Short communication

Insights into the heritable variation of hypermelanosis in Chinese tongue sole (*Cynoglossus semilaevis*): Potential for future selective breeding

Yangzhen Li [a,b,c,*], Yuanri Hu [a], Weiwei Zheng [a], Zhongkai Cui [a,b,c], Jiayu Cheng [c], Peng Cheng [a], Tingting Zhang [a]

[a] *Shandong Key Laboratory of Marine Fisheries Biotechnology and Genetic Breeding, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China*
[b] *Laboratory for Marine Fisheries Science and Food Production Processes, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao 266237, China*
[c] *Engineering and technology center for flatfish aquaculture of Tangshan, Tangshan Weizhuo Aquaculture Co., Ltd., Tangshan 063202, China*

A B S T R A C T

Flatfish is an important group worldwide aquaculture species, and hypermelanosis on blind side is one of the prevalent and economically important problem which drops market price significantly. Knowledge of whether genetic variation exists for hypermelanosis is needed in order to determine the feasibility of including this trait into the breeding goal. Therefore, the objective of this study was to detect the magnitude of the heritable variation and estimate the heritability for blind-side hypermelanosis by using Chinese tongue sole (*Cynoglossus semilaevis*). Pigmentation on blind side was defined as three types and fitted in three models respectively, i.e., (i) continuous trait (proportion of non-pigmented area to total area of the blind side) fitted in a linear model (LM); (ii) binary trait (the melanistic scored 1 and the pure white scored 0 by eyes) fitted in cross-sectional threshold model (CTM); (iii) ordinal trait (four categories based on the extent of pigmentation) fitted in ordinal threshold model (OTM). The estimated heritability on observed scale was 0.34 (LM), and on underlying scale was 0.34 and 0.38 for CTM and OTM respectively. All the heritability estimates are associated with large standard errors (SEs) and not significantly different from zero. Anyway, these results point to the existence of genetic variation of blind-side hypermelanosis and the potential for future selective breeding. The greater than zero heritability estimates imply that the selection response is expected by selective breeding in flatfish. Further studies using bigger population data (large family numbers and sample size) are highly recommended.

1. Introduction

Flatfish species, such as tongue sole (*Cynoglossidae*), sole (*Soleidae*) and flounder (*Pleuronectidae* and *Bothidae*), have been cultured worldwide, including Europe, East Asia and North America. Flatfishes are bilaterally asymmetrical, with both eyes in the same body side (ocular side) after metamorphosis when the ocular side becomes pigmented (black brown) but non-pigmented on the blind side (pure white) (Kang et al., 2011). However, in flatfish aquaculture (i.e., under hatchery rearing conditions), staining-type hypermelanosis on blind side after metamorphosis has emerged as a serious problem. In Japanese flounder (*Paralichthys olivaceus*), the pigmentation of blind side starts from the caudal peduncle area, and spread across the entire body on the blind side of the fish with distinctive appearance (Isojima et al., 2013a; Kang and

Kim, 2012a). In Chinese tongue sole (*Cynoglossus semilaevis*), it usually develops from randomly generated small speckles and then spread across the whole body of the blind side (Fig. 1).

In flatfish aquaculture, for examples in Japanese (olive) flounder in Japan and Korea (Kang and Kim, 2012a, 2012b; Isojima et al., 2013a, 2013b; Nakata et al., 2017) and tongue sole in China, a considerably high proportion (10%–90%) of individuals expresses hypermelanosis, leading to 10%–15% lower market prices due to the fact that from the consumer's point of view, appearance traits play an important role in buying decisions. For preventing the occurrence of staining, extensive attempts have been conducted in flatfish aquaculture practices from environmental, dietary and social perspectives, for example roughing the bottom of rearing tanks with sand or other substrates, adjusting light, delaying the time of feeding artificial diet, fine-tuning stocking

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density and background color (Iwata and Kikuchi, 1998; Amiya et al., 2005; Isojima et al., 2013b; Yamanome et al., 2005, 2007a; Kang et al., 2011; Kang and Kim, 2012b, 2013; Nakata et al., 2017). Moreover, studies from the endocrinological aspects revealed the possible mechanisms of hypermelanosis (Amiya et al., 2005; Yamanome et al., 2005, 2007b; Yamada et al., 2011; Kang and Kim, 2012b; Yoshikawa et al., 2013; Matsuda et al., 2018). However, these studies and practices can only diminish or alleviate the staining of pigment to a small extent, but are not permanent solutions. According to previous studies and our observations, blind side color in flatfish is highly variable and considered as a new potential breeding goal for a better commercial value. However, to date, selective breeding programs for hypermelanosis trait are still not available in flatfish. The pigmentation on blind side distributes continuously from absent to totally stained, and thus we decided to treat blind side staining as a quantitative trait under polygenic controlled.

Chinese tongue sole is an important flatfish which is widely cultured in coastal areas of China, and hypermelanosis is now a major concern under hatchery rearing conditions. By request of flatfish farmers in China, in this study, 47 tongue sole families were used to detect the heritable variation of hypermelanosis and consider a potential of genetic improvement.

2. Materials and methods

2.1. Fish material

Brood stocks were collected from our nucleus breeding population and maintained at Tangshan Weizhuo Aquaculture Co., Ltd., and individually tagged with Passive Integrated Transponders (PIT) (Qingdao Starfish Instruments Co., Ltd.). Methods of family production and maintenance were as described in previous studies (Li et al., 2019; Li et al., 2020). Finally, 47 full-sib families from 45 sires and 27 dams were produced and used in this study. The mating design is detailed in Supplementary Table 1. Although a fully hierarchical mating design was aimed at, only 15 of 27 dams were mated to more than one sire. Offspring of each family were cultured in a separate tank. The stocking density and other rearing conditions were nearly identical in the different tanks.

2.2. Data collection and trait definition

For quantifying the pigmentation on the blind side, 30 individuals were randomly sampled from each family at 110 days post hatch. Then we photographed the blind sides of these individuals using a digital camera (Huawei Mate 30) to obtain the ratio of non-pigmented area (non-pigmented area/total blind-side area) and the ratio melanistic individuals in each family (family melanistic ratio, hypermelanic fish/total fish).

Images are composed of pixels with a fixed size when pictures are

Fig. 1. Normal body color and hypermelanosis in Chinese tongue sole. *a*: normal pigmentation on the ocular side; *b*: normal (none) pigmentation on the blind side; *c*: hypermelanosis on the blind side.

taken in the same conditions with the same resolution. In this case, number of pixels can be representative of the area of the image. Accordingly, we used Photoshop 20.0 software to obtain image information. Briefly, magnetic lasso tool was used to select all the regions and darkening regions of the blind side (excluding the fins) respectively; then the pixel values can be obtained from the corresponding histograms. The proportion of non-pigmented area to total area of blind side was treated as a continuous trait (CT, ranging from 0 to 1). Furthermore, binary and categorical traits were also analyzed. Trait definitions are as follows:

Trait 1: Continuous trait (CT), where values ranged from 0 to 1 (i.e., 0 = total area is non-pigmented, 1 = total area is pigmented);

Trait 2: Binary trait (BT), where individuals were scored by eyes, 1 for melanistic and 0 for pure white;

Trait 3: Ordinal trait (OT), which is determined based on the extent of pigmentation on the blind side. Specifically, according to the proportion of non-pigmented area (p'), it was classified into four ordered categories, where $p' = 0$ scored 1, $0 < p' \leq 0.3$ scored 2, $0.3 < p' \leq 0.6$ scored 3, $0.6 < p' \leq 1$ scored 4. The ordinal data (multinomial distribution) is a special case of categorical data, where the categories can be ordered.

2.3. Genetic analysis

Estimates of variance components for the traits were analyzed by fitting three linear mixed sire-dam models using the ASReml-R 4.1 package (Butler et al., 2017). The models were defined in matrix notation below:

a. Linear model (LM) for CT:

$$y = Xb + Z_s a_s + Z_d a_d + Wc + e$$

where, y is the vector of the observed phenotypes; b is the vector of the fixed effect (batch); a_s and a_d are the vectors of the sire and the dam additive genetic effects assumed to be $\sim N(0, A\sigma_{sd}^2)$, $\sigma_{sd}^2 = \sigma_s^2 = \sigma_d^2 = 1/4\sigma_a^2$; c is the vector of common full-sib environmental effects (including tank effect) assumed to be $\sim N(0, I\sigma_c^2)$; e is a vector of random residuals assumed to be $\sim N(0, I\sigma_e^2)$; A is the relationship matrix that describes the additive genetic relationship among all individuals (sires and dams) included in a_s and a_d, and I is an identity matrix; X, Z_s, Z_d and W are the corresponding design matrices. σ_a^2, σ_s^2, σ_d^2, σ_{sd}^2, σ_c^2 and σ_e^2 is additive genetic variance, additive genetic sire variance, additive genetic dam variance, additive genetic sire-dam variance, common full-sib environmental variance and residual variance respectively.

b. Cross-sectional threshold (logit) model (CTM) for BT, and
c. Ordinal threshold (logit) model (OTM) for OT:

$$Pr(y_i = m) = \frac{exp(\lambda_i)}{1 + exp(\lambda_i)}$$

where, $\lambda = \sum_{p=0}^{N} \beta_p Z_p(m) + Z_s a_s + Z_d a_d + W_c$, $m = 1$ or (1,2,3) for CTM and OTM respectively; $N = 1$ or 3 for CTM and OTH respectively; β_p is the pth order fixed regression coefficient; $Z_p(m)$ is a pth order orthogonal polynomial of m; and the other parameters are as described above. The parameter λ_i is the expected liability of observation i, given the fixed and random effects on the underlying scale. The residual variance was assumed to be $\pi^2/3$ for CTM and OTM. For all the models, heritability (h^2) was calculated as $h^2 = 4\sigma_{sd}^2/(2\sigma_{sd}^2 + \sigma_c^2 + \sigma_e^2)$. The proportion of common full-sib environmental effect was calculated as $c^2 = \sigma_c^2/(2\sigma_{sd}^2 + \sigma_c^2 + \sigma_e^2)$. The estimated breeding values (EBVs) of parents were

estimated based on OTM using OT. The family EBV was calculated as $1/2(EBV_{Sire} + EBV_{Dam})$.

The potential selection response to one generation (R) for hypermelanosis trait was estimated as $R = i \times \sigma_P \times h^2$, predicted genetic gain in % was calculated as $\Delta G (\%) = R / \overline{X}$, where i is the selection intensity, σ_P is standard deviation, h^2 is heritability (observed scale), $\overline{X}$ is average of phenotype (Gjedrem and Olesen, 2005; Falconer and Mackay, 1996). Here, h^2 should be on the observed scale, i.e., σ_P and h^2 was estimated from LM. So, the continuous trait (CT) was used to estimated selection response.

3. Results and discussions

3.1. Descriptive statistics

Descriptive statistics were summarized in Table 1, and the average family area ratio of non-pigmentation on the blind side and family estimated breeding values (EBVs) are shown in Fig. 2. At approximate 110-day age, hypermelanosis on the blind side shows large variations, with a range of 0–97.5% darkening area individually and a range of 3.3%–100% melanistic individual ratio among families. The histograms of the frequency distributions of area ratio of pigmentation on the blind side are shown in Fig. 3. The highest frequency is non-pigmented individuals (31.1%), followed by the pigmented area ratio of 0.7–0.8 interval (12.6%), and the pigmented area ratio of 0.9–1 interval shows the lowest frequency (1.6%). In this study, the data was limited, however, when large enough, it is also not expected to be normally distributed for the data due to the nature of the trait, regardless of which trait definition was used. In future selective breeding for blind-side pigmentation trait in flatfish, adequate and sufficient data (large family numbers and sample size) are recommended. Measurements at different growth stages or in different rearing environments would likely be beneficial for selective breeding, as there is ample evidence that hypermelanosis continuously develops in flatfish aquaculture environment. Once formed, it is difficult to reverse. In other words, the larger the fish size, the worse. According to our surveys in lots of flatfish (olive flounder, tongue sole and turbot, *Scophthalmus maximus*) farms across years in China, the percentages of blind-side melanistic individuals at harvest ranging from approximate 10% to 90%. The influencing factors are still unknown or debatable.

3.2. Genetic analysis

Variance components, heritabilities and proportion of common full-sib environmental effects of pigmentation traits in different models are shown in Table 2.

The heritabilities (h^2) for pigmentation traits were 0.34 ± 0.30, 0.34 ± 0.35 and 0.38 ± 0.32 for LM, CTM and OTM respectively. All the estimates are associated with large standard errors (SEs) and not significantly different from zero. There was no certainty that h^2 was greater than 0. It is widely acknowledged that heritability is probably the most important parameter when developing a breeding program, as

Table 1
Descriptive statistics of dataset.

Item	Value
Family no.	47
Sire no.	45
Dam no.	27
Fish no.	1410
Sample size of each family	30
Range of proportion of darkening area (mean), %	0–97.5 (38.0)
Range of family melanistic ratio, %	3.3–100
Average length, cm	15.1
Average weight, g	11.4
Age, days	110
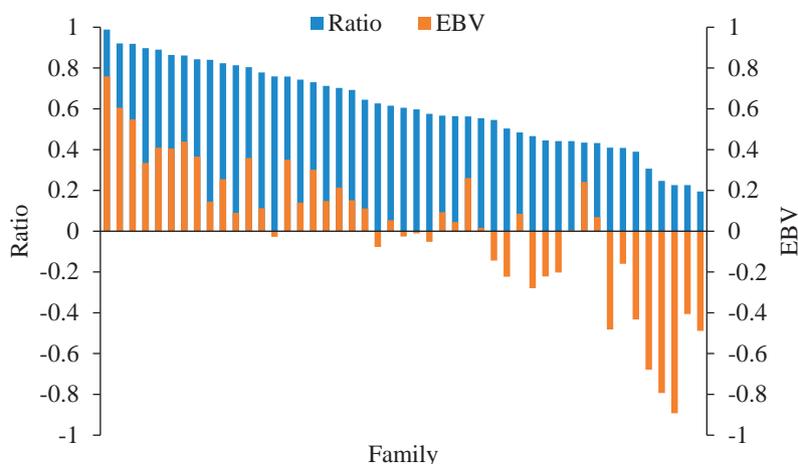
Fig. 2. The average family area ratio of non-pigmentation on the blind side and family estimated breeding values (EBVs, estimated based on ordinal threshold model by using ordinal trait definition) of tongue sole at 110-day age.
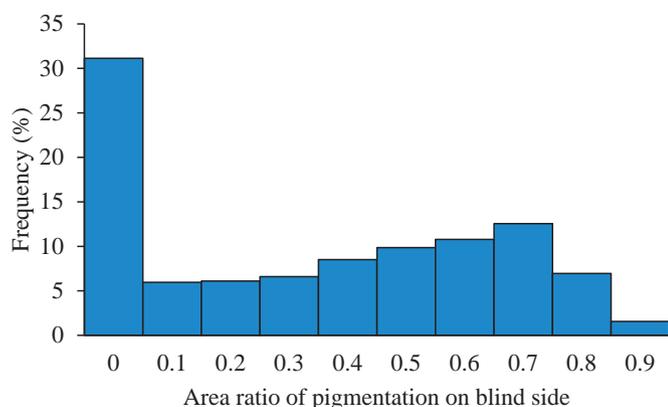
Fig. 3. The frequency of area ratio of pigmentation on the blind side among all individuals at 110-day age in tongue sole.

Table 2

Estimates of additive genetic sire-dam variance (σ_{sd}^2), common full-sib environmental variance (σ_c^2), residual variance (σ_e^2), heritabilities ($h^2 \pm$ SE) and proportion of common full-sib environmental effects ($c^2 \pm$ SE) of pigmentation traits in three models; SE: standard error; LM: Linear model; CTM: cross-sectional threshold (logit) model; OTM: ordinal threshold (logit) model.

Model	σ_{sd}^2	σ_c^2	σ_e^2	$h^2 \pm$ SE	$c^2 \pm$ SE
LM	0.008	0.024	0.056	0.34 ± 0.30	0.25 ± 0.14
CTM	0.523	1.758	3.290	0.34 ± 0.35	0.29 ± 0.16
OTM	0.561	1.426	3.290	0.38 ± 0.32	0.24 ± 0.15

they are used to estimate breeding values and to predict response to selection (Gjedrem and Baranski, 2009). High heritability implies that a large portion of the trait variation is heritable. Large SEs of heritabilities should mainly be ascribed to the limited observations (i.e., small sample size and family no.). Besides, small samples in this study also increase the stochasticity of the estimates and increase the probability to observe estimates that are larger or smaller than the true value.

The fractions of variance explained by common full-sib environmental effect (c^2) were high in all the models, accounting for 24%–29% of total phenotypic, which is clearly significantly different from zero. Can this be explained that the hypermelanosis on the blind side is influenced by environmental factors (existence of tank effect)? In the future selective breeding practices, for minimizing environmental effects, the separate rearing period should be made as short as possible, or

measuring pigmentation trait during the grow-out phases after tagging. Alternatively, genetic markers can be used for parental assignment which allows communally rearing from eggs and eliminates common environmental effects (Gjedrem and Baranski, 2009). In this study, the limited number of half sibs make it difficult to separate the environmental tank effect from the genetic effects of sib groups. Further studies with enlarged sample size and family numbers as well as a better experimental (e.g., fully hierarchical mating design with all dams mated to more than one sire) are needed to validate these reserved results.

In future selective breeding programs in Chinese tongue sole, we assume the selection proportion (*p*) of broodstocks is 30% (as indicated in Fig. 3, the frequency of blind-side non-pigmentation was approximate 30%), then the selection intensity (*i*) is 1.16. The potential selection response (*R*) for non-pigmentation trait was estimated as $R = i \times \sigma_P \times h^2$ $= 1.16 \times 0.31 \times 0.34 = 0.122$, predicted genetic gain was calculated as ΔG (%) $= R / \overline{X} = 0.122/ 0.620 = 19.7\%$, i.e., the pigmentation trait is expected to achieve an improvement of 19.7% units in next generation in Chinese tongue sole with $i = 1.16$.

In future selective breeding programs, if mass selection is performed, phenotypic variance will decrease as a larger proportion of the individuals will exhibit total depigmentation, thereby limiting the possible selection intensity. Family-based or genomic selection can help keep selection intensity to a higher level, although in the end they will also have limited efficiency when non-pigmentation reaches more than 80%. Selection response will probably slow down within several generations. Eradication of blind side hypermelanosis may require combination of selective breeding with rearing under environmental conditions that favor hypermelanosis in order to still be able to differentiate individuals and families with low hypermelanosis EBVs. Besides, how pigmentation trait possibly correlated with growth rate can be included in a breeding program, and the selection response obtained for both traits could be interesting.

4. Conclusion

The present study may indicate the existence of genetic variation of blind-side hypermelanosis in Chinese tongue sole. Moderate heritability estimates (though with large SEs and not significantly different from zero) of pigmentation traits imply response to selection is expected. Anyway, these encouraging results open up possibilities to alleviate hypermelanosis permanently by means of selective breeding for flatfish under hatchery rearing conditions. However, some caveats need to be mentioned due to the poor design and limited data. Further studies using bigger population data (more fish and families) are highly recommended. As we do not have a clear view on the environmental factors

influencing blind side hypermelanosis in Chinese tongue sole, it would also be valuable to consider data from multi-site experiments in order to study possible genotype by environment interactions, which may impact on the design of efficient breeding programs.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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