Asymmetric craniofacial remodeling and lateralized behavior in larval flatfish

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Summary

Flatfishes, such as flounder, are the world's most asymmetric vertebrates. It is unknown if the development of lateralized swimming behavior during metamorphosis is an adaptive response to bilaterally asymmetric eye positioning, or if this results from a vestibular response to thyroid hormone. This study describes larval development in left-sided, right-sided and bilaterally symmetric variants of southern flounder (Paralichthys lethostigma). Behavior and skull asymmetries precede metamorphosis, and the development of lateralized behaviors was independent of eye position in larvae treated with thyroid hormone and in symmetrical variants. Therefore, lateralized behavior is not an adaptive response to eye translocation, but rather must result from changing vestibular responses to thyroid hormone.

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Key words: thyroid hormone, metamorphosis, flounder, Paralichthys lethostigma, skull, remodeling.

Introduction

The flatfishes (Order Pleuronectiformes) have the most asymmetric shape and lateralized behavior of any vertebrate. Flatfishes are a natural paradigm for studying the development of behavioral and morphological asymmetry, as their larvae initially resemble typical fish with upright swimming and bilaterally symmetric bodies, but metamorphose abruptly into fish that swim on one side and lie on the ocean floor. Flatfish have evolved a unique developmental strategy that complements their lateralized behavior: as the larva transforms into a settled juvenile the head remodels asymmetrically and one eye is repositioned to the opposite side, giving the settled fish the distinct advantage of a broad visual panorama with two eyes on its ‘top’ side (Fig. 1). As metamorphosis progresses, flatfish larvae spend less time swimming and more time settled with their ‘blind’ side touching the ground. Like amphibians, flatfish metamorphosis is mediated entirely by thyroid hormone (TH; Inui and Miwa, 1985; Miwa et al., 1988; Schreiber and Specker, 1998).

It is not clear if flatfish tilted swimming and settling behaviors are adaptive responses to changing eye position, or if these behaviors result from a vestibular response to thyroid hormone. Unlike all other vertebrates the axes of flatfish eyes and horizontal semicircular canals of the inner ear become oriented perpendicular to each other during metamorphosis (Graf and Baker, 1983). The dorsal light reflex, which causes a typical upright swimming fish to tilt towards a lateral light source until equilibrium is established between visual and gravitational sensory input, may play a role in the development of flatfish lateralized behaviors as the migrating eye changes the perceived angle of light incidence (Graf and Baker, 1990; Neave, 1985). In adult flatfish, however, there is also evidence for asymmetry in the hindbrain (Meyer et al., 1981) and otoliths of the inner ear (Helling et al., 2005), central and peripheral vestibular regions well known to mediate vertebrate postural control. The first part of this study dissociates eye migration from tilted swimming and settling to show that these adult behaviors result from a response to thyroid hormone that is independent of eye migration in the southern flounder (Paralichthys lethostigma).

All vertebrates develop lateralized visceral organs during embryogenesis (Yost, 1995), but flatfish are virtually the only vertebrates that also undergo significant asymmetric post-embryonic development. It has generally been assumed that the unique asymmetries characteristic of adult flatfish develop during metamorphosis (Youson, 1988), and the phenomenonology of asymmetric flatfish skull development and eye migration during this period has been widely documented (Brewster, 1987; Okada et al., 2001; Okada et al., 2003a; Okada et al., 2003b; Saele et al., 2004; Wagemans et al., 1998). However, the possibility that metamorphic laterality is preceded by subtle larval asymmetry has not been explored. Flatfish species are described as either ‘sinistral’ (both eyes are on the left side of the adult head) or ‘dextral’ (both eyes on the right side). Within a species some fish may have ‘reversed’ asymmetry and appear as morphological and behavioral mirror images of their siblings (Hubbs and Hubbs, 1945; Norman, 1930; Policansky, 1982). The ontogeny of behavioral and
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craniofacial reversal has not been described, due in part to the difficulty of identifying reversed larvae before substantial eye migration has taken place. The second part of this study analyzes swimming and feeding behaviors of pre-metamorphic southern flounder larvae to predict post-metamorphic sidedness in this predominantly sinistral species. This approach, combined with a sensitive in vivo bone stain (calcein), shows that behavior and skull asymmetries present in larvae before the start of eye migration correspond with ensuing post-metamorphic laterality.

**Materials and methods**

**Fish maintenance**

Southern flounder (*Paralichthys lethostigma* L.) embryos (12–24 h post-fertilization) were obtained from the University of North Carolina Wilmington’s aquaculture facility throughout the breeding season (October–April 2004/2005) and raised at the Carnegie Institution in 40–120 l aquaria (25°C) using artificial saltwater (35‰ salinity). After hatching (48 h post-fertilization), larvae were fed live zooplankton (*Brachionus plicatilis*) and brine shrimp (*Artemia*) nauplii through the end of metamorphosis (approximately 31 days post-fertilization; d.p.f.) according to methods described (Daniels, 2000; Daniels and Watanabe, 2003). Stages of larval development were adapted from the alphabetical description (Minami, 1982) of the position of the migrating eye for the congeneric Japanese flounder (*P. olivaceus*): early (6–13 d.p.f.) and late (14–19 d.p.f.) pre-metamorphosis (D–, D+; no eye migration); pro-metamorphosis (20–23 d.p.f., E–, E, E+; start of eye migration); early, mid and late metamorphic climax (24–30 d.p.f., F, G, H; eye migrates towards dorsal midline); post-metamorphic juvenile (31 d.p.f., I; migrating eye has crossed the dorsal mid-line); sub-adult (migrating eye is located entirely on the left side of the head, and the left eye is in a more ventral position than in stage I). The development of flounder pectoral fin skeletal morphology has not been systematically described and was also used to define developmental stage in manner that was independent of eye position (see Results).

**Metamorphic variants**

Wild southern flounder are typically a sinistral (left-sided) species (Daniels, 2000). In the present study, three metamorphic variants of laboratory raised flounder are described: (1) sinistral (left-sided), (2) dextral (right-sided), and (3) bilaterally symmetric forms with either no eye migration, or migration of both eyes to the dorsal midline. Variant frequencies were determined using 3000 yolk-sac larvae (3 d.p.f.) distributed into six 120 l aquaria (500 larvae/aquaria); after completing metamorphosis the variants were counted and their settling side noted.

**Screening for lateralized behavior**

Though not previously reported, larval southern flounder exhibit tilted swimming and lateralized feeding behaviors before and during metamorphosis, most swimming with their left side tilted towards the water surface and a minority with their right side tilted upwards. To determine if pre-
metamorphic tilted swimming and feeding behaviors of individual larvae are lateralized (unidirectional) and if larval laterality corresponds with post-metamorphic laterality (tilted swimming, settling) and morphological asymmetry (eye migration, skull development), a simple procedure was developed to differentiate between left- and right-tilters. A bright light is placed above the larval aquarium and live brine shrimp are added to prompt feeding behaviors. Since brine shrimp are phototropic, they concentrate at the water surface where the larvae are now forced to feed. In the presence of brine shrimp, larvae with tilted swimming exhibit two characteristic behaviors: (1) skimming the water surface with the future eyed side (supplementary material Movie 1: Sinistral surface skimming), and (2) swimming several centimeters below the surface with the non-migratory eye facing upwards, apparently tracking brine shrimp before darting upwards to feed (supplementary material Movie 2: Sinistral tracking, and Movie 3: Dextral tracking). Because flatfish larvae have unusually narrow bodies with long dorsal–ventral profiles, these tilted feeding behaviors are easily identified when viewed from above. Younger larvae without tilted swimming do not exhibit these behaviors in the presence of brine shrimp and always swim and feed upright (supplementary material Movie 4: Upright feeding). After screening larvae for surface skimming and tracking behaviors, individuals are isolated and behavior examined in the absence of brine shrimp to estimate the default angle of swimming tilt at different developmental stages (see below).

To determine when tilted swimming begins, and to see if this behavior corresponds with post-metamorphic sidedness, the swimming behaviors of 1000 larvae living in two 1201 aquaria were observed daily from 7 d.p.f. through metamorphosis. When larvae first exhibited tilted swimming, left and right tilters (N=20) were collected for developmental staging and histological examination, and a larger number (N=50) were transferred to four behavior-segregated 40·l aquaria and raised through metamorphosis. Larvae remaining in the 1201 aquaria were collected at different stages for behavioral and histological examination.

To estimate the degree of tilt from the upright (vertical) during ordinary (non-feeding) swimming, frontal photographs were taken (n=6–10 photographs/fish, and N=3–6 fish/stage) of larvae at different developmental stages in the absence of brine shrimp. A straight line from the anteriormost tip of the jaw to the ventralmost region of the gut defined the vertical axis of the fish; these are two anatomical points that remain medial during metamorphosis. The horizontal axis of reference was a line parallel to the water surface, and the angle of tilt defined as the angle by which the fish’s vertical axis deviates from a line perpendicular to the horizontal reference. Differences in degree of tilt were analyzed using a nested factorial design (SuperANOVA, Abacus Concepts, Berkeley, CA, USA) consisting of two factors: developmental stage and individual fish. Significance was accepted when P<0.05. Fisher’s PLSD post hoc test was performed when appropriate, as indicated by significance using analysis of variance (ANOVA).

**Thyroid hormone/methimazol treatment, histology and digital morphing**

Larval fish at early pre-metamorphosis (7 d.p.f.), late pre-metamorphosis (14 d.p.f.) and pro-metamorphosis (20 d.p.f.) were induced to metamorphose by adding 3,5,3'-triiodothyronone (T3) (Sigma Chemical Co., St Louis, MO, USA) to the water (T3 final concentration, 100 nmol l⁻¹) for 3–8 days. Methimazol (0.1 mol l⁻¹; Sigma), an inhibitor of endogenous thyroid hormone (TH) production (Brown, 1997), was dissolved directly into water and administered for up to 6 weeks (starting at late pre-metamorphosis) to inhibit metamorphosis.

**In vivo** bone staining was modified from the technique described for zebrafish by Du et al. (2001): 10 mg calcein powder (Sigma Chemical Co.) was dissolved directly into 100 ml saltwater to make a 0.01% solution, then passed through a 5 µm filter to remove insoluble particles; live larvae were incubated in the solution (10–20 min), followed by three rinses (15 min each) in calcine-free saltwater. Larvae were observed live, or euthanized in tricaine-methanesulfonate (MS 222) and mounted in liquid OCT embedding compound (Electron Microscopy Sciences, Hatfield, PA, USA) for orientation and photography. Specimens were photographed using a Spot RT camera mounted onto a Leica (Deerfield, IL, USA) MZ12 fluorescent stereomicroscope using a GFP filter. At least 20 fish per developmental stage were analyzed.

For histological identification of bone and cartilage, whole-body larvae were stained with Alcian Blue and Alizarin Red as described (Klymkowsky and Hanken, 1991) with the following modifications: larvae were fixed in 4% neutral buffered paraformaldehyde for 2 days at room temperature, rinsed in water, and dehydrated through graded alcohols. Prior to staining, samples were photo-bleached for 3–12 h in 0.3% H₂O₂ in 5% KOH at room temperature.

The skull morphing movie (supplementary material Movie 7) was derived from still photographs of calcein-stained larvae at the different developmental stages depicted in Fig. 4. Images were cropped using Adobe Photoshop, aligned using Image J software for UNIX, morphed with Morph Age software for Mac OSX, and assembled using Final Cut Pro.

**Results**

**Metamorphic variants**

Of the surviving juveniles, 80% (N=1731) metamorphosed with typical (sinistral) morphology, 16% with reversed (dextral) morphology, and 4% were bilaterally symmetrical. All juveniles with sinistral and dextral morphology settled with their left and right sides (respectively) facing up. Of the symmetrically metamorphosed juveniles, 78% settled with their left side facing up (i.e. sinistral behavior), and 22% with their right side up (i.e. dextral behavior). Though very rare, approximately one in 5000 metamorphosing larvae exhibited normal sinistral morphology, but dextral behavior (settling with both eyes touching the bottom), an extreme example of the dissociation of eye position from behavior (supplementary...
material Movie 5: Sinistral morphology/dextral behavior). These juveniles feed inefficiently and usually die within the first month post-metamorphosis.

Development of lateralized behavior

Prior to metamorphosis no eye migration is observable. From hatching (2 d.p.f.) through to the end of yolk resorption (4–5 d.p.f.) the larvae did not maintain an upright swimming posture, but rather swam with random orientation in the presence of water currents caused by aeration, or drifted in the water column perpendicular to the bottom with head facing down in the absence of water flow, with occasional spontaneous bursts of non-directional swimming (not shown). Larvae began to swim with the sustained upright posture typical of most fish by 5 d.p.f. Early pre-metamorphic larvae (6–13 d.p.f.) swim with an upright posture (Fig. 1A), and late pre-metamorphic larvae (14–19 d.p.f.) swim with a 3–6° sustained right tilt (Fig. 1B). By the start of eye migration, pro-metamorphosis (20 d.p.f.), larvae swim with a 10–20° right tilt (Fig. 1C). The degree of tilt increases to 17–26° by early climax (24 d.p.f.) (Fig. 1D and supplementary material Movie 6: Climax tilt), shifts abruptly to a 50–80° tilt by late climax (26 d.p.f.; Fig. 1E), and the fish swim virtually parallel to the bottom (80–90°) by the juvenile stage (30 d.p.f.; Fig. 1F). Changes in degree of tilt per developmental stage are summarized in Table 1. Eye migration continues after metamorphosis during the juvenile stage, and by the time the fish is a sub-adult (120 d.p.f.) the right eye is located fully on the left side of the head and the left eye has moved to a more ventral position (Fig. 1G; also see Fig. 5G–H). Larvae in late pre-metamorphosis swim predominantly in the water column of the aquarium, but also occasionally settle to the bottom on their future ‘blind’ side. As metamorphosis proceeds the fish spend less time swimming and more time settled, and by mid-climax only rarely swim. Lateralized feeding and sustained tilted swimming behaviors were first evident at 14 d.p.f. at the start of late pre-metamorphosis. Larvae with these lateralized actions also displayed lateralized hiding and escape behavior: when chased with a pipet they often rapidly swim to the tank bottom where they settle with their non-migrating eye facing up (not shown). When left- and right-tilters were screened at 14 d.p.f. and raised in separate aquariums through the end of metamorphosis (N=50), 100% of left-tilters settled on their left side and 100% of right-tilters on their right side.

Craniofacial remodeling

Craniofacial morphology visualized with traditional bone (Alizarin Red) and cartilage (Alcian Blue) histology is bilaterally symmetrical through late pre-metamorphosis (Fig. 2A,A’). Craniofacial symmetry first breaks as the right (but not left) cartilaginous supraorbital bar (SB) becomes thinner during early pro-metamorphosis (not shown), and is degraded by late pro-metamorphosis (Fig. 2B,B’). The left SB appears thinner by early climax (Fig. 2C’), and by late climax (Fig. 2D’) has completely degraded. The frontal bones stain progressively with Alizarin Red in a proximal-distal manner from pro-metamorphosis through late climax (Figs 2B–D). The pseudomesial bar, a bone that forms only under the migrated eye, is visible by Alizarin Red stain in the juvenile stage 10 days after metamorphosis is completed (Fig. 2E).

The use of an in vivo fluorescent calcein stain labels bone that is unstained by traditional Alizarin Red histology, allowing early morphogenesis of the presumably weakly mineralized frontal bones to be visualized (Fig. 3). Craniofacial bone morphology appears bilaterally symmetrical at early pre-metamorphosis (Fig. 4A–A’). Asymmetry is first apparent at the start of late pre-metamorphosis (14 d.p.f.) when the right frontal bone appears slightly thinner and right parietal bone smaller than their counterparts on the left side (see Fig. 7B), and these asymmetries are exaggerated further into late pre-metamorphosis (16 d.p.f.; Fig. 4B). Although obvious asymmetries in the parietal and developing frontal bones are not clearly evident before 14 d.p.f., the presence of subtle asymmetries in size and shape cannot be ruled out. During pro-metamorphosis (Fig. 4C–C’’) the right frontal is substantially thinner than the left and both frontals and parietal bones deform slightly to the left; the dorsal fin has migrated more anterior and bends towards the right parietal bone, and the right lateral ethmoid (but not left) becomes visible. The frontal and parietal bones deform dramatically to the left, and the dorsal fin continues to migrate in an anterior/right trajectory from early to late climax (Fig. 4D–F). During climax the right lateral ethmoid elongates disproportionately compared with the left lateral ethmoid as the right eye migrates to the dorsal mid-line, and the right anterior parietal barb becomes larger and protrudes more prominently than the left (Fig. 4D’,E’,F’–D’’,E’’,F’’). By late climax both frontal bones appear similarly condensed in thickness (F’’) and the pseudomesial bar first becomes visible by calcein stain on the right side after eye migration is almost complete (Fig. 4F’’). These changes in skull morphology are summarized in supplementary material Movie 7: Skull development.

Reversed development

Larval fish with reversed tilted swimming (right side facing up; Fig. 5F) also had reversed frontal/parietal bone

### Table 1. Mean degrees of tilt away from upright (vertical axis) at different developmental stages

| Stage                  | Age (d.p.f.) | Fish (N) | Photos/ fish (n) | Tilt (degrees) |
|------------------------|-------------|----------|-----------------|----------------|
| Early pre-metamorphosis| 12          | 6        | 10              | 0±1<sup>d</sup>|
| Late pre-metamorphosis | 16          | 6        | 10              | 5±2<sup>c</sup>|
| Pro-metamorphosis      | 20          | 6        | 10              | 13±5<sup>b</sup>|
| Early climax           | 24          | 6        | 10              | 18±4<sup>b</sup>|
| Late climax            | 26          | 4        | 6               | 58±17<sup>a</sup>|
| Juvenile               | 30          | 3        | 6               | 87±4<sup>c</sup>|
| T3-treated (72 h)      | 17          | 4        | 6               | 81±9<sup>a</sup>|
| Methimazole (4 weeks)  | 42          | 5        | 6               | 8±4<sup>b,c</sup>|

Tilt values are means ± s.d. Means with the same superscript are not significantly different.
deformation during metamorphosis (Fig. 5A,B). At early climax these larvae displayed reversed eye and dorsal fin migration (Fig. 5B), greater elongation of the left lateral ethmoid and left anterior parietal barb (Fig. 5C) compared with the right (not shown), and degradation of the left cartilaginous SB prior to the right (Fig. 5D). Reversed larvae in late climax began to form the pseudomesial bar on the left side of the head under the migrated left eye (Fig. 5E). After metamorphosis, left sided (sinistral; Fig. 5G) and reversed (dextral) juvenile flounder (Fig. 5H) are morphological and behavioral mirror images of each other.

Dissociation of lateralized behavior from eye migration in thyroid hormone-treated larvae and bilaterally symmetrical metamorphic variants

A summary of results for TH induction and methimazol treatment on larval swimming and settling behaviors is shown in Fig. 6. Treatment of early pre-metamorphic larvae (7 d.p.f.) with 100 nmol l⁻¹ T₃ for 96 h induced settling behavior, but individuals settled alternately between sides and displayed no side preference (not shown). Swimming behavior was not evaluated for these fish. T₃ induction of late pre-metamorphic larvae (14 d.p.f.) for 72 h, however, was sufficient to induce

Fig. 2. Remodeling of skull bone (Alizarin Red) and cartilage (Alcian Blue) during flounder metamorphosis. Skull symmetry first breaks when the cartilaginous right supraorbital bar degrades before the left in pro-metamorphosis (B, B′), allowing passage for the migrating right eye. Right (A−D) and left (A′−D′) sides of larvae in different developmental stages. (A, A′) Early pre-metamorphosis, left (green arrow) and right (red arrow) supraorbital bars are intact; (B, B′) late pro-metamorphosis, right supraorbital bar is degrading (open red arrow) and frontal bones (orange arrowhead) begin to stain with Alizarin Red; (C, C′) early metamorphic climax, further degradation of right supraorbital bar and Alizarin Red staining of frontal bones; (D, D′) late metamorphic climax, left supraorbital bar has degraded (open green arrow); (E, E′) post-metamorphic juvenile, pseudomesial bar (above broken line) is formed. Bars, 0.3 mm.

Fig. 3. Staining for bone in vivo with calcein is more sensitive than traditional Alizarin Red histology. Note how the frontal bones (arrows), left parietal bone (p), dorsal fin rays (df), and much of the axial skeleton stain clearly with calcein, but remain unstained by traditional Alizarin Red. (A) Bone (Alizarin Red) and cartilage (Alcian Blue); (B) fluorescent calcein stain for bone. Both larvae are late pre-metamorphic (14 d.p.f.). Bars, 0.5 mm.
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lateralized settling (Fig. 7E) and tilted swimming (60–85° angle; Fig. 7F,G) (supplementary material Movie 8: TH-induced swimming and settling behaviors). Individuals consistently settled onto the same side; though they were only rarely observed swimming in the water column, they did so with their settled side facing down. The percent of larvae with reversed tilted swimming and settling behavior following T3 treatment (15%) was similar to that for spontaneous metamorphosis (16%). Interestingly, these T3-induced behavioral changes were accompanied by little to no eye migration (Fig. 7C,D). T3 treated pre-metamorphic larvae experienced a dramatic and symmetric condensation of both frontal bones without lateral deformation (Fig. 7D). Retention of the cartilaginous right supraorbital bar (SB) after 72 h T3 treatment is likely not responsible for inhibition of eye migration, as both SBs had degraded in a bilaterally symmetric manner after 6 days of treatment (not shown), yet the eyes still remained symmetrically placed with no frontal bending even after 8 days treatment (not shown). T3-treatment of pro-metamorphic larvae (which at the start of treatment already displayed some eye migration, asymmetric frontal bone condensation and bending, and right lateral ethmoid formation) induced the same behavioral changes, as well as bilaterally symmetric frontal bone condensation and eye-migration roughly commensurate with the amount of frontal bending (Fig. 7H–K).

Treatment of late pre-metamorphic larvae with 0.1 mol l⁻¹ methimazol (an inhibitor of endogenous TH production) for 6 weeks inhibited eye migration (Fig. 8B'), resorption of the right SB (Fig. 8A'), frontal bone bending (Fig. 8B) and pectoral fin remodeling (Fig. 8A), but did not inhibit bone mineralization (broad Alizarin Red staining in Fig. 8A), bilateral frontal bone condensation (Fig. 8B), or development of right parietal barb and lateral ethmoid bones (Fig. 8B'). After 4 weeks of methimazol treatment these fish still swim with a 5–15° tilt typical of larvae in late pre-/early climax (not shown). Subsequent treatment with 100 nmol l⁻¹ T3 for 1 week induced the fish to settle to the bottom of the tank. T3 treatment also induced pectoral fin remodeling (Fig. 8C; also see Fig. 10K,L) and right SB resorption (Fig. 8C'). The right eye migrated further than in fish treated only with methimazol (Fig. 8C',D'), though the frontals did not appear to bend
further (Fig. 8D). Therefore, elongation of the right lateral ethmoid in the absence of eye migration (Fig. 8B) and subsequent induction of eye migration with thyroid hormone treatment in the absence of significant frontal bone deformation (Fig. 8D, D) together suggest that asymmetric skull development alone is insufficient for eye migration.

Symmetrically metamorphosed variants all displayed normal 85–90° tilted swimming and normal settling behavior after metamorphosis (supplementary material Movie 9: Settled sinistral and symmetrical variants), despite either the lack of eye migration (Fig. 9A, A’) or having both eyes located at the dorsal mid-line (Fig. 9C, C’). In symmetrical juveniles both left and right supraorbital bar cartilages resorb normally (Fig. 9B, B’, D, D’) and pectoral fins have remodeled to their adult form (see Fig. 10M), suggesting that these variants respond to increased endogenous TH production and are not hypothyroid. Bone mineralization is not inhibited, but the frontal bones are abnormally symmetrical in shape compared with other variants: both frontals have condensed to approximately the same thickness, although the frontal bones remain medially positioned. In further contrast with the other two more common variants, both left and right lateral ethmoids appear similarly elongated in length, and the pseudomesial bar has not formed. When pro-metamorphic larvae (10–15° right or left tilt) were screened and stained with calcein, some larvae displayed bilateral frontal bone condensation with no lateralized bend and bilaterally symmetric eye position (Fig. 9E, F).

Although most of these do not survive through metamorphosis, the survivors likely develop into symmetrically metamorphosed variants.

**Pectoral fin remodeling**

Surprisingly, the extensive flatfish pectoral fin remodeling that takes place during metamorphosis has not been

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**Fig. 5.** Head remodeling in dextral flounder produces morphological and behavioral mirror images of their sinistral siblings. Asymmetric frontal bone condensation (A) and deformation (B), lateral ethmoid elongation (C), supraorbital bar resorption (D), pseudomesial bar formation (E), and tilting (F) and settling (H) behaviors are all reversed compared with typical sinistral flounder development (G; also refer to Figs 2, 4). Pro-metamorphic, frontal (A); early metamorphic climax, frontal (B), left saggital (C), right saggital (D); post-metamorphic juvenile, left saggital (E); pro-metamorphic larva with reversed tilt and eye migration (F); settled post-metamorphic juveniles with left-sided (G) and right-sided (H) morphology and behavior viewed from above. Calcein-stained bone (A–C,E); Alizarin Red (bone) and Alcian Blue (cartilage) (D). Right frontal bone (long white arrow), dorsal fin (short white arrow), left lateral ethmoid (white arrowhead), left anterior parietal barb (black arrowhead), right supraorbital bar (red arrow), left supraorbital bar (open green arrow), pseudomesial bar (above broken line), dorsal (d), ventral (v). Bars, 0.2 mm (A–F) and 1.0 cm (G, H).
Flatfish asymmetry systematically documented. Since the pectoral fins develop autonomously in response to TH, they are useful markers for classifying larval developmental stages independent of eye migration and skull morphology. Pre-metamorphic larvae have large paddle-like pectoral fins (Fig. 10A) that shrink in size systematically documented. Since the pectoral fins develop autonomously in response to TH, they are useful markers for classifying larval developmental stages independent of eye migration and skull morphology. Pre-metamorphic larvae have large paddle-like pectoral fins (Fig. 10A) that shrink in size.
during pro-metamorphosis (Fig. 10B). The long postcoracoid process is almost entirely resorbed by early climax (Fig. 10C), and four proximal radials and a distinct scapulocoracoid are formed by late climax (Fig. 10D–F). In a sub-adult, the pectoral fin marginal rays have ossified (Fig. 10G). These changes, which normally take place during metamorphic climax, can be induced in early pre-metamorphic larvae treated with 100 nmol l⁻¹ T3 in as little as 3–4 days (Fig. 10H–J). In contrast, the pectoral fins of larvae raised in 0.1 mol l⁻¹ methimazol for 6 weeks starting at late pre-metamorphosis grow abnormally large and retain their larval form entirely.
Interestingly, the pectoral fins of these methimazol-treated larvae can be induced to remodel to their juvenile form when 100 nmol l$^{-1}$ T3 is administered for an extra week in lieu of methimazol (Fig. 10L; also see Fig. 8B). The progression of pectoral fin development and responses to thyroid hormone follow the typical teleost patterns described for zebrafish (Grandel and Schulte-Merker, 1998; Brown, 1997). Although bilaterally symmetric flounder variants may exhibit no eye migration or frontal bone bending (see Fig. 9), their pectoral fins always remodel to the juvenile form (Fig. 10M), suggesting that these fish are not hypothyroid.

**Discussion**

The flatfishes are the most extreme example of behavioral and morphological lateralization in the vertebrates. The development of unique head asymmetries in response to thyroid hormone during metamorphosis make flatfish a natural paradigm for studying craniofacial remodeling and the influence of visual and gravitational sensory input on postural control. Increasing thyroid hormone levels mediate virtually every aspect of flatfish metamorphosis, similar to amphibian metamorphosis (Inui and Miwa, 1985; Miwa et al., 1988; Schreiber and Specker, 1998). However, the specific mechanisms responsible for inducing tilted swimming and settling behaviors in flatfish remain essentially unknown. In principle, the abrupt transition to lateralized behavior during metamorphosis could be influenced by asymmetric changes in eye position, inner ear morphology, and/or central changes in vestibular connectivity or activity. It is known that balance in many upright swimming fish is sustained through interactions between the directions of light and gravity such that a typical fish will tilt towards a lateral light source until a new balance with its gravistatic senses is reached (Graf and Meyer, 1983; von Holst, 1935). Indeed, pre-metamorphic larval flatfish exhibit a dorsal light response similar to typical upright fish (Neave, 1985), and as one eye begins to migrate during metamorphosis the larval tilt towards their future blind side when illuminated from above. This tilting could be attributable to a change in the angle of perceived light due to eye migration (Graf and Baker, 1990; Neave, 1985). Three findings from the present study, however, suggest that tilting and settling behaviors can be dissociated from – and thus occur independently of – asymmetric eye position: (1) lateralized feeding and sustained tilted swimming behaviors are apparent prior to eye migration as early as the start of late pre-metamorphosis (Fig. 1B) and correspond with post-metamorphic sidedness, (2) treatment of pre-metamorphic larvae with thyroid hormone induces acute tilting and settling with little to no eye migration (Fig. 7E–G), and (3) symmetrically metamorphosing flounder variants also display normal lateralized swimming and settling behaviors (Fig. 9). Interestingly, early pre-metamorphic larvae (7 d.p.f.) treated with thyroid hormone are induced to settle at the bottom of the tank, but do so without displaying a side preference. This suggests that lateralized behavioral competence is established by late pre-metamorphosis (14 d.p.f.) when larvae do respond to thyroid hormone treatment with unilateral settling. The ability to dissociate settling behaviors, skull remodeling and pectoral fin development (Fig. 10) from each other in TH-induced larvae and symmetrical variants suggests these are each autonomous developmental programs mediated by TH, similar to the autonomy of developmental programs displayed in metamorphosing frogs (Brown et al., 2005; Das et al., 2002; Schreiber and Brown, 2003; Schreiber et al., 2001). From a practical perspective, the ability to predict post-metamorphic sidedness based upon pre-metamorphic feeding and swimming behavior is a useful tool for screening dextral- from sinistral-behaving larvae for analysis during metamorphosis.

Since eye position does not influence the development of adult lateralized behavior in flatfish, changes in the peripheral or central vestibular systems must be responsible for these metamorphic changes. However, the gross anatomy and bilateral symmetry of the labyrinths in flatfish (Jacob, 1928), the distribution of the vestibular nuclear complex in the hindbrain of larval and juvenile turbot Scophthalmus maximus (Jansen and Enger, 1996), and the peripheral and central oculomotor apparatus (Graf and Baker, 1985) are bilaterally symmetrical and similar to those of other teleosts. Interestingly, all flatfish except for the most primitive (Platt, 1983) display nearly omni-directional hair-cell polarization of the inner ear’s saccular and lagener otoliths (Jorgensen, 1976; Platt, 1973). This pattern, which is unique amongst vertebrates, is thought to accommodate, but not necessarily induce, the adult flatfish posture. Other unusual features of flatfish sacculae are accessory growth centers that form at the start of metamorphosis (Jearld et al., 1990). A central regulatory feature unique to flatfish is the significant reorganization of the vestibular-ocular pathways required to stabilize eye position during head movements (Graf and Baker, 1983). These aforementioned flatfish peculiarities, however, are bilaterally symmetric phenomena and alone do not explain the development of lateralized behavior. Some evidence for asymmetric peripheral and central postural control in adults does exist, though. 2-deoxyglucose is taken up differentially by the bilateral vestibular nuclei of adult flatfish (Meyer et al., 1983), suggesting that lateralized behavior may be due to a ‘permanent imbalance in vestibular neuron activity’. Morphological asymmetries in the otoliths of flatfish have also been reported (Lychakov, 1996; Sogard, 1991), and mass asymmetries, specifically in the utricles and saccules of adult flatfish, such that the heavier otoliths are located on the bottom side (Helling et al., 2005). These asymmetries are interesting, especially considering the observation (Graf and Baker, 1990) that adult flounder exhibit different postural responses of left side vs right side utricular neurlectomy or hemilabyrinthectomy. However, it remains to be seen if these adult flatfish asymmetries actually develop during metamorphosis and induce postural change, or if they develop only after behavioral change has already been established.

Historically, bilateral symmetry in the flatfish skull has been
thought to first break at the start of eye migration during metamorphosis (Youson, 1988), the earliest reported asymmetric being differential resorption of the left and right cartilaginous supraoral bars (SB) (Wagemans et al., 1998; Williams, 1901). The present study uses an in vivo bone labeling technique (Du et al., 2001) to show that skull asymmetry is already established by the start of late pre-metamorphosis, and possibly earlier. Specifically, sinistral southern flounder have a noticeably smaller right parietal and thinner right frontal bone compared with the left side during pre-metamorphosis (Figs 4B, 7B), and these asymmetries are further exaggerated during metamorphic climax (Fig. 4C–F). In contrast, pre-metamorphic larvae with reversed parietal and frontal asymmetry metamorphose with dextral morphology (Fig. 5). These asymmetries have not been previously described, and the asymmetric architecture of the frontals before metamorphosis may facilitate their characteristic bending at climax. The cause of frontal bone bending, a unique feature of flatfish development, remains unknown. Interestingly, in bilaterally symmetric metamorphic variants both frontals are symmetrically shaped and do not develop a frontal bend (Fig. 9). The ability to dissociate eye migration from asymmetric development of the lateral ethmoid bones or frontal bone deformation in methimazol and thyroid hormone-treated larvae (Fig. 9) suggests that asymmetric skull development alone is inadequate for eye migration. A possible role for ocular muscle and orbit remodeling in facilitating eye migration cannot be ruled out.

The proportion of flatfish displaying reversed morphology and behavior in a natural population varies among species from virtually none in the tonguefishes (Cynoglossidae) (Monroe, 1996) to as high as 100% in some starry flounder (Platichthys stellatus) populations (Policansky, 1982). There are no reports of reversed southern flounder in nature, though we show 16% reversal when this species is raised in the laboratory. This suggests that reversed (dextral) southern flounder have low survival in nature, but enhanced survival in the laboratory when they are identified early and segregated from their sinistral siblings. A fundamental aberration in skull morphogenesis of dextral compared with sinistral flounders could explain their differential survival. However, this study shows that in the southern flounder, dextral skull ontogeny (SB degradation, frontal bone asymmetry, and formation of lateral ethmoid and pseudomesial bars on the blind side) proceeds as a mirror image reversal of their sinistral siblings, suggesting that factors other than skull morphology account for differential survival. For example, the optic chiasm of all dextral southern flounder and congenic summer flounder (P. dentatus) that we have observed display double-crossed optic nerves (which could impair vision) compared with their partially uncrossed sinistral siblings (A.M.S., unpublished data), an observation previously noted in other flatfish species (Parker, 1903).

The genetics of flatfish reversal are not well understood. Hashimoto et al. (2002) have isolated a Japanese flounder (Paralichthys olivaceus) clonal line (reversed, ‘rev’) whose offspring display a relatively high (20–30%) frequency of reversal. They have proposed that the directionality of metamorphic asymmetry is determined by the rev locus in a manner independent from the control of visceral asymmetry. Although the factors that mediate sinistral vs dextral skull development are not known, a differential bilateral sensitivity to thyroid hormone is likely involved. Treatment of pre-metamorphic larvae with triiodothyronine (T3) induced bilaterally symmetrical condensation of both frontal bones (Fig. 7D) and symmetrical resorption of both SBs (not shown). Eye migration in these larvae is inhibited, possibly due to failure of the symmetrical frontals to bend and allow passage for the migrating eye, as may be the case for the aforementioned symmetrical metamorphic variants. Interestingly, the occurrence of bilaterally symmetrical flatfish variants in aquaculture has been reported (Okada et al., 2003b; Pittman et al., 1998), and symmetrical variants appear similar to symmetrical spotted halibut (Verasper variegatus) juveniles that were artificially produced by treating early pre-metamorphic larvae with thyroxin (Tagawa and Aritaki, 2005). Therefore, a symmetrical condensation of the frontals due either to early TH treatment or abnormal bilateral sensitivity to endogenous TH during metamorphosis may explain this unusual phenotype. TH receptors are expressed in cartilage and presumptive osteoblasts of Japanese flounder larvae (Yamano and Miwa, 1998), and asymmetric sensitivity to TH in flatfish could, in principle, be regulated by differential expression of thyroid hormone receptor levels or isoforms, transcriptional coregulators, or types I, II or III deiodinase activity.

In summary, the main findings of this study are that (1) lateraled swimming behaviors and eye migration in larval flatfish both develop in response to thyroid hormone during metamorphosis, but are independent of each other, and (2) behavioral and craniofacial asymmetries are present before metamorphosis and can be used to predict post-metamorphic sidedness. Therefore, the abrupt 90° change in body orientation with respect to gravity during metamorphosis most likely results from asymmetric remodeling of central vestibular connectivity (hindbrain) and/or peripheral sensory organs (inner ear) in response to thyroid hormone. The thyroid hormone-responsive genes that ultimately mediate asymmetric skull and vestibular/inner ear remodeling remain to be identified.

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