An effective and practical method of net settings in rearing tank to suppress hypermelanosis in Japanese flounder

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Abstract

In Japanese flounder aquaculture, hypermelanosis occurs widely on the blind side. Rearing flounders in a net-lined tank was recently reported to prevent hypermelanosis. To effectively apply this method to larger tanks for aquaculture farming, the net setting method was examined. Juvenile flounders without darkened areas on the blind side (total length TL=13 cm) were selected, and reared for 6 months (TL = 32 cm). In the control tank without a net, the median value of darkened area ratio (darkened area / blind side area) was 46%. By only covering the tank bottom with slack-net, darkened area ratio was suppressed to 8%, less than 1/5 of that in the control tank. at the end of experiment Bottom coverage ratios of 0%, 10%, 30%, 50%, and 100% revealed a negative correlation between bottom net coverage and darkened area ratio. In this experiment, the darkening area in the tank with 50% bottom net coverage reduced to 1/3 of the control. Although the occurrence of hypermelanosis differs depending on the production lot, these results are expected to be a reference to select the suitable net size to meet the level of clearness of the blind side.

Keywords: Color abnormality・Hypermelanosis・Flatfish・Japanese flounder aquaculture・Bottom net・Covering ratio・Net-lined tank

Introduction

Japanese flounder *Paralichthys olivaceus* belongs to Pleuronectiformes, a large group of Heterosomata, and has a bilaterally asymmetrical body. The left side (ocular side) has two eyes, and the right side (blind side) has no eyes. On the ocular side, melanophores and xanthophores of the adult type appear after the normal completion of eye relocation, while they do not appear on the blind side. Consequently, the ocular side appears brown, and the blind side appears white (Seikai et al. 1987; Nakamura et al. 2010).

Japanese flounder is an important species in Japanese and Korean aquaculture and Japanese coastal fisheries. Notably, the aquaculture production of the species was 2,186 t, which is approximately 1/4 of the total yield in Japan in 2017 (Statistical survey of seawater fishery production 2018). Therefore, Japanese flounder is a species that has been successfully used in aquaculture production at the industrial level.

However, there are various unsolved problems in Japanese flounder aquaculture. One of the problems is color abnormality on the blind side; the white skin of the blind side gradually turns dark after the normal completion of eye relocation (see reviews, Aritaki and Seikai 2017; Tagawa 2017). This phenomenon is called hypermelanosis, and darkened flounders are sold at lower prices than normal (Kaji et al. 1999; Aritaki 2004). Therefore, establishing a method for preventing hypermelanosis is required, and various basic and applied studies have been conducted. For example, the contribution of chromatophore-related hormones, the stimulating effect of melanophore-stimulating hormone (Yamanome et al. 2007b; Kang and Kim 2012, 2015; Matsuda et al. 2018a), and the suppressing effect of melanin-concentrating hormone (Yamanome et al. 2005, 2007a; Kang and Kim 2012, 2013a) have been clarified. Higher rearing density (Fukunaga 1998) and cortisol (Matsuda et al. 2018b), a stress-responsive hormone, also increase hypermelanosis. Vitamin A (Miwa and Yamano 1990; Tarui et al. 2006) and D (Haga et al. 2004) have been shown to stimulate hypermelanosis. Although the control mechanisms of hypermelanosis have been gradually clarified, a practical method that has strong reproducibility and easy applicability to aquaculture is still lacking.

Interestingly, the light-colored bottom of rearing tanks has been reported to decrease or delay hypermelanosis (Amiya et al. 2005; Yamanome et al. 2005, 2007a; Nakata et al. 2017). However, in Kang and Kim (2013a, 2013b), the light-colored bottom of rearing tanks did not suppress hypermelanosis. Therefore, the effectiveness of the light color could be unstable depending on unknown factors. On the other hand, rearing flounders in tanks with bottom sand can prevent hypermelanosis (Seikai 1991; Iwata and Kikuchi 1998; Kang and Kim 2012, 2013b; Isojima et al. 2013a, 2014). As shown by several independent groups, bottom sand strongly prevents hypermelanosis with higher reproducibility. However, such a method of rearing has not been applied in aquaculture farm because the bottom sand is an obstacle for tank cleaning, and uncleaned particles, remnant food, and excretes are deleterious to water and bottom quality, as well as to fish health. Among various characteristics of bottom sand, Nakata et al. (2017) pointed out the importance of undulation of the bottom of the tank for the prevention of hypermelanosis, and further demonstrated that rearing juveniles in net-lined tanks also effectively prevented hypermelanosis “expansion.” Mizutani et al. (2020), using flounders before the first appearance of hypermelanosis, proved that rearing in net-lined tanks was also effective in preventing the “appearance” of hypermelanosis. From these two studies, a net-lined tank is a strong candidate for a practical method for the prevention of hypermelanosis.

Much larger tanks (several kL or more) than experimental tanks (mostly less than 1 kL) are used in the aquaculture farm of Japanese flounder. It is difficult to install a net that covers the entire inner surface of a large tank. In addition, tank cleaning is not easy because of the difficulty to remove the large net without damaging the juvenile flounders inside of the net. Therefore, we conducted experiment to find a better way of net settings that is easier to install and therefore more practical for larger tanks, and determine the suitable net size.

Materials and methods

Fish rearing

The rearing experiment was conducted at the Nagasaki Prefectural Institute of Fisheries. Juvenile Japanese flounders (total length, TL, = 12.82 ± 1.59 cm, mean ± SD) without hypermelanosis or negligible hypermelanosis (<1% of the darkened area ratio, see below in detail) on the blind side were selected and kindly provided by a private hatchery (Ootawa Shubyo, Saikai, Nagasaki, Japan). They were randomly allocated to seven experimental tanks (500 L circular tank, bottom diameter = 97.5 cm, transparent polycarbonate) with the following characteristics: (1) net-lined tank (net-lined); (2) 100% coverage bottom-net without slack (100% tight net); (3) 100% coverage bottom-net with slack (100% loose net); (4) 50% coverage bottom-net with slack (50% loose net); (5) 30% coverage bottom net with slack (30% loose net); (6) 10% coverage bottom-net with slack (10% loose net); and (7) without net (control).

In treatment (1) net-lined tank, white net (mesh size 12 mm, Russell knitting, polyethylene, Dionet; Dio Chemicals Co., Ltd., Tokyo, Japan) was processed into a pouch shape of a size approximately similar to the tank, and loosely set inside the tank, consequently covering the entire inner surface of the tank with undulated net material. In treatment (2) 100% tight net tank, a framed round net (diameter = 97.5 cm, the same size as the bottom of the tank, consequently covering 100% of the tank bottom) was made by fixing the same net material without undulation to a circular white frame (fiber-reinforced plastics, Ø = 7 mm, GF-7; Yamaten Co., Ltd., Osaka, Japan), and further fixed to the tank bottom. In treatment (3) 100% loose net tank, a framed round net of the same size was made, but with 10% larger net material, resulting in an undulated net surface with approximately 5 cm bumping. In treatments (4) 50% loose net, (5) 30% loose net, and (6) 10% loose net tanks, a similar undulated framed net was made, but with smaller round frames (diameter = 68.9 cm, 53.4 cm and 30.8 cm, respectively) in order to adjust the bottom covering ratio [= (area of the framed net)/(bottom area of the tank)×100] to 50, 30 and 10%, respectively. All framed nets were fixed on the bottom with a silicone sealant (Bus Bond Q Clear, Konishi Co., Ltd., Osaka, Japan) to prevent floating up from the bottom.

All rearing tanks were placed indoors and only received indirect and weak sunlight, even during the daytime (max 10 lx; measured about 20 cm above the water surface). The rearing tanks were placed on a white Styrofoam sheet, and the side walls of the tanks were covered with the same Styrofoam sheet on the outside. Since the rearing tanks were transparent, the entire inner surface of the tank was white including the area that uncovered by white net. On April 6, 2018, 30 juveniles were stocked in each experimental tank. Ultraviolet-sterilized natural seawater was supplied at a rate of ten rotations per day, and an air stone was set at the center of each tank. The water temperatures at the beginning and end of the experiment were 19.5°C and 22.7°C, respectively. The lowest and highest water temperatures during the experiment were 18.0°C (April 28, 2018) and 28.5°C (September 7, 2018), respectively.

Flounders were fed commercial pellets (initially Hirame EPF-2 and later Hirame EPF-3 according to fish size, Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) every morning. Starting from April 27, the total body weight of all flounders in each experimental tank was weighed once a month, and the pellet (0.7-3% of the total body weight of the tank) was fed for the next month. Diet was not supplied from one day before measurement and taking photographs, but was supplied in the evening after the manipulation. Remnant diet and excretes in tanks were discarded once a day before feeding with a syphon tube. On October 26, 2018, the rearing experiment was finished.

Photography and image analyses

Approximately once a month, all flounders in each experimental tank were anesthetized with 200 ppm 2-

phenoxyethanol, and photographs of the blind side were individually taken with a ruler using a digital camera (α550, DSLR-550, Sony Marketing Inc., Tokyo, Lens; AF 50/2.8 macro, MINOLTA Co., Ltd., Osaka, Japan, Photographic conditions; ISO = 200, aperture = 22 for 0 month and 6.3-8 for others, shutter speed = 1/125 for 0 month and 1/2-1/15 for others). Using ImageJ (National Institute of Health, USA, https://imagej.nih.gov/ij/. Accessed 4/6/2021), standard length (from the tip of the lower jaw to the posterior end of the hypural), head length (to the posterior end of the operculum), body depth (maximum length in the dorsal-ventral axis), blind side area (excluding fins), and darkened area of the blind side were measured manually from the pictures of the blind side. Although the hypural itself was not visible on the pictures, standard length could be determined with sufficient accuracy for the purpose of this study.

The darkened area ratio was calculated as follows:

$$\text{(darkened area ratio)} = \text{(darkened area)} / \text{(blind side area)}.$$

The fish coverage ratio of the tank was calculated as follows:

$$\text{(fish coverage ratio)}$$
$$= \text{(sum of blind side area of all individuals in each tank)} / \text{(bottom area of the tank)}$$

Because single value of fish coverage ratio is available from one tank, statistical analysis was not carried out for this value.

To determine the timing suitable for comparison among groups, we first examined the time course changes in the darkened area ratio in the following two tanks: control as the most heavily darkened, and

30% loose net tank as mildly darkened. The lightly darkened group (net-lined tank) was not examined at this step because almost no darkening was observed even at the end of the experiment. In addition, changes in the standard length and fish coverage ratio were examined in the two tanks.

Blood sampling and cortisol measurement

After taking the final photographs at the end of the experiment, blood samples were collected from fish in the control tank and net-lined tank, from the tail vein using a heparinized syringe under anesthesia with 200 ppm 2-phenoxyethanol. To minimize the influence of stress-induced increase of cortisol on the comparison between the tanks, blood samples were collected from three individuals at once (scooped and anesthetized together) from each tank, and alternately from control and net-lined tanks. Blood samples were then centrifuged, and the separated plasma was frozen at -20°C, and the cortisol concentration was measured by a specific radioimmunoassay after diethyl ether extraction and carbon tetrachloride washing following the methods of Hiroi et al. (1997).

Statistical analysis

All statistical analyses were performed using EZR (Kanda 2013; available at https://www.jichi.ac.jp/saitama-sct/ Accessed 4/6/2021, Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (R Core Team 2014). The significance level was set at *p* <0.05. First, the normality of data was confirmed using the Shapiro-Wilk test. When normality was not rejected, the equality of variance of the data was confirmed using the Bartlett test. When both normality of the data and equality of variance were not rejected, the statistical differences among the averages of the data values were parametrically examined by one-way analysis of variance followed by the Tukey method. When either normality or equality of the variance was rejected, the statistical differences among the averages of the data values were non-parametrically examined by the Mann-Whitney *U* test (two groups) or Kruskal-Wallis test followed by the Steel-Dwass method (three groups or more). For the survival of juveniles, statistical significance was tested by the Fisher's exact test with significance level adjusted by the Bonferroni method.

Results

Time course changes in the darkened area ratio and body size

To determine the timing suitable for comparison among groups, we first examined the time course changes in the darkened area ratio in control and 30% loose net tank as described in materials and methods section. Although the darkened area ratio was higher in the control tank, as expected, the value continued to increase and did not saturate even at the end of the sixth month in both tanks (Fig. 1a). As shown in the figure, the 30% loose net tank showed a significantly lower darkened area ratio consistently after 4 months (Mann-Whitney *U* test, p<0.05, n=29-30). In addition, the first appearance of the significantly darkened area looked similar in the two tanks; almost no darkened area in the first month, appeared in the second month, and significantly increased in the third month. The standard length (Fig. 1b) and fish coverage ratio (Fig. 1c) increased similarly between the two tanks, with slightly higher values in the control tank. For standard length, statistical differences were detected at three time points (Mann-Whitney *U* test, $p<0.05$, $n=29\text{-}30$).

Comparison of survival, body size and body shape at the sixth month

As basic information, survival of the juveniles, body size and body shape were compared among treatments. The survival of juveniles at the sixth month were not statistically different among experimental tanks (Fisher's exact test, $p>0.05$, net-lined; $n=29$, 100% tight net; $n=29$, 100% loose net; $n=30$, 50% loose net; $n=28$, 30% loose net; $n=29$, 10% loose net; control; $n=30$).

For the standard length of all tanks, as shown in Figure 2a, there was no statistical difference at the sixth month (Kruskal-Wallis test, $p>0.05$, $n=28\text{-}30$). However, statistical differences were detected in head length ratio (100% loose net < 30% loose net, Fig. 2b) and body depth/standard length (100% tight net < net lined, 30% loose net, 10% loose net and control, Fig. 2c) (Tukey method, $p<0.05$, $n=28\text{-}30$).

Comparisons of the darkened area ratio at the sixth month

At first, to find a better way of net settings the effects of the net-setting method are examined (Figure 3 and 4). The darkened area ratios in the tank with 100% tight net (median = 14%) and 100% loose net (8%)

were significantly lower than, and approximately 1/3 and 1/5, respectively, of the control tank (46%) (Steel-Dwass method, $p<0.05$, $n=28\text{-}30$). However, these values were significantly higher, approximately three times and two times higher, respectively, than those in the net lined tank (4%) (Steel-Dwass method, $p<0.05$, $n=28\text{-}30$). Although the difference of darkened area ratio between the net-lined tank and the 100% loose net was statistically significant, the blind side appearance of 50% individuals was not much different by the naked eye (Fig. 4).

Because a strong suppression effect was observed in the tank with a loose net on the tank bottom, next, using loose net, we examined the effect of the bottom covering ratio on the darkened area ratio at the sixth month (Figs. 5 and 6). There was a clear dose-response relationship; a smaller darkened area ratio was attained in the tank with a higher bottom covering ratio.

Plasma cortisol concentration in control tank and net-lined tank at the sixth month

To examine the possible contribution of stress-cortisol axis to the hypermelanosis prevention by the net, plasma cortisol concentration was measured in the juveniles in control tank and net-lined tank. However, there was no significant difference in the plasma cortisol concentration between the control tank and net-lined tank (Fig. 7, Mann-Whitney *U* test, $p>0.05$, $n=29$ and 28, respectively).

Discussion

Although the occurrence of the hypermelanosis is known to different among the production lot of flounders, loosely installing net material covering only 50% of the bottom area significantly suppressed hypermelanosis, as low as 1/3 of the control tank (ordinary flat bottom tank), in this specific experiment. In addition, a strong negative correlation was found between bottom net coverage ratio with slack-net and darkened area ratio. Because the juvenile flounders of about 13 cm in standard length without hypermelanosis were selectively used in this experiment, there is a limitation of the applicability of results, individual values of net size and darkened area ratio, for example. But effectiveness of loosely-installed bottom net, together with the negative correlation between bottom net size and severity of hypermelanosis, were clearly indicated in the present study.

Changes in darkened area ratio and body size during experiment

This study was the first trial to examine the time course of the appearance and expansion of hypermelanosis using a net material. During the planning of the experiment, we expected that the increase in the darkened area ratio would stop in the second month, because stasis of darkening expansion was previously observed at about 60-80 days (Seikai 1991) or two months (Isojima et al. 2013b) of rearing without bottom sand. Although the experimental period was extended to six months, the longest possible period due to the restriction of experimental equipment, the darkening area expansion did not stop until the sixth month in this study. Therefore, comparisons among experimental groups were conducted using the data obtained in the sixth month. Between preceding studies and the present experiments, various factors are different: the origin of flounder juveniles, initial size, and experimental season. At present, it is unclear why the hypermelanosis expansion did not stop even after sixth months. It would be helpful to understand the control mechanism of darkening expansion by further accumulating the information.

On the other hand, the appearance timing of the darkened area were similar between the control tank and the 30% loose net tank. This result indicates that the effect of installing a net material does not delay the appearance timing, but reduces the expansion speed. In addition, in the second month, the fish coverage ratio of both tanks increased from approximately 0.3 to approximately 0.5. This is reasonable because the growth speed was similar between the two tanks. Although we cannot exclude the possible "spontaneous" appearance of hypermelanosis according to time, the appearance of darkened area is possibly "triggered" by the fish coverage ratio of over 0.3-0.4, at least in the individuals used in the present experiment.

As shown in Fig. 1, the darkened area ratio at zero months was statistically different; average, not median, was higher in the 30% loose tank. This was probably due to unexpected deviations in the random allocation of individuals. Since all the individuals at zero months had smaller values of the darkened area ratio (less than 1%), it is highly possible that this difference affected the comparison among treatments at the sixth month.

The speculated mechanism(s) of the appearance and expansion of darkened area during the experiment is 1) increase in melanin contents on non-pigmented cells on the skin, 2) increase in number of melanophores potentially differentiated from melanoblasts, 3) both of them. However, we did not examine the changes in melanin contents and number of melanophores on the blind side skin. Future studies on these points will provide fundamental understanding on the staining type hypermelanosis.

Comparison of standard length and body shape at the sixth month

Nakata et al. (2017) and Mizutani et al. (2020) reported that the standard length of flounders reared in net-lined tanks was significantly smaller than that in control tanks. From the results of the first experiment by Mizutani et al. (2020), they attributed the reduced growth to the lower food availability due to the inaccessible pellets between the net and the tank bottom, because pellets of the sinking type were offered, and some of them sink through the mesh before consumption. However, in the second experiment, although floating-type pellets were used, similar results were obtained. Therefore, Mizutani et al. (2020) suggested the presence of other possible factors for reduced growth in net-lined tanks. In addition, body depth/standard length in the net-lined tank was significantly lower than that in the control tank and was closer to that of wild-caught flounders in Mizutani et al. (2020).

In the present study, we used floating-type pellets; however, there was no significant difference in the standard length among the seven experimental tanks. In addition, regarding body depth/standard length and head length ratio, although statistical differences were observed among several tanks, we could not find any simple and logical explanations for the results. Therefore, to reduce growth and alter body shape, further research is needed to clarify the effect of net-lined tanks, starting with the confirmation of the reproducibility of the phenomena.

Comparisons of the darkened area ratio at the sixth month

In this study, the darkening suppression effect was first compared among various methods of net setting, including a net-lined tank (covering the entire inner surface of the tank), in which the prevention effect has been proven in our previous studies (Nakata et al. 2017; Mizutani et al. 2020), and a bottom net, in which the setting of the net is expected to be easier than the net-lining when applied to much larger tanks of actual aquaculture scene.

The median darkened area ratio was 4% in the net-lined tank, which was the smallest among all the experimental tanks. The value was 9% in tanks with a 100% loose net, though larger than net-lined, but significantly less than, and only approximately 1/5 of the control tank (46%). In the net-lined tank, it is clear that the bottom portion of the net has a major effect, and the vertical portion has a minor effect on the prevention of hypermelanosis. In our observation, some individuals occasionally locate themselves on the side wall of the tank, attaching themselves vertically. Therefore, for fish in the net-lined tank, there is a higher probability of being able to contact the net material.

The tension of the bottom net is another contributing factor. Although there was no significant difference, the loose net (9%) had a slightly stronger effect than tight nets (14%). Together with the smaller variance of the darkening area ratio, it is better to loosely set the bottom net, giving undulation on the net surface.

This conclusion is reasonable because the undulated surface increases the contact area between the blind side of the flounder and the net, and therefore effectively prevents hypermelanosis (Nakata et al., 2017). As shown in the photograph, although the net-lined tank seems to be the best, the 100% loose net has practically comparable prevention effect.

For the effect of the bottom covering ratio on the darkened area ratio, we initially expected the presence of a net size of “necessary and sufficient”, in a range less than 100% bottom coverage. However, as shown in Figure 5, the higher the coverage, the lower the ratio of the darkening area. Therefore, for application to actual aquaculture scenarios, it is necessary to first know the required clean level of the blind side from the aquaculture farm. Next, based on the required clean level in terms of the darkened area ratio, the suitable bottom covering rate by the net can be proposed. Although it seems difficult to decrease the darkened area ratio of the “darkest” fish, a 50% loose net may be a candidate for practical application in aquaculture (Fig.6).

Among the factors contributing for hypermelanosis suppression, tank color (Amiya et al. 2005; Yamanome et al. 2005, 2007a; Nakata et al. 2017) and undulation of the bottom (Nakata et al. 2017) are important. In this experiment, to have a similar color condition among tanks, the experimental tanks were transparent and covered with white Styrofoam sheet, and color of the net was also white. In addition, to have a similar undulation among different size nets, all loose nets were made with net materials having 10% larger size than their frames. Therefore, color condition of the tank and degree of undulation of the net are expected to be similar among experimental tanks, and the difference among the tanks are expected to be due to the difference of the net size. In this experiment, all darkened areas on the blind side were measured together without considering the location. Hypermelanosis preventing effect of net-lined tank was different depending on the location, minor on the darkened area at the base of pectoral fin and stronger on those at the base of dorsal and anal fins (Mizutani et al., 2020). As shown in figure 6, similar tendency was found in this experiment. Because the darkened areas at the base of dorsal and anal fins are larger than those at other locations, it is possible that loose net on the tank bottom exerted its hypermelanosis-preventing effect mainly through these areas.

Because the bases of dorsal and anal fins have different chance to contact with bottom substrate between flat bottom and loose net, contact stimulation is a candidate as a direct inhibitor of the hypermelanosis in net tanks, as suggested for the undulated bottom by Nakata et al. (2017). This idea could be supported by the discussion on the relationship between the net size and the fish coverage rate (Online Resource 1). Staying time of individual juveniles on the net and on the flat surface possibly affects the effectiveness of hypermelanosis prevention but was not examined in this experiment. Therefore, this point should be clarified in future. If the contact stimulation is the main inhibitor of the hypermelanosis, the prevention effectiveness of the loose net may not be limited for circular tanks used in this experiment, and probably also effective for larger rectangular concrete tanks. This point should be examined in the next step for the application of loose net in aquaculture farms at industrial scale. More detailed mechanisms of hypermelanosis prevention by loose net should be examined in future.

Blood cortisol levels of the fish reared in the control tank and net-lined tank at the sixth month

Cortisol is secreted by stress (Pickering and Pottinger 1989). Addition of cortisol in rearing water promotes hypermelanosis in spotted halibut (Yamada et al. 2011) and cortisol supplementation in diet also promotes hypermelanosis in Japanese flounder (Matsuda et al. 2018b). Therefore, in tanks with net materials on the bottom, one of the possible mechanisms for hypermelanosis prevention is the lowered cortisol level caused by possible stress reduction due to the comfortable environment. However, as shown in Figure 7, there was no significant difference in the blood cortisol concentration at the end of experiment between the most heavily darkened group (control tank) and the least darkened group (net-lined tank). This finding does not support the contribution of stress-cortisol axis to the hypermelanosis prevention by the net. We could not find information on the contribution of upstream regulatory hormone of cortisol, adrenocorticotropic hormone (ACTH), for hypermelanosis. But in the two papers on the enhancing effect of cortisol on hypermelanosis (Yamada et al. 2011; Matsuda et al. 2018b), ACTH levels are expected to be decreased because of negative feedback. Hypermelanosis enhancing effect of ACTH should be minor compared to cortisol.

It was better to collect blood samples several times during the experiment to cover the progression period of hypermelanosis, not one time at the end of experiment. However, to avoid additional stress and damage to juveniles, and not to affect the result of hypermelanosis, blood was collected only at the end of the experiment. In order to examine the presence or absence of cortisol contribution on spontaneously progressing hypermelanosis, further experiment is required.

In conclusion, this study demonstrated that loosely set bottom nets, an easier method to be applied to larger tanks, can prevent hypermelanosis with effectiveness comparable to that of a net-lined tank. In addition, covering 50% of the bottom significantly reduced the darkening area at approximately 1/3 of the ordinary flat bottom tank in this experiment. When lesser darkening is required, the use of a larger bottom net with a higher coverage ratio is recommended. For effective hypermelanosis prevention at the industrial scale, the use of a rearing tank with a loosely set bottom net is a strong alternative for introducing bottom sand for flounder aquaculture.

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Figure captions

Fig. 1 Time course changes in darkened area ratio (a), standard length (b), and fish coverage ratio (c)

Open circles, 30% loose net; closed circle, control. Data are presented as median values with 25th and 75th percentiles. Asterisks indicate statistical difference between the groups (Mann-Whitney *U* test, $p<0.05$, *n*=29 - 30)

Fig. 2 Comparisons of standard length (a), head length ratio (b), and body depth / standard length (c), at the sixth month

The upper and lower ends of the vertical bars show the maximum and minimum values, respectively. The top and bottom of the boxes show the 75 and 25% values, respectively, and the horizontal line in the box shows the median value. The different lowercase letters indicate statistical difference between the groups (Kruskal-Wallis test, $p<0.05$, $n=28$ - 30)

Fig. 3 Comparison of darkened area ratio among different net settings at sixth month

Different letters indicate statistical significance among groups (Steel-Dwass method, $p<0.05$, $n=28$ - 30). The upper and lower ends of the vertical bars show the maximum and minimum values, respectively. The top and bottom of the boxes show the 75 and 25% values, respectively, and the horizontal line in the box shows the median value

Fig. 4 Blind side photographs of flounders reared in tanks with different net settings at the sixth month

The upper photographs are individuals with 75% values, the middle with median, and the lower with 25%

values in each tank

Fig. 5 Comparison of darkened area ratio among tanks having different bottom covering ratio with net at the sixth month

Different letters indicate statistical significance between groups (Steel-Dwass method, $p<0.05$, $n=28$ - 30). The upper and lower ends of the vertical bars show the maximum and minimum values, respectively. The top and bottom of the boxes show the 75 and 25% values, respectively, and the horizontal line in the box shows the median value. Dots represent individual values

Fig. 6 Blind side photographs of flounders reared in tanks having different bottom covering ratio with net at the sixth month

The upper photographs are individuals with 75% values, the middle with median, and the lower with 25% values in each tank

Fig. 7 Comparison of plasma cortisol concentrations between control and net-lined tanks at the sixth month

There was no statistical difference (Mann-Whitney *U* test, $p=0.08$; control: $n=29$, net-lined: $n=28$). The upper and lower ends of the vertical bars show the maximum and minimum values, respectively. The top and bottom of the boxes show the 75 and 25% values, respectively, and the horizontal line in the box shows the median value

Fig. 1

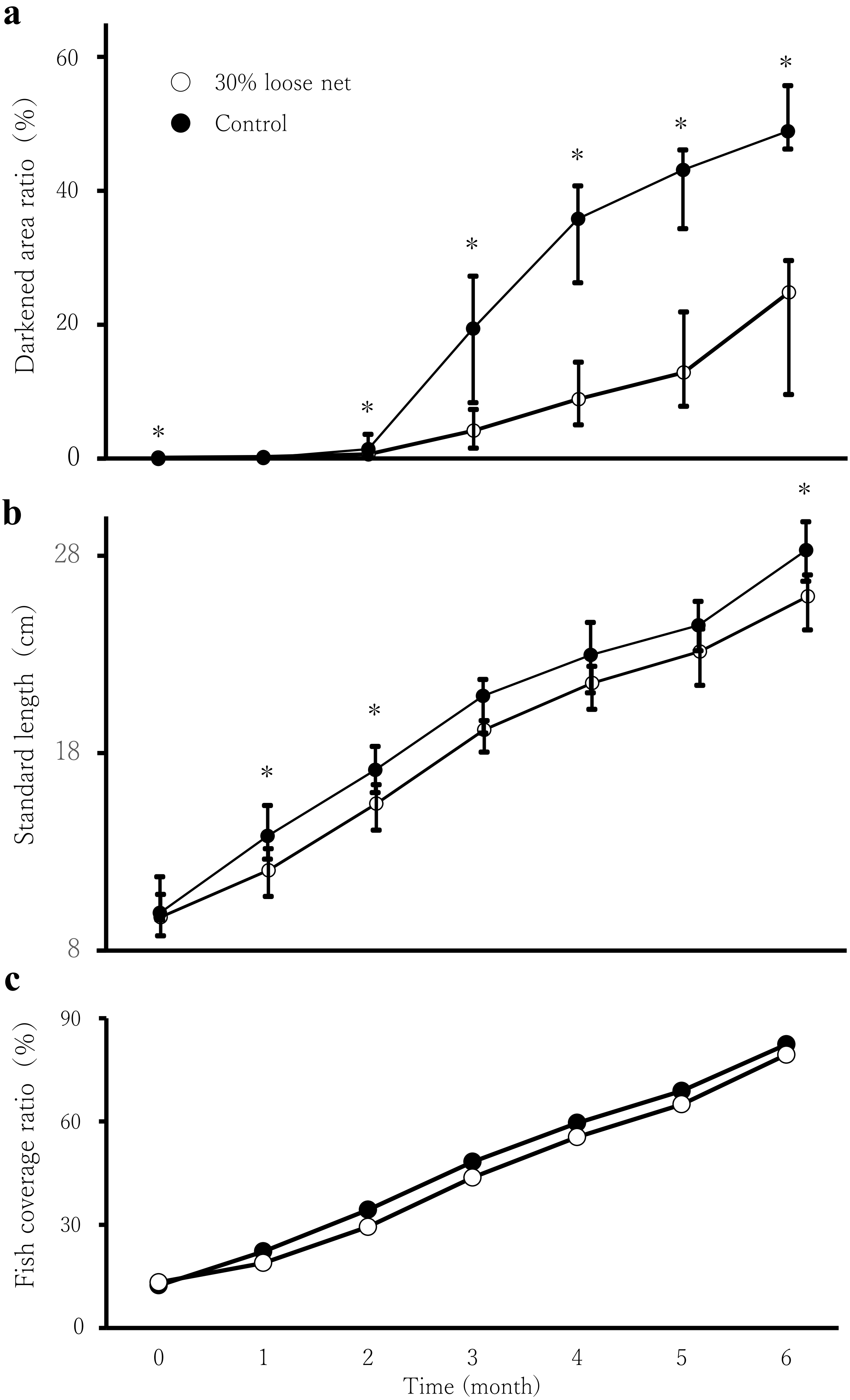

a
60
40
20
0
Darkened area ratio（%）
○ 30% loose net
● Control
*
*
*
*
*
*
b
28
18
8
Standard length（cm）
*
*
*
c
90
60
30
0
Fish coverage ratio（%）
0
1
2
3
4
5
6
Time (month)

Fig. 2

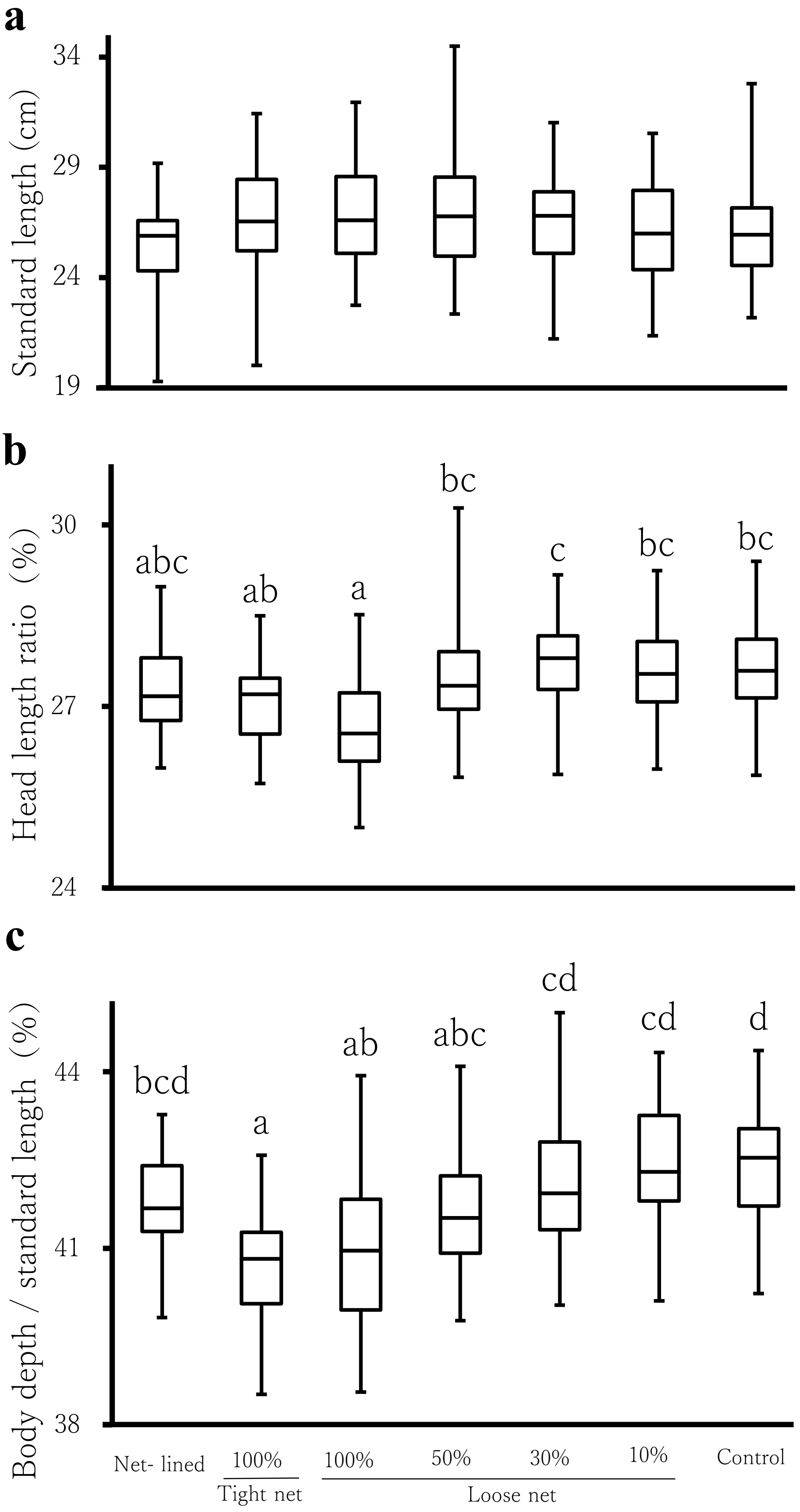

a
34
29
24
19
Standard length (cm)
b
abc
ab
a
bc
c
bc
bc
30
27
24
Head length ratio (%)
c
bcd
a
ab
abc
cd
cd
d
44
41
38
Body depth / standard length (%)
Net- lined
100%
Tight net
100%
50%
30%
10%
Loose net
Control

Fig. 3

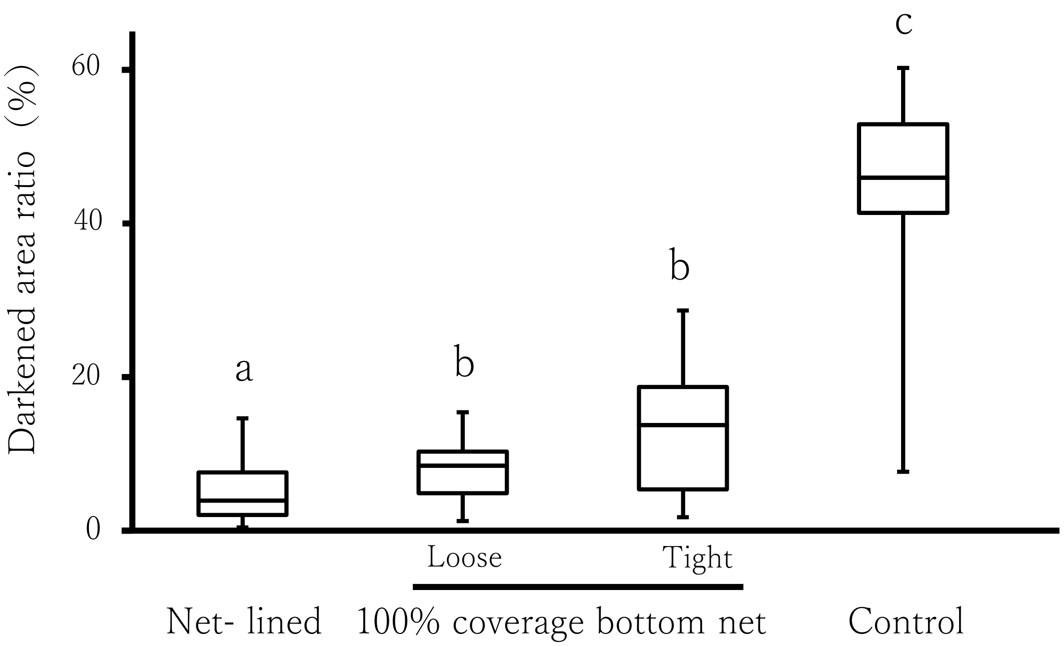

c
60
40
b
20
a
b
0
Darkened area ratio（%）
Loose
Tight
Net- lined
100% coverage bottom net
Control

Fig. 4

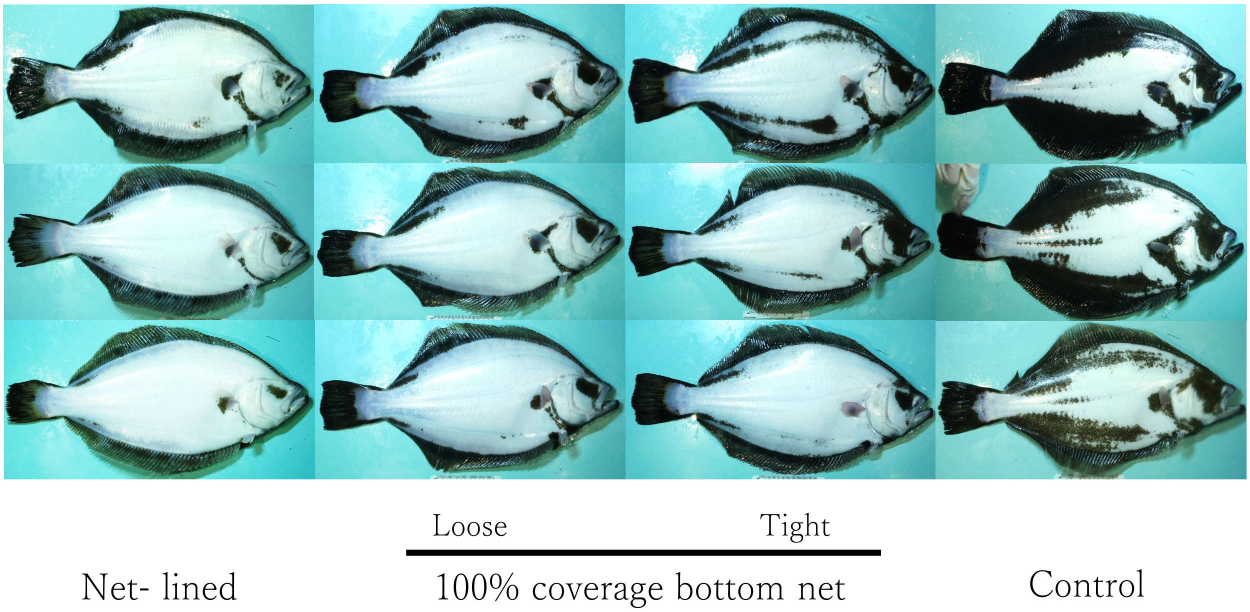
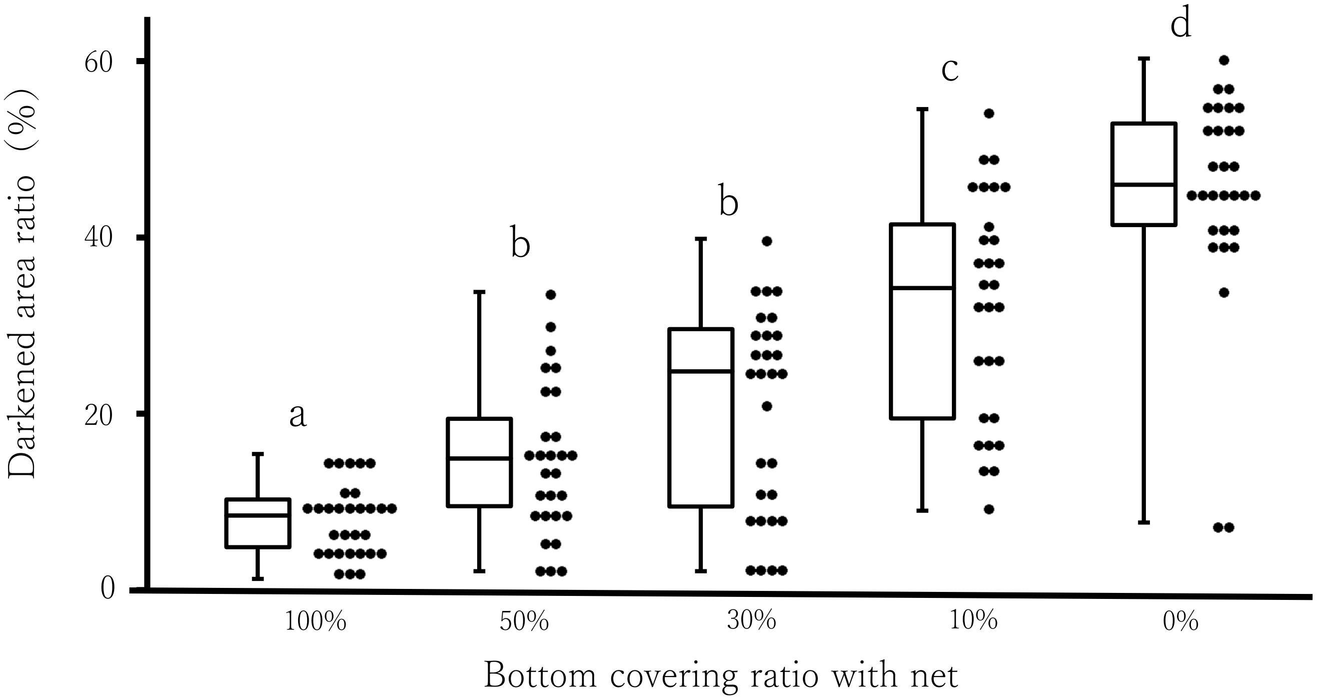
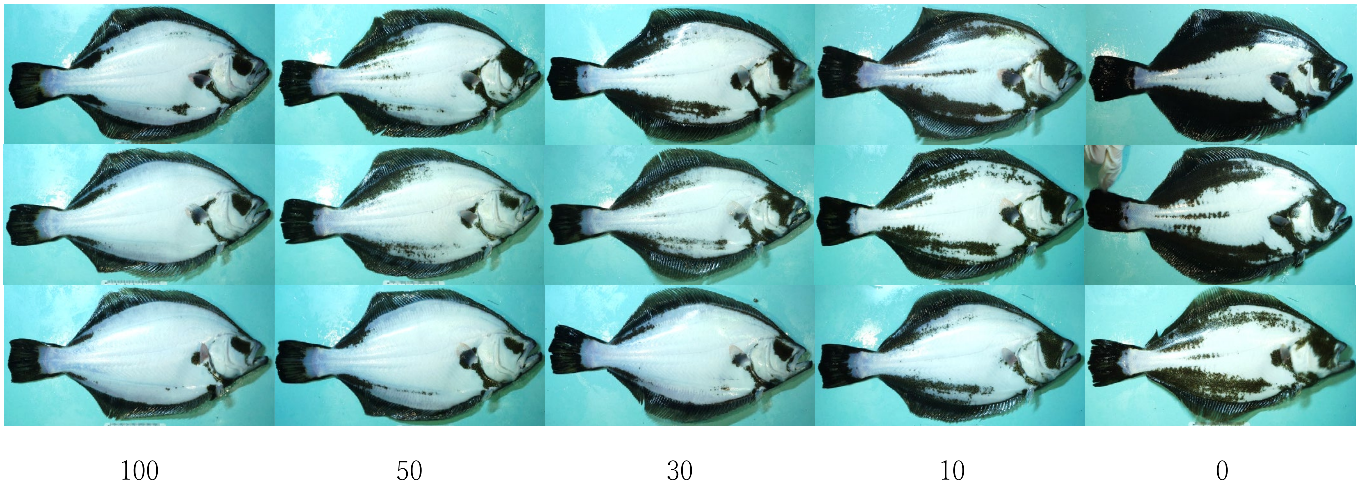


Fig. 5



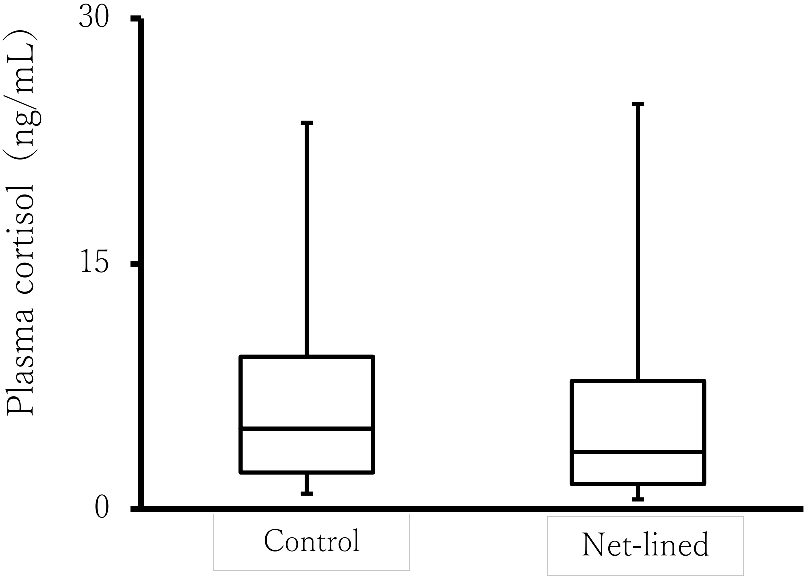
Darkened area ratio（%）
60
40
20
0
a
b
b
c
d
100%
50%
30%
10%
0%
Bottom covering ratio with net

Fig. 6



Bottom covering ratio with net（%）

Fig. 7



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The fish covering ratio of the tank bottom was approximately 80% at the sixth month, as shown in Figure 1c. Therefore, some individuals cannot lie on the net in tanks having the net size of, and the size less than, 50%. The number of individuals that could not lie on the net (therefore on flat bottom, or possibly on other individuals) in each tank was calculated from the bottom coverage ratios of the net and the fish at the sixth month, and compared with the observed number of individuals with a greater darkened area ratio than the darkest individual in the 100% loose net tank, in which all individuals theoretically can fit on the net (Table 1 in this Online Resource 1). In this study, the two numbers matched fairly well. Although we cannot exclude the possibility of coincidence, this observation may suggest the possible prediction of the effectiveness of hypermelanosis prevention from the relationship between the total fish coverage and the net size at a certain time. In addition, it may be possible that individuals with a lower darkened area ratio "prefer" to be located on the bottom net, and spend more time on it. This point should be examined in behavioral studies, with individual identification of experimental juveniles as the next step in this line of research.

Table 1. Comparison of the calculated number of individuals unable to lie on the net material due to insufficient area and the observed number of individuals having a higher darkened area ratio than the maximum value in the 100% loose net tank

group	calculated number	observed number
50% loose net	9.25	13
30% loose net	17.75	17
10% loose net	26.25	26
control	30	28