Difference in body proportion

Wild-caught juveniles of 40 – 110 mm SL are known to have a constant body depth/SL value of approximately 0.38 (Yoshimura and Kawashita 2003), which is smaller than that of artificially raised juveniles (Nihira 1990; Yoshimura and Kawashita 2003). In the present study, the body depth/SL value was significantly lower in tanks with net linings, and these values in Exp. 2 (Fig. 8b) are similar to that of wild-caught juveniles (0.39) of a smaller size. Similar to the net-lined tanks, but inconsistent to the sand tank of the present study, juveniles reared in tanks with bottom sand or sand-pasted bottom displayed significantly smaller body depth/SL ratio (0.37-0.38) than those reared in control tank (0.39-0.40) (Kawana and Namba 2004). In addition, the present study showed differing values between Exp. 1 and Exp. 2; the body depth ratios of the control groups were 0.44 and 0.42, and those of the net groups were 0.42 and 0.39, respectively, the trend being roughly similar to that of the SL of each group. Although contact stimulation from the bottom substrate is possibly involved, growth-dependent changes in body shape cannot be excluded as a reason for the difference. Further study is required to elucidate this point.

Optimal application of the net-lining method

In this study, the larger mesh size (12 mm) showed better results in terms of less hypermelanosis in the axilla area, while the results of total hypermelanosis suppression were comparable to the 4-mm mesh treatment. Theoretically, a larger mesh should be less effective due to less skin contact with the net; the contact frequency of a given skin area is expected to be proportional to the length of the strands constituting each unit area of the net. For example, when using a mesh size of 12 mm the frequency of contact with the skin is expected to decrease to 1/3rd of that when using a 4 mm mesh. It is also possible that a smaller mesh size provides a smoother touch and, therefore, less stimulation to the skin. In addition, the mesh size of the net lining must be small enough not to let the juveniles through, and large enough to allow excrement through. It is still unknown whether different mesh sizes would be optimal for differently sized juveniles or whether a single optimal mesh size applies for all fish size classes. For factors other than mesh size, it is also to be examined whether net of other type (other knitting methods than Raschel, and other materials than polyethylene, for example) also prevents hypermelanosis. If we are able to answer these questions and if we improve the installation methods for net linings in larger tanks, then the utilization of nets as a bottom substrate may become an excellent solution for the prevention of hypermelanosis in flatfish aquaculture.

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Figure captions

Fig. 1 Schematic illustrations of the experimental tanks. a: Experiment 1 (effect of a net-lined rearing tank on juveniles before the appearance of hypermelanosis), b: Experiment 2 (hypermelanosis suppression in the axilla area). *X* on the blind side indicates pectoral fin ablation. See text for details

Fig. 2 Typical darkened areas on the blind side of Japanese flounder. Hypermelanosis typically emerges in four areas independently on the blind side, *a*, the marginal area; *b*, the axilla area; *c*, the abdominal area; and *d*, the head area. Other darkened areas (near the caudal fin, for example) were also measured and included in total darkened area, but the occurrence was not separately counted in Table 1.

Fig. 3 Photographs of the blind side of juveniles reared in the (a) control tank and (b) net-lined tank in Experiment 1. The blind sides of 24 randomly selected juveniles are shown

Fig. 4 Total-darkened-area ratio of juveniles reared in the control and net-lined tanks in Experiment 1. Different letters indicate statistical significance between groups (Mann-Whitney *U*-test, $p < 0.05$). In the figure, the upper and lower ends of the vertical bars show the maximum and minimum values, respectively. The upper and lower ends of the boxes show 75% and 25% values, respectively, and the horizontal line in the box shows the median value

Fig. 5 Standard length (SL) of juveniles reared in the control and net-lined tanks in Experiment 1. In the figure, the upper and lower ends of the vertical bars show the maximum and minimum values, respectively. The upper and lower ends of the boxes show 75% and 25% values, respectively, and the horizontal line in the box shows the median value

Fig. 6 Total-darkened-area ratio in Experiment 2. Different letters indicate statistical significance between groups (Steel-Dwass test, $p < 0.05$). The number of individuals was 18 - 19. In the figure, the upper and lower end of the vertical bars show the maximum and minimum values, respectively. The upper and lower end of the boxes show 75% and 25% values, respectively, and the horizontal line in the box shows the median value

Fig. 7 Axilla-darkened-area ratio in Experiment 2. Different letters indicate statistical significance between groups (Steel-Dwass test, $p < 0.05$). The number of individuals was 18 - 19. In the figure, the upper and lower end of the vertical bar show the maximum and minimum values, respectively. The upper and lower end of the boxes show 75% and 25% values, respectively, and the horizontal line in the box shows the median value

Fig. 8 Standard length (SL) and body proportion in Experiment 2. a: SL, b: body depth/SL. The number of individuals was 18 – 19. Values obtained from 15 wild-caught juveniles were added to the graphs, but not included in the statistical test. Different letters indicate statistical significance between groups (Steel-Dwass test, $p < 0.05$). The upper and lower end of the vertical bars show the maximum and minimum values, respectively. The upper and lower end of the boxes show 75% and 25% values, respectively, and the horizontal line in the box shows the median value

Table 1 Number of individuals having darkening at various areas of the blind side in Exp. 1

group	marginal	axilla	abdominal	head	(Total)
control	66^a	58^a	52^a	40^a	66
net	1^b	57^a	28^b	14^b	68

Different *uppercase letters* indicate statistical significance between groups (Fisher exact test, $p < 0.05$)

Fig. 1

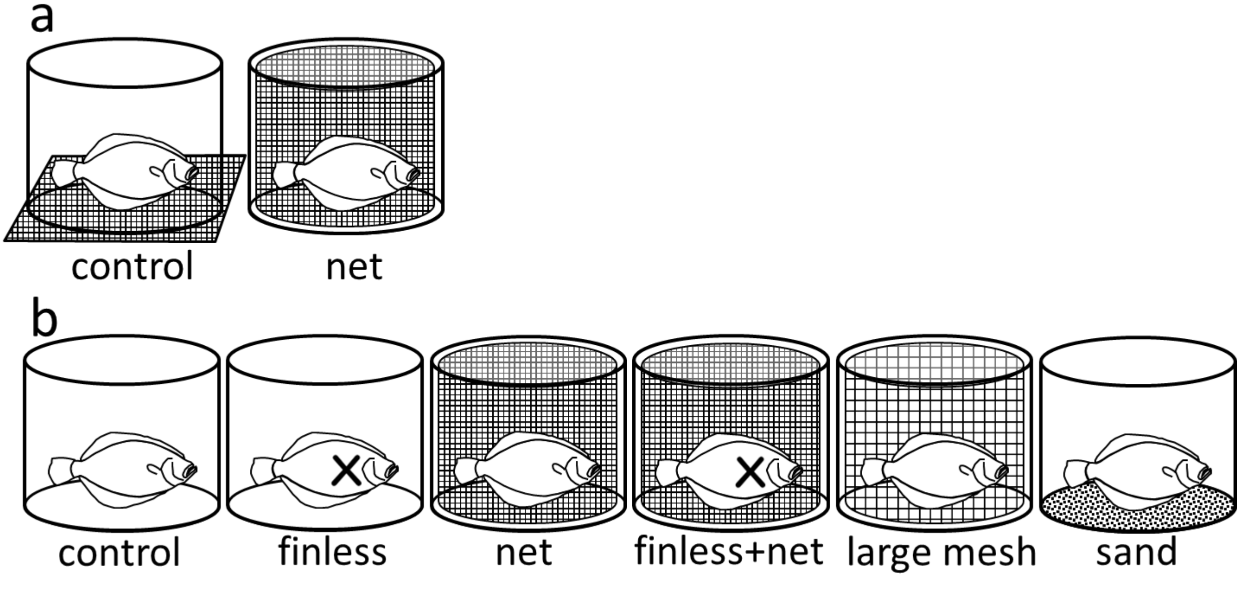
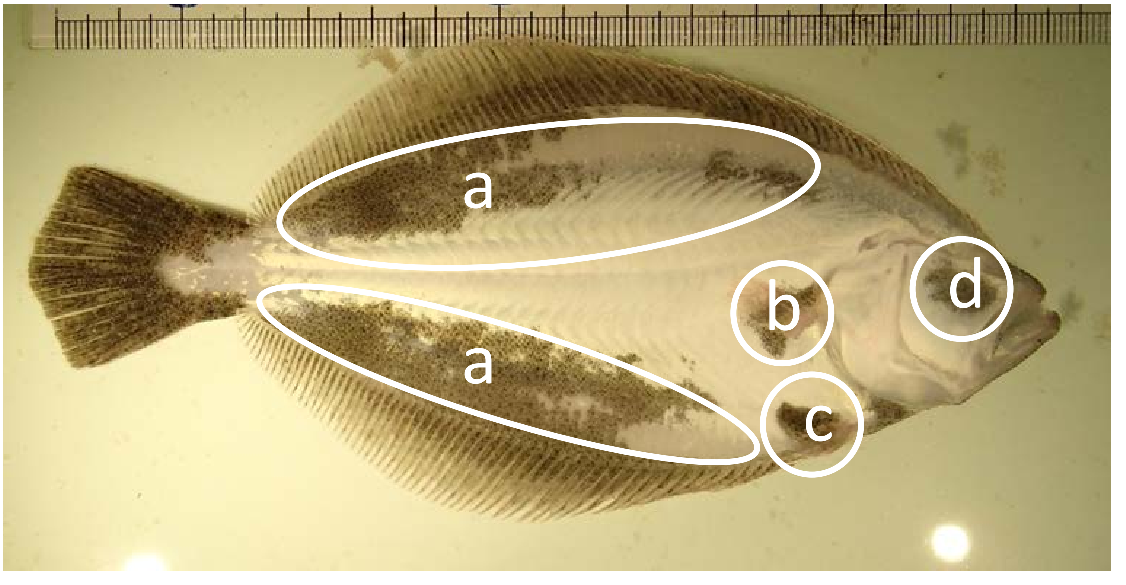

a
control
net
b
control
finless
net
finless+net
large mesh
sand

Fig. 2



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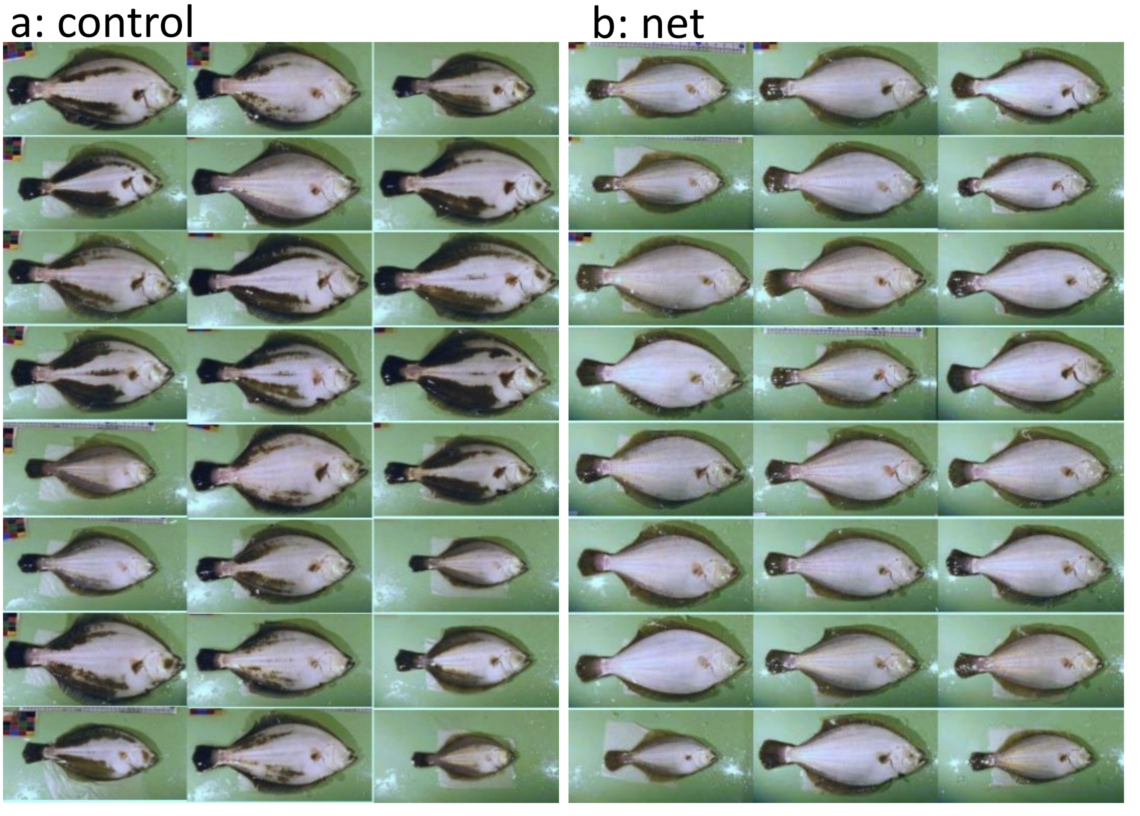

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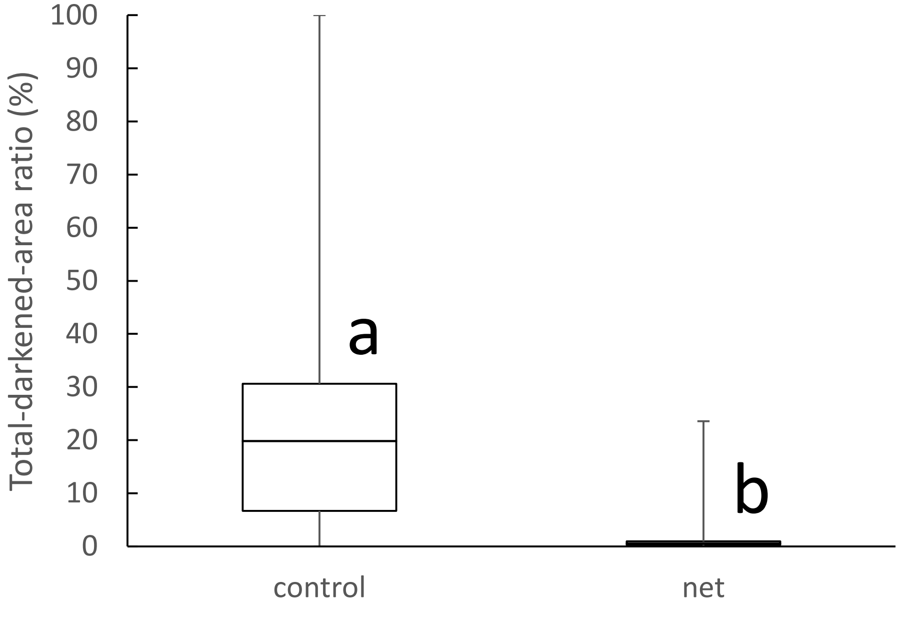
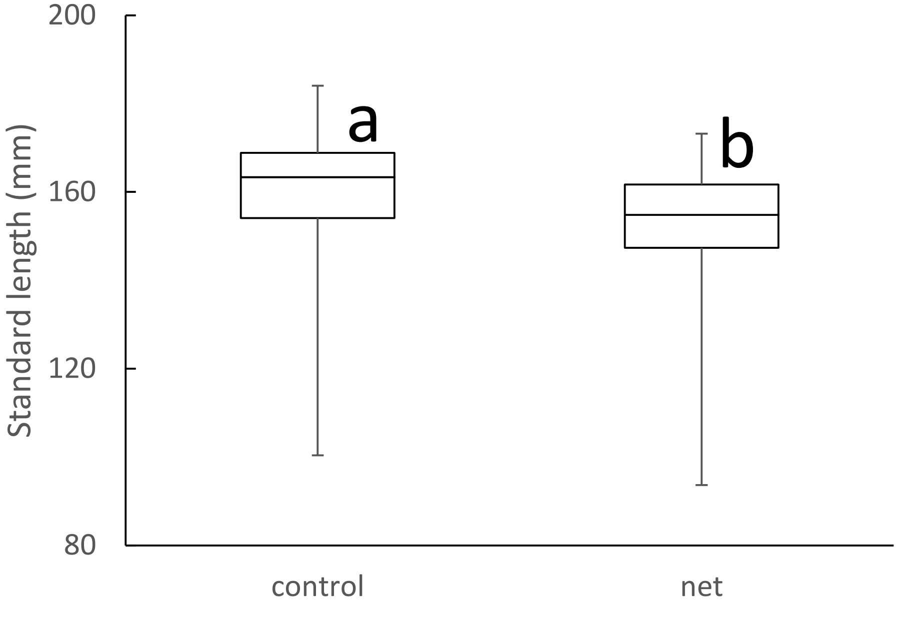


Total-darkened-area ratio (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
control
net

Fig. 5



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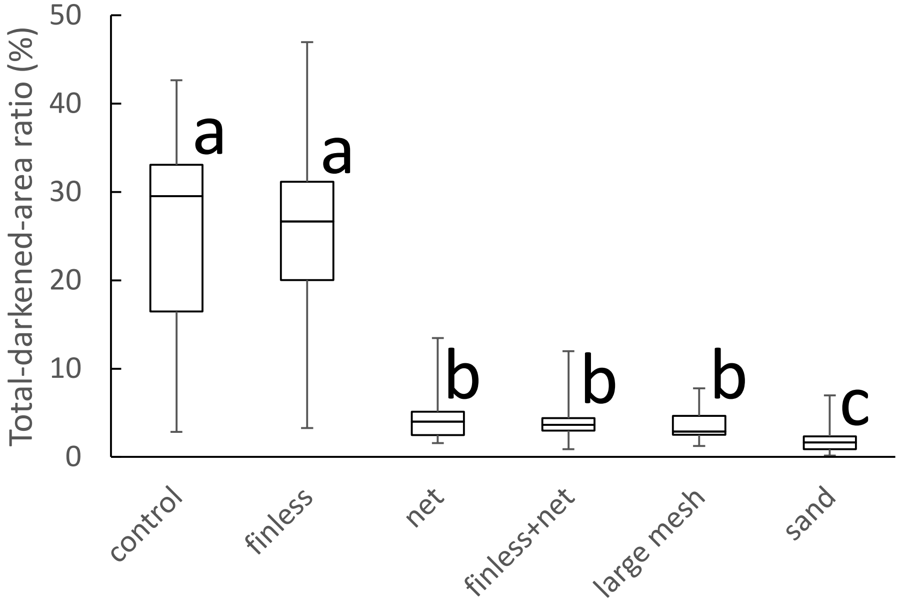


Total-darkened-area ratio (%)
50
40
30
20
10
0
a
a
b
b
b
c
control
finless
net
finless+net
large mesh
sand

Fig. 7

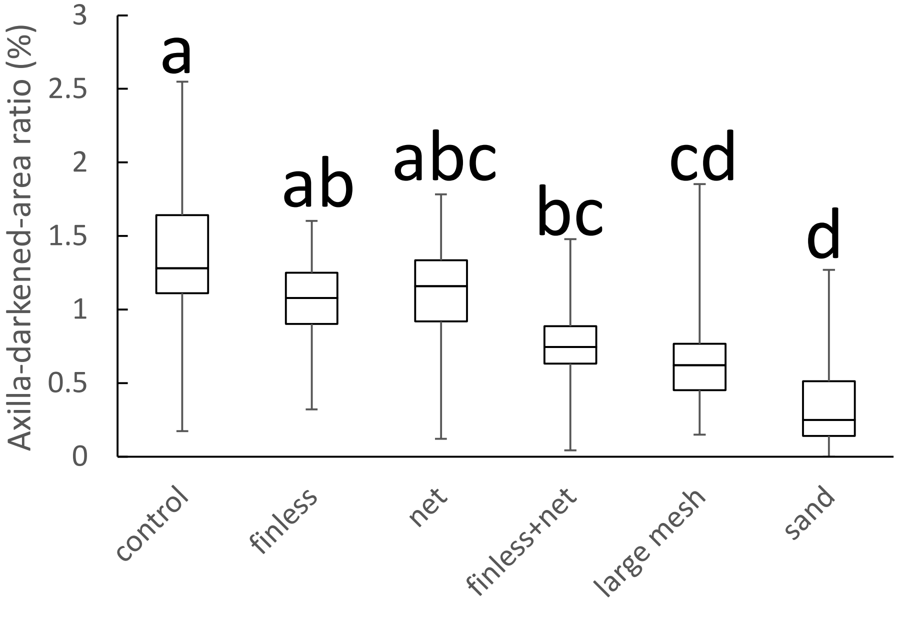
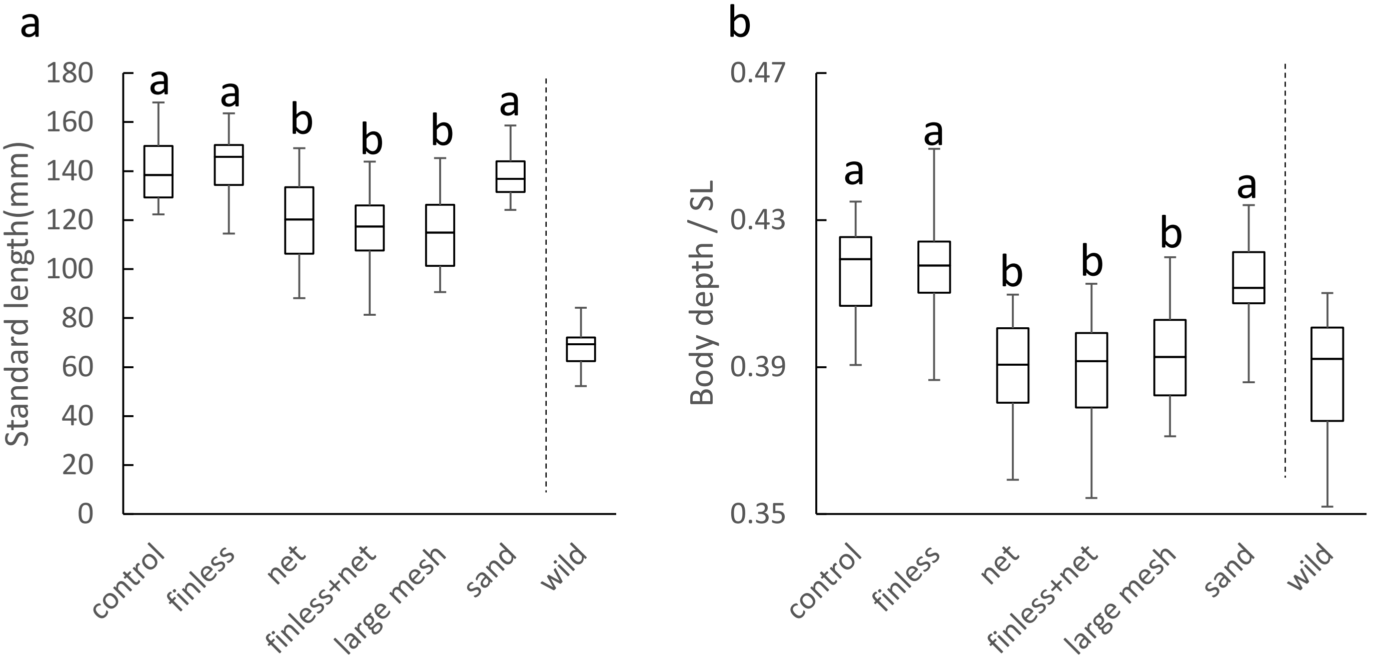

3
2.5
2
1.5
1
0.5
0
Axilla-darkened-area ratio (%)
a
ab
abc
bc
cd
d
control
finless
net
finless+net
large mesh
sand

Fig. 8



a
b
Standard length(mm)
180
160
140
120
100
80
60
40
20
0
a
a
b
b
b
a
control
finless
net
finless+net
large mesh
sand
wild
Body depth / SL
0.47
0.43
0.39
0.35
a
a
b
b
b
a
control
finless
net
finless+net
large mesh
sand
wild