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Thyroid hormone-dependent adult pigment cell lineage and pattern in zebrafish

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Abstract

Pigment patterns are useful for elucidating fundamental mechanisms of pattern formation and how these mechanisms evolve. In zebrafish, several pigment cell classes interact to generate stripes, yet the developmental requirements and origins of these cells remain poorly understood. Using zebrafish and a related species, we identified roles for thyroid hormone (TH) in pigment cell development and patterning, and in post-embryonic development more generally. We show that adult pigment cells arise from distinct lineages having distinct requirements for TH, and that differential TH-dependence can evolve within lineages. Our findings demonstrate critical functions for TH in determining pigment pattern phenotype and highlight the potential for evolutionary diversification at the intersection of developmental and endocrine mechanisms.

Vertebrates exhibit a stunning variety of pigment patterns yet the mechanisms underlying pattern development and evolution are only beginning to be discovered. Among the most conspicuous and elaborate patterns are those of teleost fishes, which function in mate choice, shoaling, camouflage, and speciation (1–3). In zebrafish *Danio rerio*, the adult pattern comprises dark stripes of black melanophores and a few iridescent iridophores, alternating with light "interstripes" of yellow/orange xanthophores and abundant iridophores, all within the hypodermis, between the epidermis and myotome (4) (Fig. 1A). Short-range and long-range interactions among pigment cells are critical for patterning (5–9) and dynamics of some of these interactions resemble those predicted by Turing models of pattern formation (10, 11). Despite the emphasis on "local" pigment cell autonomous interactions, tissue-specific positional information (7, 8) and "global" endocrine factors are likely required as well.

Adult pattern development involves post-embryonic differentiation and morphogenesis, potentially subject to hormonal control. By ~4 days post-fertilization (dpf) an embryonic/

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early larval (EL) pattern has formed with melanophore stripes and widespread, autofluorescing xanthophores (12) that subsequently disappear (fig. S1). Then, over ~4 weeks the pattern transforms into that of the adult (13) (movie S1). Adult melanophores and iridophores arise from "extra-hypodermal" peripheral nerve-associated precursors that migrate to the hypodermis (14–16). Iridophores differentiate in the interstripe, establishing the adult pattern; melanophores initiate their differentiation more widely over the flank and adult interstripe xanthophores appear later, in association with iridophores. Stripes gradually become more distinct as adult and residual EL melanophores in the interstripe die, are covered by iridophores, or join adult stripes, and as remaining melanophores expand and align at stripe edges (7, 14, 16–20). We hypothesized that the morphogenesis and differentiation of pigment cells during adult stripe development requires thyroid hormone (TH), which regulates metabolism, has cell-type specific effects and is essential for the abrupt metamorphoses of amphibians and flatfishes (21–25).

To test TH-responsiveness of pigment cells, we reared larvae in the tetra-iodinated form of TH, T4, and observed a marked xanthophore excess and melanophore deficiency (fig. S2). These phenotypes resembled the mutant *opallus*^{b1071}, which we identified as harboring a missense mutation (D632Y) in *thyroid stimulating hormone receptor* (*tshr*) identical to a human mutation causing constitutive Tshr activity and hyperthyroidism (26) (Fig. 1A; fig. S3). *opallus* mutants had elevated expression of the TH precursor gene *thyroglobulin* (*tg*) and T4, more xanthophores, and fewer melanophores (Fig. 1B,C,F; movies S1, S2). Although xanthophores were the last adult pigment cell type to differentiate on the lateral flank of wild-type (WT) fish, they were the first to differentiate in *opallus* mutants (Fig. 1F; fig. S4).

To determine if TH is required for pattern development, we sought to abolish TH production and so generated a transgenic line, *Tg(tg:nVenus-2a-nfnB)*$^{wp.rt8}$, to ablate the thyroid by metronidazole (Mtz) treatment (27). Fish thyroid-ablated at 4 dpf exhibited no *tg* mRNA or T4 expression as juveniles, lacked adult body xanthophores and had excess melanophores (Fig. 1A,D,E,G; movies S3, S4). Although some xanthophores developed eventually, they were delayed relative to stage progression, which was itself retarded (figs. S4, S5). Hypothyroid fish also had craniofacial and other defects, resembling the mutant *manet*$^{wp.r23e1}$, which we identified as a hypomorphic allele of *tshr* expressing *tg* mRNA at levels 7% of WT (fig. S6).

To better understand the TH-dependence of xanthophores, we first identified their origins. Adult fin xanthophores and melanophores arise from stem cells (28), whereas adult body melanophores and iridophores arise from extra-hypodermal, peripheral nerve associated precursors (14, 15, 16); fate mapping has not revealed an extra-hypodermal source of body xanthophores. We hypothesized that xanthophore precursors are present already in the hypodermis, and, specifically, that EL xanthophores give rise to adult xanthophores. Consistent with this idea, EL xanthophore ablation resulted in adult xanthophore deficiency (as well as defects in adult melanophore patterning; fig. S7).

To test whether EL xanthophores are a source of adult xanthophores, we fate-mapped these cells with nuclear localizing photoconvertible Eos (nEos) driven mosaically by the promoter

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of the xanthophore pigment synthesis gene *aox3* (17). EL xanthophores over the lateral myotomes lost their pigment by ~8 dpf yet persisted and proliferated (Fig. 2A; fig. S8; movie S5). Later, photoconverted cells were found within the interstripe, where many reacquired pigment; others, in the interstripe or in melanophore stripes, failed to reacquire pigment. Thus, some adult xanthophores originate directly from neural crest-derived EL xanthophores: these cells enter a cryptic period, in which they lose pigment and proliferate; some of these cells, localizing in the interstripe, then redifferentiate during adult pattern formation. Additional cells initiated *aox3* expression only after early larval stages—and thus exhibited only unconverted nEos in the juvenile—indicating a source of adult xanthophores independent of EL xanthophores as well (Fig. 2B).

In thyroid-ablated fish, EL xanthophores persisted, and new *aox3*$^{+}$ cells developed, but none acquired pigment by juvenile stages (Fig. 2C). Thus, TH is not essential for survival of xanthophore lineage cells but instead promotes their differentiation. In hyperthyroid *opallus* mutants, xanthophores were 40-fold more likely to divide as compared to WT (fig. S9; movie S6), and differentiated not only in the hypodermis but also extra-hypodermally (Fig. 3A). Stable *aox3:GFP* reporter lines confirmed the presence of unpigmented precursors in hypothyroid larvae, and the presence of these cells within melanophore stripes of euthyroid larvae (fig. S10); *aox3*:GFP^{+} cells were also observed extra-hypodermally even in WT (Fig. 3B). Thus, although interstripe xanthophores are the last adult pigment cell to differentiate (7; fig. S4), xanthophore precursors are present throughout pattern formation, suggesting that interactions between melanophores and xanthophores that contribute to stripe patterning (5, 6, 11) depend on the differentiative states of the interacting cells. Additionally, because xanthophore precursors were widely distributed but differentiated principally in the interstripe, where their densities increased relative to stripes (fig. S10), a close interplay of differentiation and migration is likely important for establishing and maintaining pattern.

Effects of TH on zebrafish xanthophores led us to ask whether xanthophores of other species are TH-dependent as well. Thus, we examined *D. albolineatus*, which has an evolutionarily derived pattern in which differentiated xanthophores are intermingled with melanophores (Fig. 3C) (29). In this species, we found extra-hypodermal xanthophores similar to hyperthyroid *D. rerio* (Fig. 3A,B; fig. S11). To see if TH requirements are conserved, we ablated thyroids of *D. albolineatus*. These fish had hypothyroid phenotypes, yet still developed extra-hypodermal xanthophores and some hypodermal xanthophores (Fig. 3D; fig. S12), suggesting a partial evolutionary decoupling of xanthophore development from TH in *D. albolineatus*.

In contrast to xanthophores, melanophores were more numerous in hypothyroid fish of both species, and this was particularly evident in the interstripe of *D. rerio* (Fig. 1G). To test if TH represses proliferation or survival of these cells, we treated fish with N-phenylthiourea to retard melanization of new melanophores and facilitate tracking previously melanized cells (28) (fig. S13). In euthyroid larvae, melanophores rarely divided and often died in the interstripe. In hypothyroid larvae, these cells divided frequently and were more likely to survive, whereas in hyperthyroid larvae, melanophores differentiated but many died (Fig. 4A; movies S3, S4, S7, S8). To assess roles for TH in earlier melanophore lineage development we examined *mitfa:GFP*^{w47}-expressing presumptive melanophore precursors

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(14). *mitfa*:GFP$^+$ cells of hypothyroid larvae were less likely to differentiate, arrive at the hypodermis, or survive (fig. S14). These defects may indicate a metabolically compromised state in the absence of TH. Thus, TH promotes hypodermal melanophore precursor abundance and differentiation, while simultaneously having a greater, repressive effect on melanophore proliferation and survival.

The preceding analyses focused on pattern development, but mechanisms are also needed for pattern maintenance (5, 10, 11) and it remains unclear if patterning mechanisms at one stage may or may not be relevant at another. To test if TH is required for homeostasis, we ablated thyroids after the adult pattern had formed. Six months later, these fish had extra melanophores, wider stripes and craniofacial defects, yet xanthophore numbers were unchanged (Fig. 4B, fig. S15). Thus, TH limits melanophore population expansion homeostatically but is not required by xanthophores once they have differentiated. Roles for TH in amniote melanocytes have not been well-studied but our finding that TH limits melanophore numbers may be translationally relevant as hypothyroidism is especially prevalent among melanoma patients (30, 31).

We have identified origins of adult xanthophores distinct from those of melanophores and iridophores, revealing a previously unappreciated diversity in post-embryonic neural crest lineages underlying adult pigment pattern (fig. S16A). We also found that TH represses melanophore numbers and promotes xanthophore development, yet this latter dependency has been reduced in *D. albolineatus*, raising the possibility of a new compensatory factor in this species (fig. S16B). Our results indicate that responsiveness to “global” factors such as TH should be considered along with modifications to “local” interactions in attempting to understand pattern development and evolution. The different TH-dependencies of these variously colored pigment cells (fig. S5C) further suggests the potential for complex selection involving behavior, endocrine mechanisms and diverse pigment cell lineages arising from different sources and having different fate restrictions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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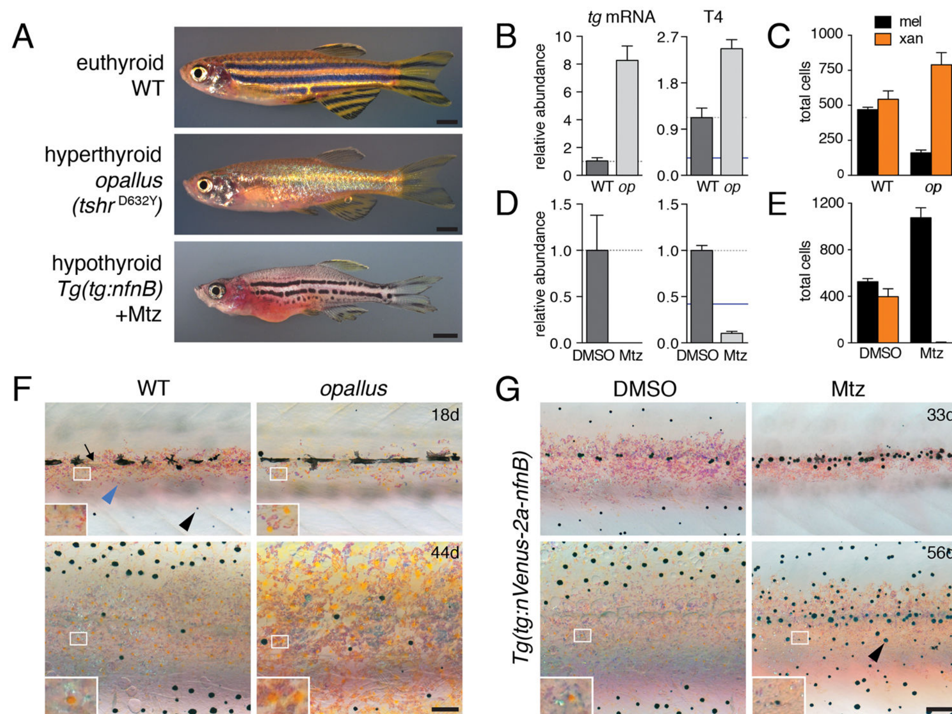

Fig. 1. TH is required for xanthophore development and melanophore repression

(A) WT, hyperthyroid and hypothyroid phenotypes. **(B, C)** In *opallus*, *tg* transcript and T4 were increased ($F_{1,4}$= 46, P<0.005; $F_{1,6}$=36, P<0.001; blue line, ELISA detection limit) and there were more xanthophores but fewer melanophores [$F_{1,4}$= 46, P<0.005; $F_{1,6}$=36, P<0.001; 17–22 mm standard length (SL), stage J++ (13)]. **(D, E)** In thyroid-ablated fish, *tg* mRNA and T4 were undetectable ($F_{1,4}$= 13, P<0.05; $F_{1,6}$=100, P<0.0001); xanthophores were missing but there were extra melanophores ($F_{1,14}$=29, P<0.0001; $F_{1,14}$=184, P<0.0001; 17–18 SL). **(F)** At 18 dpf (top), interstripe xanthophores had not yet differentiated in WT but were abundant in *opallus* (insets). Blue arrowhead, iridophores; black arrowhead, adult melanophore. By 44 dpf, *opallus* exhibited a gross xanthophore excess and severe melanophore deficiency. Fish were treated with epinephrine to contract pigment granules and facilitate counting. **(G)** Hypothyroid fish lacked xanthophores (insets) and had ectopic melanophores (black arrowhead). Note that development rate and xanthophore numbers were decreased even in DMSO controls owing to a TH-free diet. Scale bars: 2 mm (A); 100 μm (F, G).

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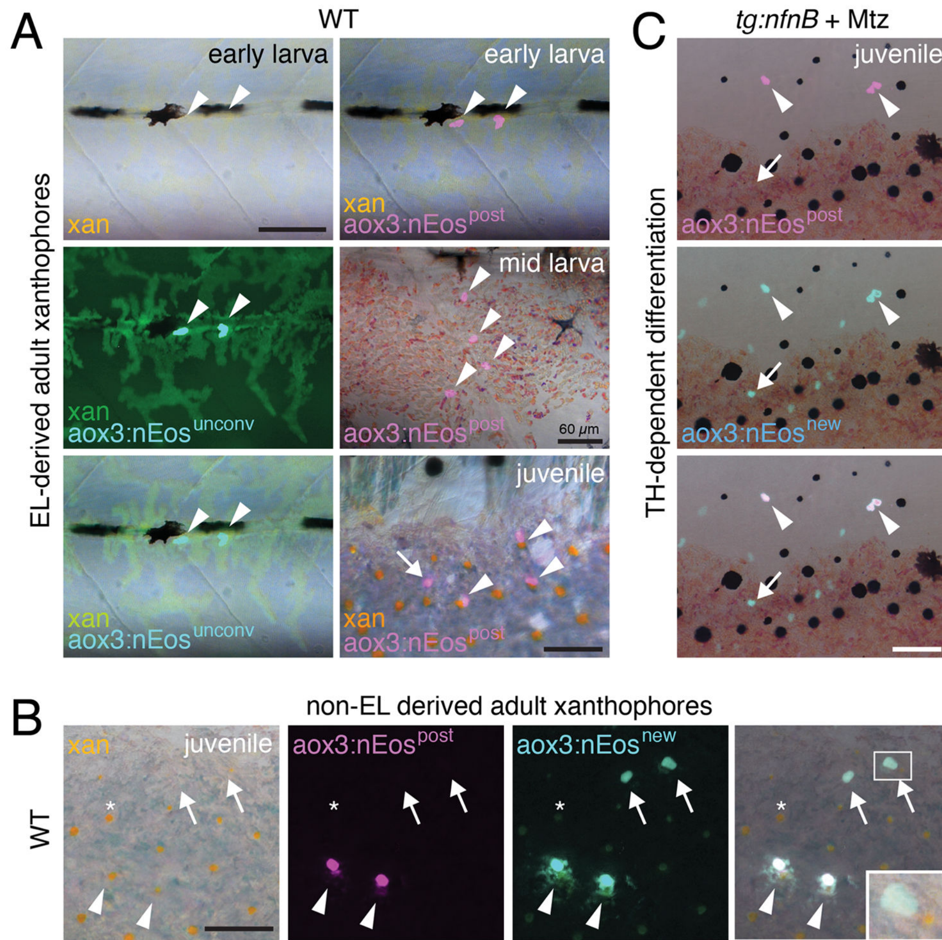

Fig. 2. Hypodermal xanthophore origins and TH-dependence

(A) Autofluorescing *aox3*:nEos+ EL xanthophores (left panels; post-photoconversion: upper right). Later, yellow pigment was absent and each cell had divided (middle right). In the juvenile, 3 cells redifferentiated (arrowheads) and one remained unpigmented (arrow). Overall, 86 photoconverted EL xanthophores generated 71 adult xanthophores and 78 unpigmented cells (*n*=44 larvae); including all stages examined, marked cells had a 59% probability of dividing (*n*=82 larvae, 190 EL xanthophores, 283 cells later). **(B)** In a juvenile in which all *aox3*+ cells had been photoconverted at 5 dpf, two photoconverted EL-derived xanthophores were visible (arrowheads) but so were cells expressing only unconverted nEos (arrows), one of which had differentiated (inset); 9 of 15 juveniles had unconverted cells that developed after 5 dpf (5.8±1.9, range 1–19 cells), of which 54% had differentiated as xanthophores. **(C)** In thyroid-ablated fish, the likelihood of photoconverted EL xanthophores (arrowheads) persisting into the juvenile did not differ from controls (X^2=0.01, d.f.=1, *P*=0.9; Mtz, *n*=18 larvae; DMSO, *n*=13 larvae, 20 EL xanthophores); some cells initiated *aox3*:nEos expression only after 5 dpf (e.g., arrow). Scale bars: 60 μm.

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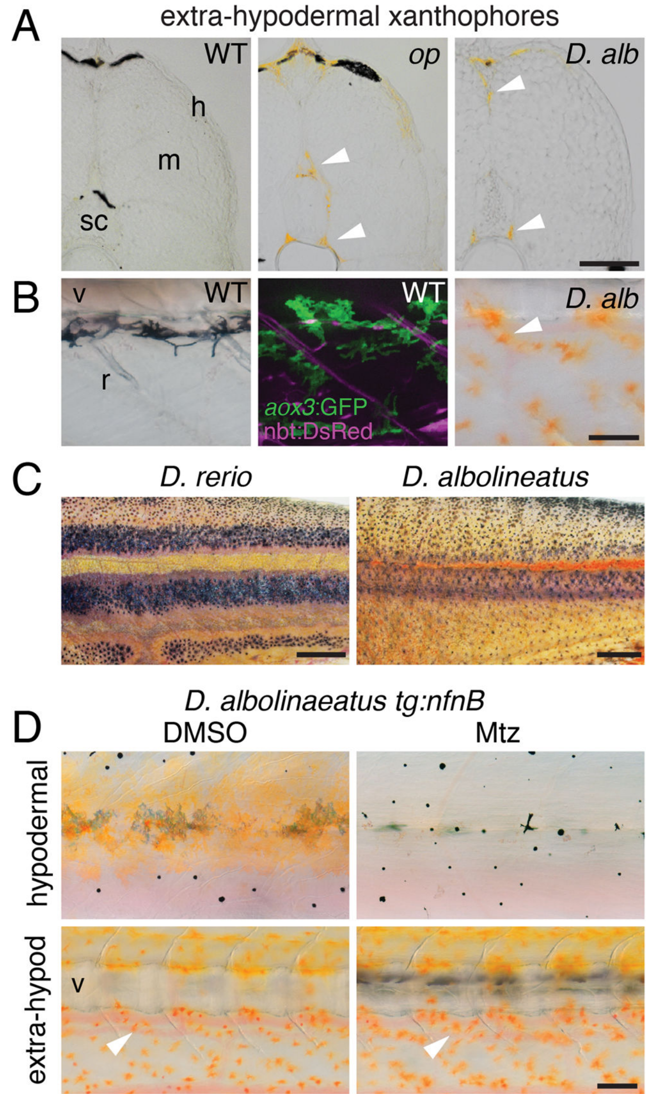

Fig. 3. Extra-hypodermal xanthophores and evolution of TH-dependence

(A) Hyperthyroid *D. rerio* (*op*) had extra-hypodermal xanthophores (arrowheads). These cells were present in wild-type *D. albolineatus* (*D. alb*) as well (stage AR). sc, spinal cord. m, myotome. h, hypodermis. **(B)** Medial *aox3*:GFP^{+} cells relative to *nbt*:DsRed^{+} peripheral nerves in WT *D. rerio* (left and middle) and xanthophores (right, arrowhead) in *D. albolineatus*. v, vertebral column; r, rib. **(C)** Adult *D. rerio* and *D. albolineatus*. **(D)** Thyroid-ablated *D. albolineatus* retained extra-hypodermal xanthophores (arrowheads), despite lacking most hypodermal xanthophores (fig. S12). Scale bars: 60 μm (A, B); 1 mm (C); 100 μm (D).

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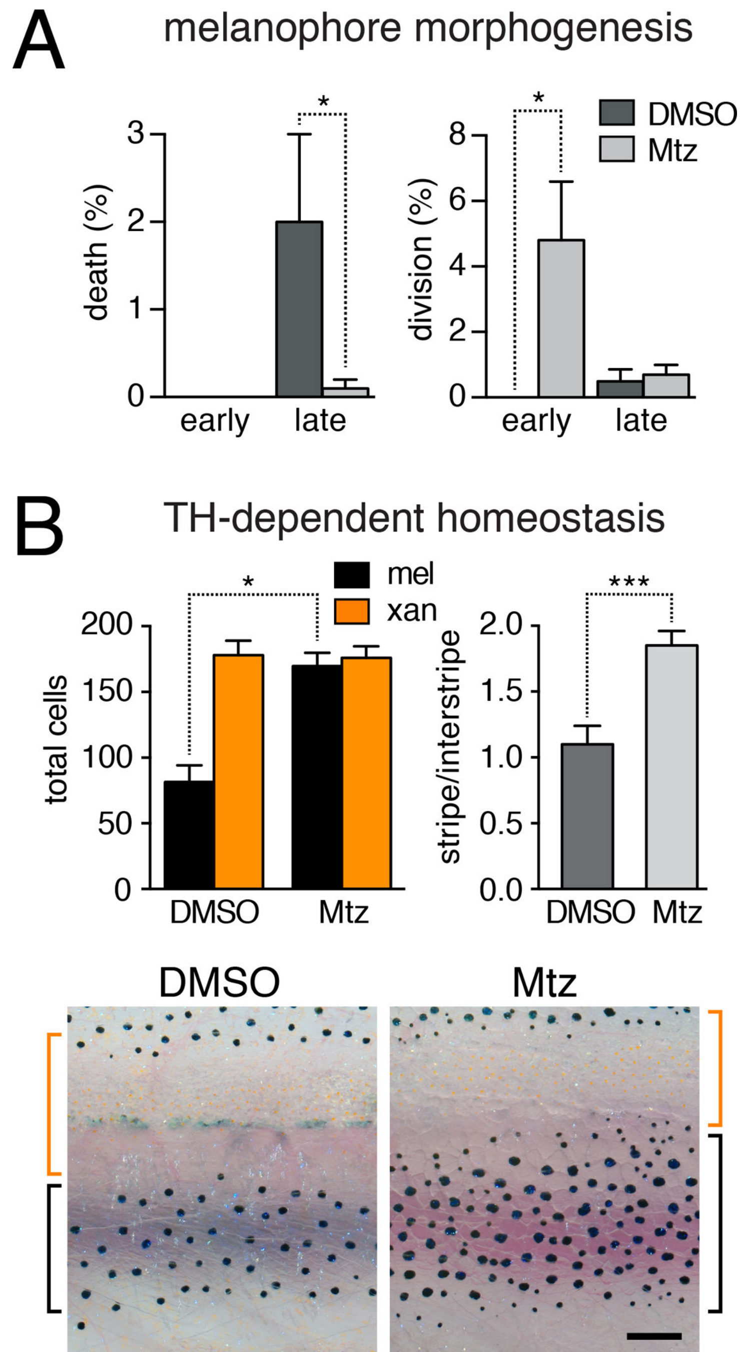

Fig. 4. TH-repression of melanophores

(A) Melanophores of thyroid-ablated fish were less likely to die at a late stage of pattern formation (PB+; X^2=35.7, d.f.=1, P<0.0001) and more likely to divide at an early stage (DR; genotype, X^2=10.5, d.f.=1, P<0.005; N=45 larvae, 2510 melanophores). *, Tukey Kramer *post hoc* P<0.05. **(B)** Thyroid ablation of juveniles resulted in more melanophores ($F_{1,19}$=50, P<0.0001; *P<0.05, Tukey-Kramer post hoc test) and wider stripes ($F_{1,19}$=19, P<0.0001, ***). Scale bar: 200 μm.