

Eye location, cranial asymmetry, and swimming behavior of different variants of *Solea senegalensis*

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ABSTRACT

Many flatfish species, such as *Scophthalmus maximus*, *Paralichthys olivaceus*, *Solea senegalensis*, are extensively cultured worldwide. In flatfishes, eye migration varies among different species and even within the same species. The genetic mechanism underlying eye migration is still unclear, and it is the key to improve metamorphosis in flatfish aquaculture and understand flatfish evolution. In this study, the high frequency of eye location variants in an artificial *S. senegalensis* population allowed us to investigate the eye preference during migration; completeness of the eye migration; and other related left-right asymmetric characteristics, such as asymmetrical skull remodeling and lying down on one side. The different frequencies of eye preference during migration, completeness of the eye migration, and settling side suggest that they are determined by different genotypes and imply independent evolutionary events during flatfish evolution. We found that the swimming time may be related to the completeness of eye migration, and further the migration of the eye, the more time the fish spent at the sea bottom. The left-right asymmetric degrees of not only the frontal bones and lateral ethmoids but also the jaw bone, including the premaxillary, maxillary, and dentary bones, depend on the completeness of eye migration. Finally, we proposed a hypothesis for the asymmetric origin of flatfish that provides reasonable explanations for the selective advantages of primitive flatfish species.

1. Introduction

Flatfishes (Pleuronectiformes) are generally deep-bodied and laterally compressed fishes, and they represent a diverse order of marine and estuarine fishes. Around 716 flatfish species can be found worldwide, and they comprise a significant proportion of the groundfish catch (Gibson, 2005). Flatfishes are among the most popular and valuable fishes used for human consumption; therefore, they are attractive candidates for both stock enhancement and intensive cultivation. For example, the turbot (*Scophthalmus maximus*), olive flounder (*Paralichthys olivaceus*), and Senegalese sole (*Solea senegalensis*) have become important aquaculture species worldwide.

During the aquaculture of flatfishes, inherent variations in growth and settling behavior during metamorphosis may lead to cannibalism and necessitate increased labor due to grading (Gavlik, Albino, & Specker, 2002; Gavlik & Specker, 2004). To produce a larger and more uniformly sized cohort of juvenile flatfishes, methods such as salinity

change and thyroid hormone manipulation have been used to synchronize the settling behavior, growth, and development of the summer flounder, *Paralichthys dentatus*, and spotted halibut, *Verasper variegatus* (Gavlik et al., 2002; Gavlik & Specker, 2004; Tagawa & Aritaki, 2005). Understanding the regulation mechanism of flatfish metamorphosis is the key for synchronizing these metamorphic events in artificial populations.

All flatfishes begin their life as pelagic, bilaterally symmetrical fishes. However, during larval development, flatfishes undergo a spectacular ontogenetic metamorphosis where one eye migrates from one side of the head to the other (Brewster, 1987). After eye migration, flatfishes settle out of the water column and assume a benthic lifestyle. Recently, studies on the metamorphosis of flatfishes have reported information on the regulation of the signal pathway for eye migration, frontal bone deformation, dorsal fin elongation, and changes in body depth during metamorphosis and mechanism underlying the establishment of left/right asymmetric pigmentation after metamorphosis in

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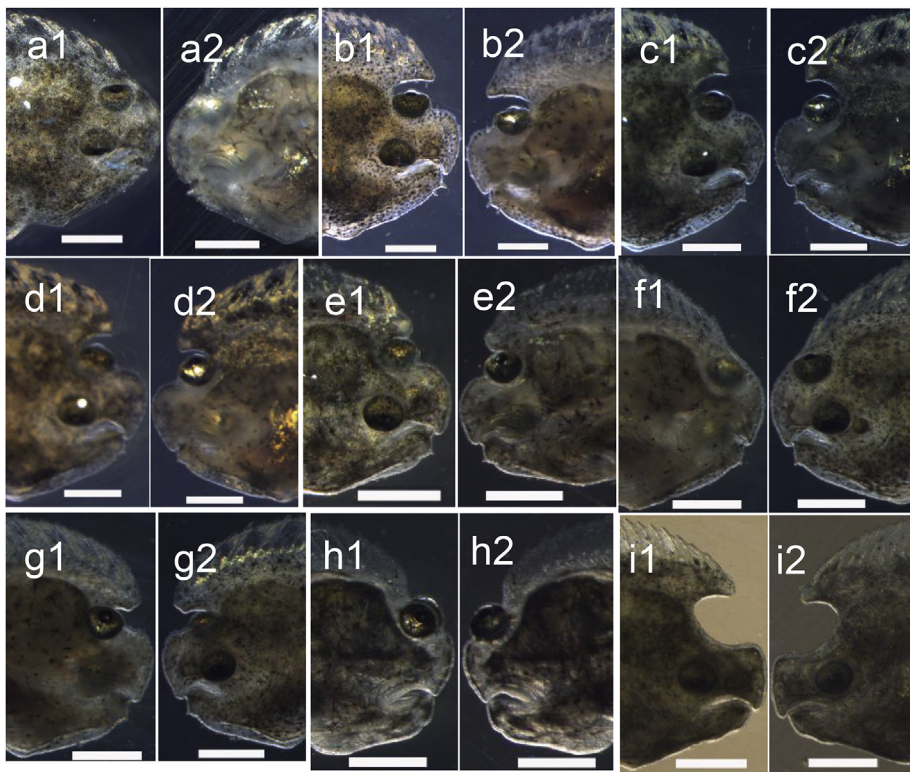


Fig. 1. Variants with different eye locations in the artificial *S. senegalensis* population. a, Normal juvenile with a left eye that has reached the target location. (a1), Right view; (a2), Left view. b, Type 1-A variant in which only the left eye has experienced migration and just passed over the dorsal midline. (b1), Right view; (b2), Left view. c, Type 1-B variant in which only the left eye has experienced migration and finally located on the dorsal midline. (c1), Right view; (c2), Left view. d, Type 1-C variant in which only the left eye has experienced migration and not passed the midline. (d1), Right view; (d2), Left view. e, Type 1-C' variant in which only the left eye has experienced migration and not passed the midline, but the body settles on the right side. (e1), Right view; (e2), Left view. f, Type 2-A variant (reversed eye variant) in which only the right eye has experienced migration and passed over the dorsal midline and finally located very close to the target site. (f1), Right view; (f2), Left view. g, Type 2-B variant (reversed eye variant) in which only the right eye has experienced migration and not passed the midline. (g1), Right view; (g2), Left view. h, Type 3 variant in which both eyes have experienced migration and finally located close to the dorsal midline. (h1), Right view; (h2), Left view. i, Type 4 variant in which both eyes have not experienced migration. (i1), Right view; (i2), Left view. Bars, 1.0 mm.

flatfish (Bao et al., 2011; Campinho et al., 2018; Chen, Liu, Yao, Gao, & Bao, 2017; Li, Zheng, Bao, & Berendzen, 2013; Shao et al., 2017; Sun et al., 2015; Wei, Chen, Chen, & Bao, 2017; Xu, Ke, Xia, He, & Bao, 2016). However, the genetic basis for eye location and settling side in flatfishes is still unclear. In this study, the relatively high diversity of eye location and settling side in an artificial population of *S. senegalensis* helped us to understand the relationships among eye preference during migration, completeness of eye migration, and settling side.

2. Materials and methods

2.1. Fish maintenance

Artificially fertilized eggs were collected from farm-raised Senegalese soles (*S. senegalensis*) at Dahua Fisheries Farm (Laizhou, Shandong, China). Hatching and larval rearing occurred at 20 °C in saltwater with 30 salinity. After 3 days after hatching (DAH), the larvae started to feed on the rotifer *Brachionus plicatilis*. The larvae at 9 DAH were transferred to our lab at Shanghai Ocean University (Shanghai, China) and maintained in saltwater at 20 °C and 30 salinity. The larvae were fed with live brine shrimp (*Artemia*) nauplii until 32 DAH. All individuals (total 1396) at 24 DAH had completed metamorphosis and developed into juveniles with a bottom-dwelling lifestyle.

2.2. Classification of *S. senegalensis* individuals

Normal *S. senegalensis* is typically dextral (right-sided), and only the left eye shows migration (Dinis, Ribeiro, Soares, & Sarasquete, 1999). In the present study, four types of *S. senegalensis* were recorded on the basis of which eye migrated, final position of the movable eye, and settling side: Type 1: left eye migrated, including Type 1-A (left eye moved just over the dorsal midline, and body laid down on the left side), Type 1-B (left eye stopped at the dorsal midline, and body laid down on the left side), Type 1-C (left eye stopped below the dorsal midline, and body laid down on the left side), and Type 1-C' (left eye stopped below the dorsal midline, and body laid down on the right

side). Type 2: reversed eye variants with right eye migration, including Type 2-A (right eye migrated over the dorsal midline, very close to the target location, and body laid down on the right side) and Type 2-B (right eye stayed below the dorsal midline, and body laid down on the right side). Type 3: both eyes migrated and close to the dorsal midline, and body laid down on the left side. Type 4: both eyes experienced no migration, and body laid down on the left side. The frequencies of the various asymmetric phenotypes were determined by counting the individuals at the post-metamorphosis stage (28 DAH).

2.3. Swimming behavior

The post-metamorphic juveniles of *S. senegalensis* were maintained in glass tanks (40 cm × 30 cm × 25 cm) to observe their swimming behavior with a digital camera (SONY). The videos were edited using the software Avidemux 2.5.

2.4. Skeletal staining and photography

The post-metamorphic juveniles of *S. senegalensis* were fixed in 4% paraformaldehyde for at least 24 h. Alcian blue 8GX (A3157; Amresco) was used for cartilage staining, and alizarin red S (A5533; Amresco) for bone staining. The staining procedures were performed as described previously (Gavaia, Sarasquete, & Cancela, 1999). The samples were observed under a dissecting microscope (SZX7; Olympus) and documented using the Image-Pro Plus 6.0 software (Media Cybernetics). The scale on each photograph was autogenerated by Image-Pro Plus 6.0 software on the basis of the magnification.

3. Results

3.1. Frequencies of the asymmetric phenotypes in the artificial *S. senegalensis* population

Representative individuals with different eye location and settling side in the artificial *S. senegalensis* population are shown in Fig. 1. The

Table 1The frequency of various variants in the artificial *S. senegalensis* population.

Type	Frequency	Settling side	Eye preference during migration	Final location of the migrated eye
Wild	926.22‰ (1293)	left	left	Target location
Type 1-A	51.58‰ (72)	left	left	Just over the dorsal midline
Type 1-B		left	left	Dorsal midline
Type 1-C	9.31‰ (13)	left	left	Below the dorsal midline
Type 1-C'	0.72‰ (1)	right	left	Below the dorsal midline
Type 2-A	7.16‰ (10)	right	right	Target location
Type 2-B	0.72‰ (1)	right	right	Below the dorsal midline
Type 3	1.43‰ (2)	left	Left and Right	Below the dorsal midline
Type 4	2.87‰ (4)	left	none	Original site

frequencies of each phenotype in a total of 1396 juveniles are listed in Table 1. The frequency of settling side different from that of the normal juvenile was 8.6‰, including Type 2-A, Type 2-B and Type 1-C' variants, whereas the frequency of specific eye migration different from that of the normal juvenile was 9.3‰, including Type 2 and Type 3 variants. The variety in the final location of the migrated eye can reflect the completeness of eye migration. The phenotype of incomplete eye migration was 66.63‰, after excluding the Type 2-A variants.

3.2. Cranial asymmetry in the different eye variants

In the *S. senegalensis* wild type, some pair of bones located in the front part of the head, such as the frontal bones and lateral ethmoid, premaxillary, maxillary, dentary, lachrymal, entopterygoid, and ectopterygoid bones, are distributed with a left-right asymmetry (Fig. 2). In Type 2-A (reverse eye variants), these pairs of bones also present left-right asymmetry, but as a mirror image of the wild type (Fig. 2).

The left-right asymmetric degree of some pairs of bones seems to be related to the migration distance of the eye. Some pairs of bones

presented lesser left-right asymmetry than those of the wild type. In Type 1-A and Type 1-B variants with one eye migrated on or over the dorsal midline, bones such as the premaxillary, maxillary, and dentary presented a left-right asymmetry that was very similar to that of the wild type (Fig. 2). In Types 1-C and 4, whose eyes do not move or reach the dorsal midline, these three pairs of bones present almost left-right symmetry (Fig. 2). Although the pair of entopterygoids present left-right asymmetry in the wild type, it should be noted that the pair of bones seem to be no business with eye asymmetry, because they present a similar degree of left-right asymmetry in variants with different types of eye location (Types 1-A, 1-B, 1-C, and 4) (Fig. 2).

3.3. Different variants with different activities in the artificial *S. senegalensis* population

The behaviors of the different variants seem to be related to the migration distance of the eye. The normal juveniles spent most of their time (94.1%) at the bottom on their eyeless body side (left side; Table 2). The Type 1 variants (Type 1-A, Type 1-B, and Type 1-C), with

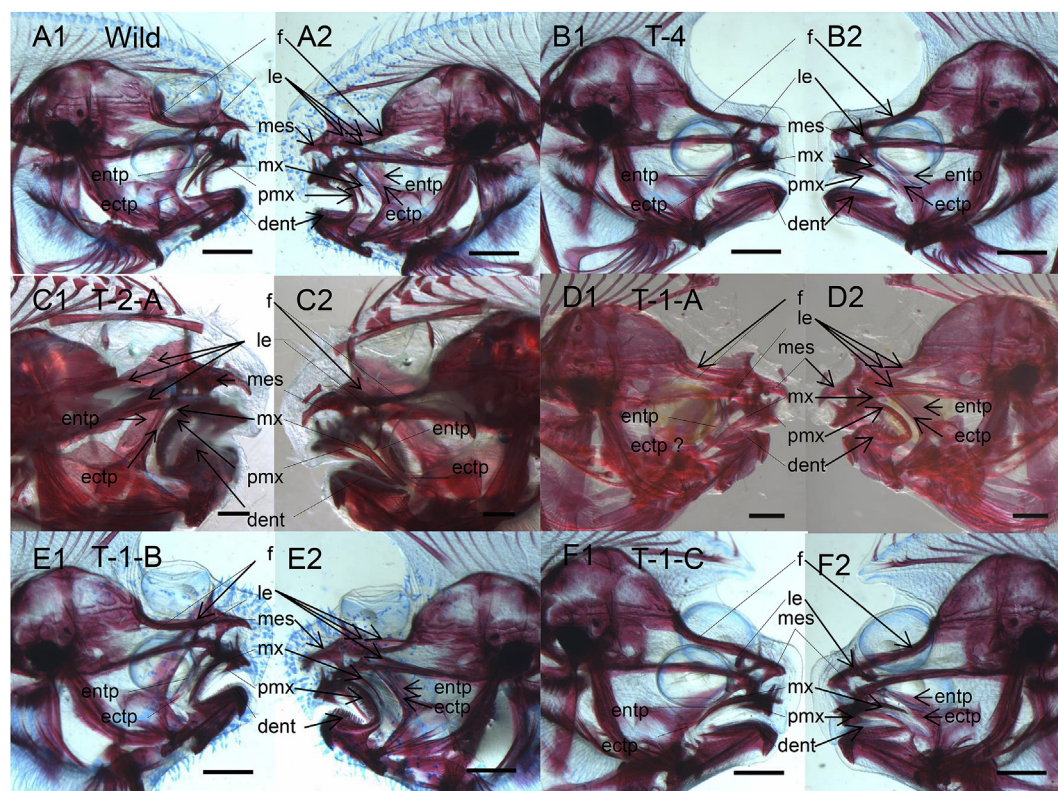


Fig. 2. Skull staining for different variants with different eye locations. A, Wild type, (A1) Right view, (A2) Left view; B, Type 4, (B1) Right view, (B2) Left view; C, Type 2-A, (C1) Right view, (C2) Left view; D, Type 1-A, (D1) Right view, (D2) Left view; E, Type 1-B, (E1) Right view, (E2) Left view; F, Type 1-C, (F1) Right view, (F2) Left view. Bars, 1.0 mm; f, frontal; le, lateral ethmoid; mes, mesethmoid; pmx, premaxillary; mx, maxillary; dent, dentary; entp, entopterygoid; ectp, ectopterygoid. Lines represent pairs of bones on the ocular side, and arrows represent pairs of bones on the blind side. Calcified bone is red, and cartilage is blue.

Table 2

Different variants with different activities in the artificial *S. senegalensis* population.

Type		Number of individuals	Total video record time (in seconds)	Percent of swimming time in water column	Percent of rest time at the bottom
Normal		8	511	5.9%	94.1%
Type 1 variant	Type 1-A	2	269	2.6%	97.4%
	Type 1-B	4	203	56.7%	43.3%
	Type 1-C	5	235	28.9%	71.1%
	total	11	707	26.9%	73.1%
Type 2 variant	Type 2-A	11	537	18.1%	81.9%
	Type 2-B	1	35	77.1%	22.9%
	total	12	572	21.7%	78.3%
Type 3 variant			85	84.7%	15.3%
Type 4 variant			51	94.1%	5.9%

less left-eye migration, lay on the left side, swam upwards more often, and spent more time (26.9%) in water column (Table 2). The Type 2 variants (Type 2-A and Type 2-B), with less right-eye migration, lay on the right side and swam upwards more often (21.7%) in water column (Table 2). Type 1-A, with eye migrated over the dorsal midline, spent 97.4% of the time at the bottom, whereas Type 1-C, whose eye did not reach the dorsal midline, spent less time (71.1%) at the bottom (Table 2). The Type 3 variants, with both eyes close to the dorsal midline, spent most of the time (84.7%) in water column and only 15.3% of the time at the bottom. The Type 4 variants, with almost no eye migration, spent 94.1% of the time swimming (Table 2).

4. Discussion

In the artificial *S. senegalensis* population, the frequency of variants with incomplete eye migration was quite different from that of the variants with an eye preference for migration or different settling sides. Moreover, a Type 1-C' individual with mismatched eye migration and body settling directions showed that settling side and eye preference for migration are not determined by the same genotype, and even the frequency of variants with an eye preference for migration or different settling side was close. It is clear that these three phenotypes are determined by different genotypes and could be the result of independent evolutionary events during flatfish evolution. Schreiber (2006) suggested that tilting and settling behaviors are independent of asymmetric eye position on the basis of southern flounder variants produced by disrupting the thyroid hormone before or during metamorphosis. In our previous study (Bao et al., 2011), we proved that settling side is independent of eye migration in eye-symmetric *S. senegalensis*, *Cynoglossus semilaevis*, and *P. olivaceus* juveniles with a bottom-dwelling lifestyle; the specimens were produced artificially by inhibiting cell proliferation in the suborbital area on the blind side.

Swimming behavior can be affected by incomplete eye migration. The variants with incomplete eye migration, especially with eyes on both sides, might not live well in the wild because the swimming behavior of *S. senegalensis* was observed to be impaired by incomplete eye migration. Such specimens spent more time in water column than the normal individuals, suggesting that they would not adapt well to a benthic lifestyle. In contrast, the swimming behavior of reversed eye variants that showed complete eye migration (Type 2-A) was similar to that of normal individuals (that spend most of their time at the bottom), indicating these variants could adapt to a benthic lifestyle like the normal individuals.

Besides eye migration and settling behaviors, cranial asymmetry during flatfish metamorphosis is a striking asymmetric development in the vertebrates. Cranial asymmetry involves several bones, such as the frontal bone, lateral ethmoid, and pseudomesial bar (in some species)

(Brewster, 1987; Okada, Takagi, Seikai, Tanaka, & Tagawa, 2001; Wagemans, Focant, & Vandewalle, 1998). In the Japanese flounder, distortion of the frontal bone and left-right asymmetric enlargement of the lateral ethmoids during metamorphosis depend on eye migration (Bao et al., 2011; Sun et al., 2015). This study has also shown that, in *S. senegalensis*, left-right asymmetry of the frontal bone and lateral ethmoids is related to the migration distance of the eye and left-right asymmetric degree of the jaw, including the premaxillary, maxillary, and dentary bones, depends on eye migration.

The findings of this study suggest that the incompleteness of eye migration and settling side may play important roles in the evolution of flatfishes because the adaptive ability for a benthic ground lifestyle is mainly related to the completion of eye migration and flatfishes with the wrong settling side will not survive in the wild. Therefore, here we propose a hypothesis on the asymmetric origin of flatfish evolution.

It is unclear which living species is the closest to the ancestor of flatfishes; Perciformes fish species with compressiform bodies are supposed to be close to the ancestor of flatfishes (Dawkins, 1986; Zimmer, 2008). Upper binocular vision would be a very useful trait for a bottom-dwelling species (Janvier, 2008). A new species with asymmetric eyes, even if the eyes are still on both sides, could obtain upper binocular vision (Fig. 3), like the fossil species *Heteronectes* and *Amphistium* with a possibly equal frequency of dextral and sinistral individuals (Friedman, 2008). Obviously, the species with asymmetric eyes would have a more selective advantage than its ancestor with symmetric eyes. Regardless of which eye migrated, if the distance of eye migration is the same, the selective advantage should be the same. Therefore, the right or left eye could have equal opportunity to migrate in the same species. A species with one eye located at the dorsal midline, such as the spiny turbot (genus *Psettodes*; the most primitive living group that belongs to Pleuronectiformes) often lying on one side on sand and mud bottoms (Hussain, 1990), acquires wider upper binocular vision and a more selective advantage than *Heteronectes* or *Amphistium* with eyes on both sides (Fig. 3). *Psettodes* species spend most of their time lying at the bottom to maximize upper binocular vision and maintain front

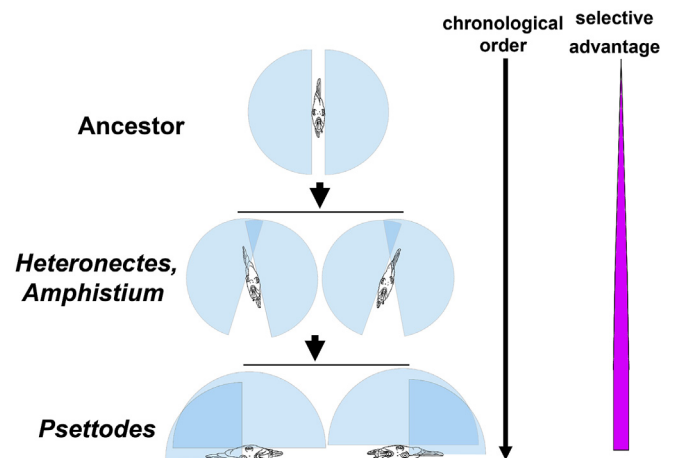


Fig. 3. Scheme for the evolutionary origin of flatfish asymmetry. The ancestor of flatfishes from Perciformes is supposed to be compressiform with symmetric eyes. The upper vision field in each eye (light blue in the figure) is supposed to be 180°. *Heteronectes* and *Amphistium* species with asymmetric eyes that were still on both sides could have evolved from the ancestor because they obtained upper binocular vision (blue) and, accordingly, more selective advantage than their ancestor. The size of the pink area on the right side of the figure represents the selective advantage. *Heteronectes* and *Amphistium* could adjust the front binocular vision at the horizontal level via tilted swimming. The eye on the left or right side was upper lined with equal frequency in the same species, for example, *Heteronectes*, *Amphistium*, and *Psettodes* species. One eye located at the dorsal midline would help *Psettodes* species obtain a much wider binocular vision and, accordingly, selective advantage.

binocular vision at the horizontal level. In *Psettodes erumei*, the frequency of both eyes on the left or right side is similar in wild populations (Hussain, 1990), suggesting that the preference and distance of eye migration are attributable to different genes.

Ethical standards

The experiments comply with the current laws of China.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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References

- Bao, B., Ke, Z., Xing, J., Peatman, E., Liu, Z., Xie, C., et al. (2011). Proliferating cells in suborbital tissue drive eye migration in flatfish. *Developmental Biology*, 351(1), 200–207.
- Brewster, B. (1987). Eye migration and cranial development during flatfish metamorphosis: A reappraisal (teleostei: Pleuronectiformes). *Journal of Fish Biology*, 31, 805–834.
- Campinho, M. A., Silva, N., Martins, G. G., Anjos, L., Florindo, C., Roman-Padilla, J., et al. (2018). A thyroid hormone regulated asymmetric responsive centre is correlated with eye migration during flatfish metamorphosis. *Scientific Reports*, 8, 12267.
- Chen, J., Liu, X., Yao, X., Gao, F., & Bao, B. (2017). Dorsal fin development in flounder, *Paralichthys olivaceus*: Bud formation and its cellular origin. *Gene Expression Patterns*, 25–26, 22–28 2017.
- Dawkins, R. (1986). *The blind watchmaker: Why the evidence of evolution reveals a universe without design*. New York: Norton.
- Dinis, M. T., Ribeiro, L., Soares, F., & Sarasquete, C. (1999). A review on the cultivation potential of *Solea senegalensis* in Spain and Portugal. *Aquaculture*, 176(1/2), 27–38.
- Friedman, M. (2008). The evolutionary origin of flatfish asymmetry. *Nature*, 454(7201), 209–212.
- Gavaia, P. J., Sarasquete, C., & Cancela, M. L. (1999). Detection of mineralized structures in early stages of development of marine Teleostei using a modified alcian blue-alizarin red double staining technique for bone and cartilage. *Biotechnic & Histochemistry*, 75, 79–84.
- Gavlik, S., Albino, M., & Specker, J. L. (2002). Metamorphosis in summer flounder: Manipulation of thyroid status to synchronize settling behavior, growth, and development. *Aquaculture*, 203(3–4), 359–373.
- Gavlik, S., & Specker, J. L. (2004). Metamorphosis in summer flounder: Manipulation of rearing salinity to synchronize settling behavior, growth and development. *Aquaculture*, 240(1–4), 543–559.
- Gibson, R. N. (2005). *Flatfishes: Biology and exploitation*. Oxford: Blackwell Science.
- Hussain, S. M. (1990). Biology of *Psettodes erumei* (Schneider, 1801) and *Pseudorhombus arsius* (Hamilton, 1822) from the northern arabian sea. *Indian Journal of Fisheries*, 37(1), 63–66.
- Janvier, P. (2008). Squint of the fossil flatfish. *Nature*, 454(7201), 169–170.
- Li, L., Zheng, J., Bao, B., & Berendzen, P. B. (2013). Change of eye shape during the metamorphosis in two flatfishes *Paralichthys olivaceus* and *Solea senegalensis*, with comparison of eye shape within the Pleuronectiformes. *Ichthyological Research*, 60(2), 178–183.
- Okada, N., Takagi, Y., Seikai, T., Tanaka, M., & Tagawa, M. (2001). Asymmetrical development of bones and soft tissues during eye migration of metamorphosing Japanese flounder *Paralichthys olivaceus*. *Cell and Tissue Research*, 304, 59–66.
- Schreiber, A. M. (2006). Asymmetric craniofacial remodeling and lateralized behavior in larval flatfish. *Journal of Experimental Biology*, 209(4), 610–621.
- Shao, C., Bao, B., Xie, Z., Chen, X., Li, B., Jia, X., et al. (2017). The genome and transcriptome of Japanese flounder provide insights into flatfish asymmetry. *Nature Genetics*, 49, 119–124.
- Sun, M., Wei, F., Li, H., Xu, J., Chen, X., Gong, X., et al. (2015). Distortion of frontal bones results from cell apoptosis by the mechanical force from the up-migrating eye during metamorphosis in *Paralichthys olivaceus*. *Mechanisms of Development*, 136, 87–98.
- Tagawa, M., & Aritaki, M. (2005). Production of symmetrical flatfish by controlling the timing of thyroid hormone treatment in spotted halibut. *Verasper variegates*. *General and Comparative Endocrinology*, 141, 184–189 2005.
- Wagemans, F., Focant, B., & Vandewalle, P. (1998). Early development of the cephalic skeleton in the turbot. *Journal of Fish Biology*, 52, 166–204.
- Wei, F., Chen, J., Chen, X., & Bao, B. (2017). Comparative analysis with the neurula transcriptomes of two species of flatfishes *Platichthys stellatus* and *Paralichthys olivaceus*. *Gene*, 596, 147–153 2017.
- Xu, J., Ke, Z., Xia, J., He, F., & Bao, B. (2016). Change of body height is regulated by thyroid hormone during metamorphosis in flatfishes and zebrafish. *General and Comparative Endocrinology*, 236, 9–16 2016.
- Zimmer, C. (2008). The evolution of extraordinary eyes: The cases of flatfishes and stalk-eyed flies. *Evolution: Education and Outreach*, 1(4), 487–492.