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Development of the posterior lateral line system in *Thunnus thynnus*, the atlantic blue-fin tuna, and in its close relative *Sarda sarda*

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ABSTRACT The lateral line system of amphibians and fish comprises a large number of individual mechanosensory organs, the neuromasts, and their sensory neurons. The pattern of neuromasts varies markedly between species, yet the embryonic pattern is highly conserved from the relatively basal zebrafish, *Danio rerio*, to more derived species. Here we examine in more detail the development of the posterior lateral line (PLL) in embryos and early larvae of one of the most derived fish species, the blue-fin tuna *Thunnus thynnus*, and of its close relative, the Atlantic bonito *Sarda sarda*. We show that the basic features of embryonic PLL development, including the migratory properties of the PLL primordium, the patterning of neuromasts and their innervation, are largely conserved between zebrafish and tuna. However, *Thunnus* and *Sarda* embryos differ from *Danio* in three respects: the larger size of the neuromast cupula, the capability of mature neuromasts to migrate dorsally, and the presence of a single, precisely located terminal neuromast.

KEY WORDS: *neuromast, hair cell, planar polarity, cupula, migrating primordium*

The lateral line sensory system is central to many aspects of fish behavior (Coombs and Montgomery, 1999). It comprises a set of discrete sensory organs, the neuromasts. At the core of each neuromast lies a group of mechanosensory hair cells that provide information about local water flow. The system is thought to mediate a sense of "touch-at-a-distance" (Dijkgraaf, 1963) whereby fish can "feel" their surroundings within a radius close to their own body length.

The neuromasts on the body and tail form the posterior lateral line system (PLL). The embryonic PLL is formed by a primordium which migrates from head to tail and deposits small groups of cells, each one a prospective neuromast, in its wake (Metcalfe *et al.*, 1985). In zebrafish, the embryonic PLL comprises 5 neuromasts regularly spaced along the body, and 2-3 terminal neuromasts (Gompel *et al.*, 2001). This pattern is highly conserved in several species of the more derived acanthopterygian group (Pichon and Ghysen, 2004), although adult patterns vary widely across teleosts (Webb, 1989). The developmental mechanisms underlying the formation of the zebrafish PLL have been extensively studied over the past 10 years (Ghysen and Dambly-Chaudière, 2007). It is not known if and to what extent the same mechanisms operate in other teleosts.

Here we report our observations on the embryonic and early larval development of the PLL in the Atlantic blue-fin tuna, *Thunnus thynnus,* an acanthopterygian that shows highly derived features, e.g., endothermy (rev. in Stevens and Neill, 1978) or cooperative hunting (Partridge al., 2004). Some observations were also done on *Thunnus'* close relative, the bonito (*Sarda sarda*). Our aim was double: first, to determine to what extent the observed conservation of pattern reflects a conservation of developmental mechanisms, and second, to provide background infor-

Abbreviations used in this paper: PLL, posterior lateral line.

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#Note: These authors performed the experimental work; the other authors provided the experimental material used in this report.

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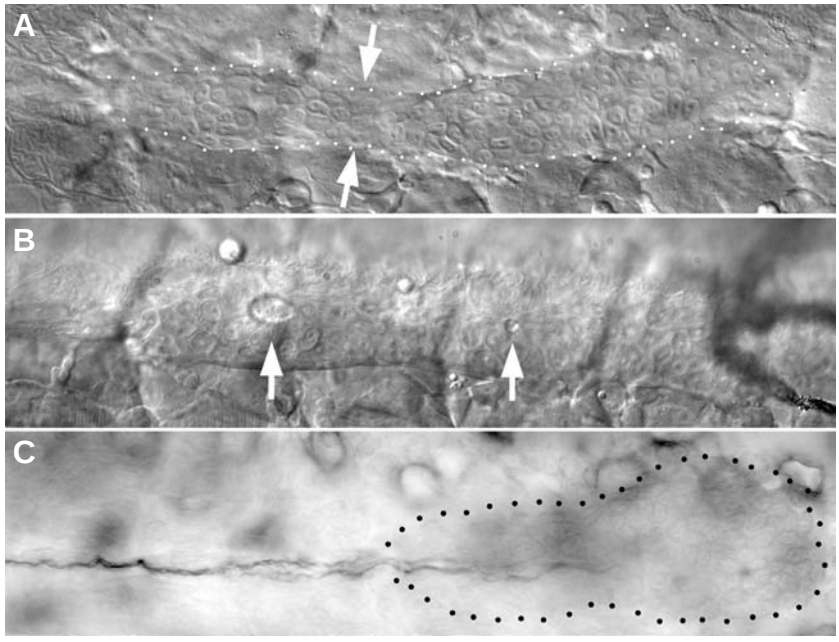

Fig. 1. Migrating posterior lateral line (PLL) primordium in pre-hatch *Thunnus* embryos. (A) *Primordium in the process of depositing a neuromast; arrows indicate the thinning between the migrating cells of the primordium, and the cells that are slowing down. The primordium is migrating to the right.* **(B)** *Cavitation of two rosettes (arrows) during primordium migration.* **(C)** *Towing of sensory axons revealed by anti-acetylated tubulin immunolabeling. The outline of the primordium is dotted. In all figures, dorsal is up, anterior to the left.*

mation about sensory development in blue fin tuna, possibly leading to improvements in the handling of larvae of a threatened species.

Results

Posterior lateral line primordium and nerve

We observed a primordium migrating posteriorwards along the horizontal myoseptum in pre-hatch, 28-34hpf (hours post-fertilization) *Thunnus thynnus* embryos (Fig. 1A and Supp. Movie 1). The size and shape of the primordium, and the process of neuromast deposition, resemble very much those documented in zebrafish (Fig. 1A). The organization of the primordium is also very similar in the two species, with 2-3 rosettes in the trailing region prefiguring the next neuromasts to be deposited (Fig. 1B).

It has been proposed in amphibians (Harrison, 1904), and confirmed in zebrafish (Metcalfe, 1985), that growth cones of sensory neurons accompany the primordium, and are guided by its migration (Gilmour, 2004). We labeled *Thunnus* axons with anti-acetylated tubulin and observed a close association between PLL axons and migrating primordium (Fig. 1C), suggesting that guidance of sensory axons by the primordium also takes place in *Thunnus*.

Neuromast differentiation

A few hours after neuromasts have been deposited, cells at their center begin to take up the hair cell specific dye, DiAsp (Fig. 2A). The number of hair cells increases rapidly (Fig. 2B). The neuromasts appear very fragile, however, and labeling often reveals a punctate pattern suggestive of hair cell death (Fig. 2C).

Cilia protruding through the pore of the neuromast can be detected under Nomarski optics (Fig. 2D-F). At late stages of neuromast differentiation, the number of hair cells increases to several tens (Fig. 2F). Labeling the stereocilia with fluorescent phalloidin demonstrates that hair cells are polarized along the antero-posterior axis (Fig. 2G-I). Young hair cells tend to appear as pairs of unpolarized cells (Fig. 2I, arrows), suggesting that, as in zebrafish, interaction between the two daughters of a hair cell precursor cell leads to their polarization towards each other, thereby generating pairs of cells with opposite polarity (Lopez-Schier and Hudspeth, 2006).

The cilia of hair cells are embedded in a gelatinous cupula, which can occasionally be seen under Nomarski optics (Fig. 3A-C). Cupulae are much longer in tuna than in zebrafish (as already reported by Kawamura *et al.*, 2003), and reach in excess of 130μm, as opposed to an average length of 20-40μm for zebrafish neuromasts (van Trump and McHenry, 2008).

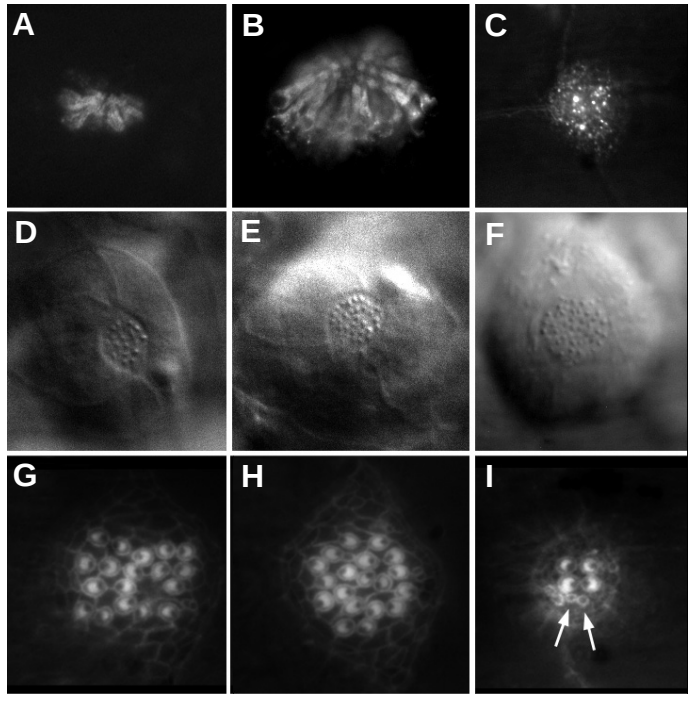

Fig. 2. Neuromast hair cells. (A-C) *Vital fluorescent labeling of hair cells in* Thunnus *neuromasts.* **(D-F)** *Apical surface of hair cells in neuromasts that comprise respectively 18-20, 30-32 and close to 50 hair cells. D and E are neuromasts L2 and L1 of the same* Thunnus *larva, F shows a neuromast in* Sarda. **(G-H)** *Antero-posterior polarity of hair cells visualized with fluorescein-coupled phalloidin in neuromasts L2, L3 and L4 respectively, of the same* Thunnus *embryo. Arrows: see text.*

Strands of cells extend between neuromasts (Fig. 3B-E, arrowheads), similar to the stripe of interneuromast cells observed in zebrafish (Grant *et al.*, Lopez-Schier and Hudspeth, 2005). Due to the dorsal migration of L1 (see below), this stripe is best observed between L1 and L2 (Fig. 3F, arrowheads). It seems plausible that this line of cells is at the origin of at least some of the many additional neuromasts observed on adult animals.

Hair cell cilia extend into the surrounding water through a pore in the skin (Fig. 3G, arrow) formed by 3-4 specialized peridermal cells (Fig. 3G, asterisks). In zebrafish, the migration of mature neuromasts involves a translocation of these specialized peridermal cells among their neighbors (Sapède *et al.*, 2002).

Pattern of the embryonic PLL

Thunnus thynnus embryos have about 40 somites, vs. 32 somites for zebrafish. Their embryonic PLL, as visualized by DiAsp labeling (Fig. 4A) comprises five neuromasts (L1-L5) followed by a terminal one near the tip of the body. *Sarda sarda* embryos have about 55 somites; their PLL has 6-7 neuromasts, and a terminal one (Supp. Fig. 1A,B). Neuromasts and neuromast innervation can also be visualized by anti-acetylated tubulin immunolabeling (Fig. 4B).

The first PLL neuromast occupies a dorsal position (Fig. 4A,B, Supp Fig. 1). We wondered whether this neuromast might actually correspond to D1, a dorsal neuromast deposited by a later dorsal primordium in zebrafish (Sapède *et al.*, 2001). We settled this question by examining pre-hatch embryos. We found cases where L1 was positioned along the myoseptum (Supp. Fig. 2A). Those were just beginning to differentiate, as evidenced by the presence of only two hair cells. In other cases, L1 was found at more dorsal positions (Supp. Fig. 2B,C), and had an increased number of hair cells. We conclude that L1 is deposited along the horizontal myoseptum, consistent with the observation that the nerve branch to L1 exits from the PLL nerve (Fig. 4B), and migrates soon after deposition to reach a dorsal position after one day (Fig. 4A-C). The migration of L1 is followed later by a less extensive dorsal migration of L2, and then by L3. Intriguingly, L2 first migrates ventrally as it does in zebrafish, and subsequently migrates dorsally (Supp. Fig. 2 D,E).

The early migration of L1, and resulting extension of its nerve branch, occasionally reveals a splitting of this branch in two tracts (Supp. Fig. 2 F,G). Splitting of the L1 branch, as well as of part of the PLL nerve, was also observed at a later stage, in one out of five cases where we labeled the PLL nerve by an application of the lipophilic dye, DiI, rostral to L1 (Supp. Fig. 2H).

The position of PLL neuromasts on the two sides of the body rarely coincides (Supp. Fig. 1B), indicating that the deposition is probably not triggered by spatial landmarks. The distribution of neuromast positions in both *Thunnus* and *Sarda* embryos (Fig. 4E,F) shows an increasing dispersion of positions from anterior to posterior neuromasts, as also reported in zebrafish (Gompel *et al.*, 2001), consistent with the idea that neuromast deposition results from a cyclic process that is intrinsic to the primordium.

Early larval development of the terminal system

A single terminal neuromast, ter1, is found about halfway between the tip of the tail and the posteriormost pigment cell, at the end of *Thunnus* (Fig. 4A,B) and *Sarda* embryogenesis (Supp. Fig. 1). The position of ter1 is precisely determined in both species, as the neuromasts on the two sides of the body always overlap by more than 50% (see e.g. Fig. 5B-E). A second neuromast, ter2, appears in a more anterior position early during larval life, at 5-6 days post-hatch (dph). ter2 is invariably located close to the posterior edge of a pigment cell which itself occupies a reproducible location on the ventral side of the tail (Fig. 5C, 5mm larva).

A third neuromast, ter3, forms just posterior to ter2 when the larva reaches 6mm (7dph, Fig. 5D). The initial position of ter3 next to ter2 (Fig. 5D), and the fact that its nerve branch always arises from ter2 (Fig. 5F), suggests that ter3 is formed by budding from

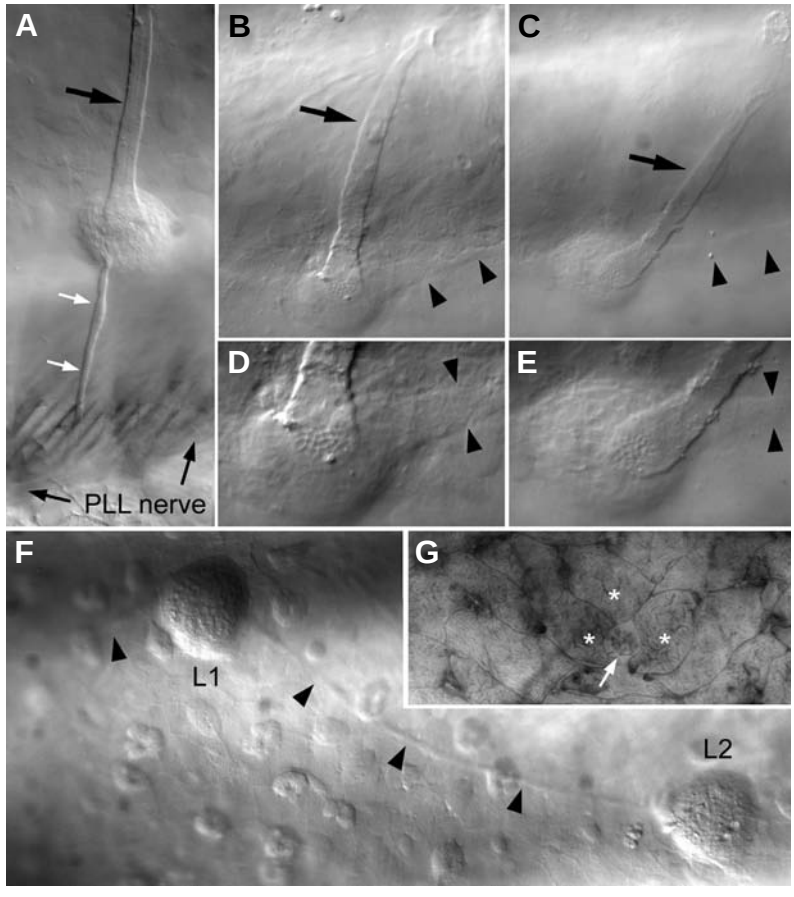

Fig. 3. Embryonic posterior lateral line (PLL) of *Sarda* embryos. (A-C) *neuromasts L1, L2 and L3 of the same embryo. Large arrows indicate the cupula, arrowheads indicate the strand of interneuromast cells, and white arrows indicate the branch of the PLL nerve extending to neuromast L1.* **(D,E)** *Higher magnification of panels B and C respectively, showing the tightly packed apical surfaces of hair cells at the base of the cupula.* **(F)** *Continuous strand of interneuromast cells extending between L1 and L2.* **(G)** *Three peridermal cells (asterisks) form the pore (arrow) through which hair bundles extend into the surrounding water.*

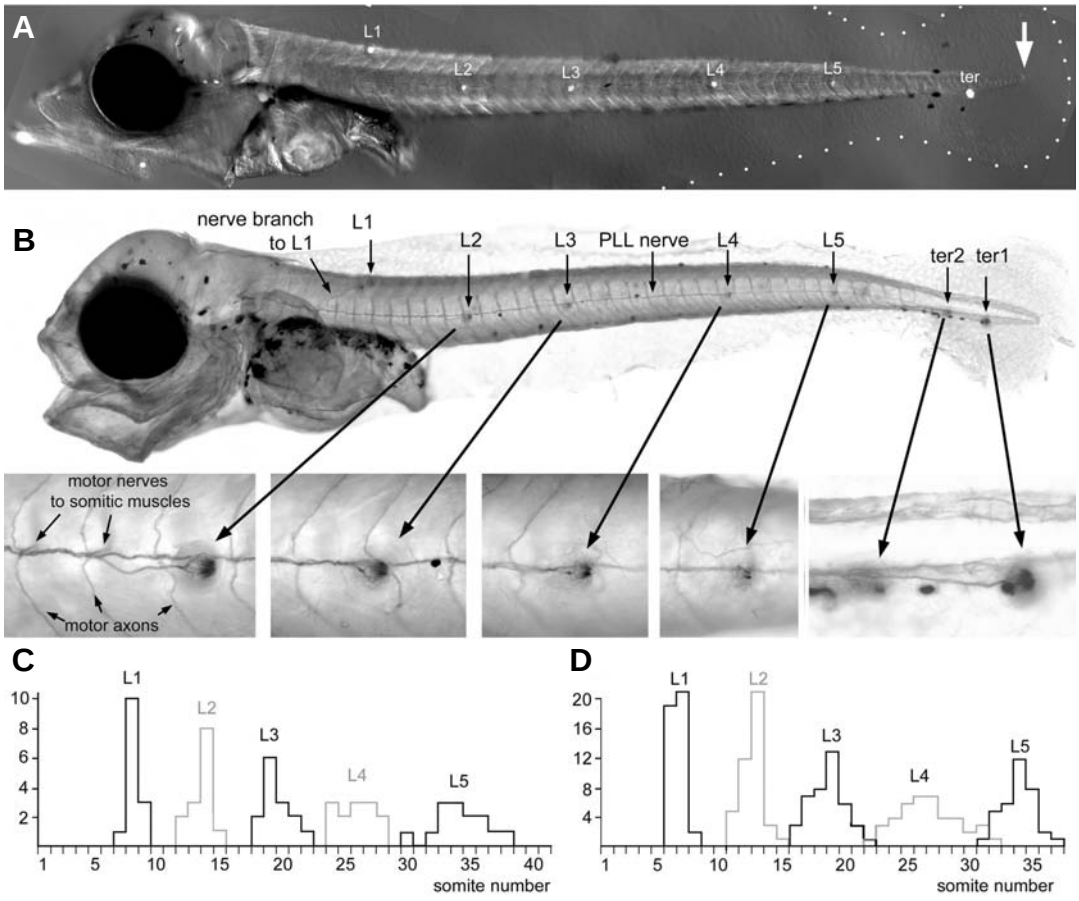

Fig. 4. Posterior lateral line (PLL) pattern. (A) *As visualized by DiAsp fluorescent labeling in a 3dph larva. The white arrow marks the tip of the tail.* **(B)** *As visualized by anti-acetylated tubulin immunolabeling, in a 3.9mm* Thunnus *larva: notice that the post-embryonic neuromast ter2 is beginning to differentiate. The number of hair cells is >6 in L2, 6 in L3, 4 in L4 and 2 in L5.* **(C,D)** *Positions of the first five neuromasts in* Sarda *and* Thunnus *early larvae, respectively.*

ter2 (Wada *et al.*, 2010). ter3 migrates ventro-posteriorly (Fig. 5E,F) above the first hypural plate, H1, which corresponds to the ventral part of the forming caudal fin (Supp. Fig. 4A). Ter3 eventually buds off a fourth neuromast, ter 4, when the larva reaches 8 mm (9 dph, Fig. 5F). There is no further development of the system up to two weeks (10mm larvae, Supp. Figs. 3 and 4B). At this stage, as expected from their position relative to the hypural plates, ter3 and ter4 are located in the ventral half of the well developed caudal fin, whereas ter1 is located in the dorsal half (Supp. Fig. 4B).

Discussion

The embryonic PLL of *Thunnus* is strikingly similar to that of the relatively basal *Danio rerio*, confirming the conservation of embryonic PLL patterns among teleosts (Pichon and Ghysen, 2004). Here we show that not only the embryonic pattern, but essential aspects of its development, are also conserved: the migrating primordium is accompanied by sensory axons, presents 2-3 rosettes in its trailing region, and deposits neuromasts within which hair cells appear as pairs of opposite polarities, as well as interneuromast cells. These similarities suggest that the major features of PLL development, including the molecular bases of primordium organization and migration, and of neuromast differentiation and innervation, have been conserved throughout teleosts.

We will discuss separately three differences between *Thunnus* and *Danio*: the large size of the cupulae, the dorsal migration of L1, and the early larval development of the terminal system.

The cupulae of tuna embryos reach in excess of 130μm, as opposed to an average length of 20-40μm for zebrafish neuromasts. Extrapolating results on the dynamic properties of zebrafish cupulae (van Trump and McHenry, 2008), tuna cupulae should be ten times more sensitive, and have a much lower frequency cut-off value, possibly as low or lower than 1Hz. This suggests that tuna cupulae are exceedingly sensitive, but unable to provide a frequency analysis of the stimulus. The PLL system would thus mostly be used as trigger for a strike reaction during early larval life: a highly sensitive water movement detector may allow the hatched larva to feed quickly, an essential requirement given that the yolk is completely used one day post-hatch (as opposed to 3-4 days in zebrafish larvae). The size of their cupulae may make tuna neuromasts highly susceptible to shearing forces, which obviously do not exist in their normal environment. The fragility of large cupulae could contribute to the extreme sensitivity of tuna larvae to handling, and may also explain why most

neuromasts show badly damaged hair cells just after transfer to the DiAsp solution (Fig. 2C), as this transfer necessitates pipetting of the larvae.

Zebrafish neuromasts migrate ventrally, starting with L2, whereas in tuna they migrate dorsally, except for L2 which first migrates ventrally and subsequently veers dorsally. The intriguing behavior of L2 suggests that dorsal migration of PLL neuromasts in tuna has been superimposed on an ancestral tendency to migrate ventrally. The dorsal migration of neuromasts is not specific for perciforms, as dorsally migrated neuromasts have also been reported in medaka (*Oryzias latipes*, Sapède *et al.*, 2001), which occupies an intermediate level between the basal ostariophysians and the derived perciforms. More species need obviously to be sampled before we can evaluate whether dorsal migration is a monophyletic feature, and at what stage in teleost evolution it is likely to have appeared.

Terminal neuromasts are distinct from the other PLL neuromasts in several respects: not only do they mark the posterior end of the PLL, but in addition they are the starting point of the caudal lines that will later extend along the caudal fin (Wada *et al.*, 2006), and they are deposited ventrally rather than laterally, as the primordium veers ventrally and migrates along the ventral midline shortly before terminal deposition (Gompel *et al.*, 2001). In zebrafish the number of terminal neuromasts is variable (2-3, more rarely 4), as is their position (Wada *et al.*, 2008). Terminal neuromasts undergo a massive rearrangement associated with the dorsal flexure of the notochord, and end up as a vertical row of three neuromasts, T1 to T3 from dorsal to ventral, located on the three caudalmost scales (Wada *et al.*, 2008). T1 and T3 are at the origin of all caudal fin lines in several teleost species, whereas T2, whose position corresponds to the middle of the caudal fin, does not form any caudal fin line (Wada *et al.*, 2008). Similar roles could be played by ter1-3 in tuna, since ter1 ends up associated to the dorsal part of the caudal fin, whereas the migration of ter3 accompanies the expansion of hypural plate H1, corresponding to the ventral half of the developing caudal fin. However, whereas in *Danio* ter2-3 are deposited by the primordium, in *Thunnus* and *Sarda* ter2 is formed post-embryonically, and quickly generates ter3 to complete the terminal pattern. Thus similar larval patterns are achieved by different mechanisms in *Danio* and in *Thunnus/Sarda.* It seems conceivable that the highly reproducible sequence of formation of ter1, ter2 and ter3 in tuna embryos ensures that precursor neuromasts T1 and T3 will unerringly form the dorsal and ventral branches of the caudal fin lines. In zebrafish, the ventral branch is occasionally missing, probably due to imprecision in the mechanism of ter neuromast deposition (Wada *et al.*, 2008).

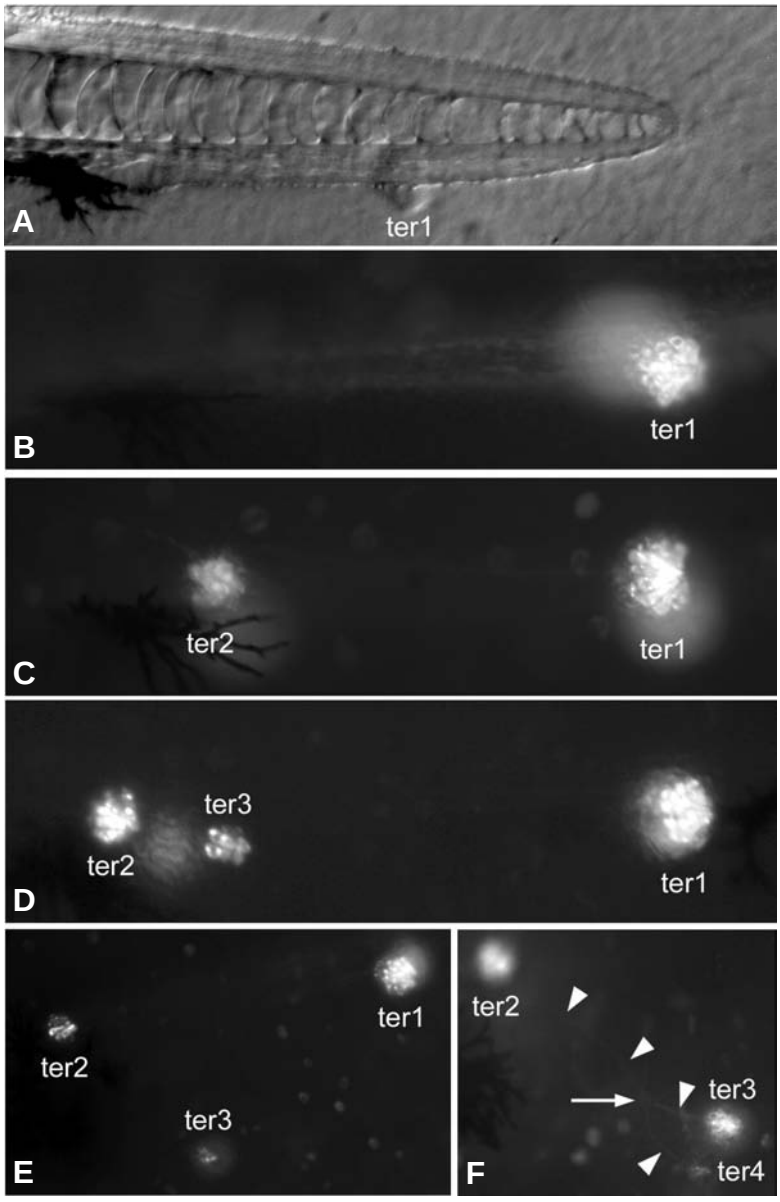

Fig. 5. Development of the terminal pattern. (A) *Position of ter1 in a* Sarda *embryo.* **(B)** *At 3dph, only ter1 is present.* **(C)** *At 5dph, ter2 is forming just posterior to a pigment cell.* **(D)** *At 7dph, in a 5.9mm larva, ter3 is appearing next to ter2.* **(E)** *At 8 dph, in a 7.6mm larva, ter3 is migrating away from ter2.* **(F)** *At 9dph, in a 8.1mm larva, ter4 has budded off from ter3. arrowheads: afferent fibers; arrow: place where ter4 split off from ter3, as evidenced by the branching of the afferent fibers (Wada* et al.*, 2010).*

Materials and Methods

Egg collection

The bluefin tuna broodstocks were composed of 35 fish with an estimated mean body weight of 100 kg. They had been kept in captivity for 3 years in a floating cage, at El Gorguel (Cartagena, Spain). Fifteen fish were induced to spawn using delivery systems loaded with GnRHa (Mylonas *et al.*,£2007) on June 26 and 27, and an egg collector was placed around the cage. Beginning 48-72h later, massive spawning occurred. 2.3 million eggs were collected at dawn on July 30, with sea water temperature at 23.5°C. Collected eggs were transported in a 500l plastic tank supplied with pure oxygen to the IEO facilities at Puerto de Mazarrón, and washed with sterilized sea water. Egg diameter was 1032.13 ± 32.89 µ. Each egg had 1.28 ± 0.52 oil globules and its dry weight was 61.82 ± 1.05 µg. The exact age of the embryos is not known, as the time of spawning was not precisely determined, but they must have been around 28-34hpf (hours post-fertilization), assuming they were laid between midnight and 6AM. The onset of primordium migration

must therefore be close to 24 hpf, as in zebrafish.

Atlantic bonito eggs were obtained from captive breeders stocked in IEO land based facility at Puerto de Mazarron, Spain (11 fish with a weight between 2 and 3 kg). Natural spawning started on May 13 and extended over more than fifty days. Fish spawned at dusk, between 19 and 22 h. Eggs were collected in the early morning and were washed with sterilized water, cleaned and counted. Fertilization rate was 62%, egg diameter was 1280.88 ± 35.33 µm, and each egg had 4.47 ± 1.41 oil globules. Temperature was 22°C.

Cleaned eggs were reared in the IEO facilities at Puerto de Mazarron, or transported by car to IFREMER facilities in Palavas, France, and by air to HCMR facilities in Heraklion, Crete. Rearing conditions ranged from the semi-extensive mesocosm (at HCMR) to the pseudo green water (at IEO and HCMR) and clear water methods (at IFREMER and IEO), with no detectable difference in the PLL pattern. Surface skimmers were installed between 2-4 and 10 days post-hatch (dph) to maintain a clean surface and facilitate swim bladder inflation. Temperature, salinity and dissolved oxygen were monitored daily in the tank. Phytoplankton was added twice daily in the tanks with mesocosm and pseudo-green conditions, in order to maintain a green medium until 14 dph. Feeding was based on enriched rotifers, beginning at 2 dph, followed by enriched 2d instar *Artemia* nauplii at 9dph, and by newly hatched larvae at 15dph. Weaning to minced fish was initiated at 23 dph in pseudo-green and 35dph in mesocosm conditions.

Neuromast and nerve labeling

For live fluorescent labeling of the hair cells, larvae were transfered as gently as possible to a solution of DiAsp (4-(4-diethylaminostyryl)-N-methylpryridinium iodide (Sigma D-3418), 5 µM in sea water, and left in the solution for 15-20 min (or more, to achieve afferent neurite labeling). The larvae were anesthetized in 0.5 mM tricaine (3-aminobenzoic acid ethyl ester) in sea water, and examined under epifluorescence.

For anti-acetylated tubulin immunolabeling, larvae were fixed in 2%TCA (trichloracetic acid) in sea water, rinsed in phosphate-buffered saline (PBS), and with PBS with 0.7% triton X-100 (PBT), 10min each. They were then treated for 2 min on ice with pre-chilled 0.25% trypsin. After two rinses with pre-chilled phosphate buffer, the larvae were post-fixed for 5min with 4% paraformaldehyde. They were rinsed with PBT, preincubated in PBS with 1% bovin serumalbumin and 10% lamb serum, incubated overnight at 4° in anti-acetylated tubulin from Sigma, diluted 1:1000, rinsed for 1 h in PBT, incubated 8-10 h in HRP-coupled secondary antibody diluted 1:1000, and processed for HRP reaction.

Fluorescent phalloidin labeling of hair cell microvilli was done as described in Lopez-Schier and Hudspeth, 2006. DiI labeling of the PLL nerve was done by iontophoretic application as described in Fame *et al.*, 2006.

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