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Local reorganization of xanthophores fine-tunes and colors the striped pattern of zebrafish

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The pattern of alternating blue and golden stripes displayed by adult zebrafish is composed of three kinds of pigment cells - black melanophores, yellow xanthophores and silvery/blue iridophores. Here, we analyzed the dynamics of xanthophores during stripe morphogenesis in vivo with long-term time-lapse imaging. Larval xanthophores start to proliferate at the onset of metamorphosis and give rise to adult xanthophores covering the flank before the arrival of stem-cell-derived iridophores and melanophores. Xanthophores compact to densely cover the iridophores forming the interstripe, and acquire a loose stellate shape over the melanophores in the stripes. Thus, xanthophores, attracted by iridophores and repelling melanophores, sharpen and color the pattern. Variations on these cell behaviors could be at play in generating the great diversity of color patterns in fish.

Many animals have evolved a fascinating diversity in their color patterns, which serve as an essential component of their survival strategy. Replacing a much simpler larval pattern (Fig. 1, A and B), the zebrafish acquires a stereotypic pattern of four to five longitudinal dark stripes and four light interstripes (Fig. 1C) during the metamorphic period [20 days post fertilization (dpf) - 45 dpf]. The stripes consist of melanophores covered by a thin layer of iridophores and xanthophores, whereas interstripes are composed of iridophores covered by xanthophores (*1*–*4*). Iridophores come in two morphologically distinct shapes that are clonally related: dense iridophores in interstripes, and loose blue iridophores in stripes. The first light interstripe is formed by dense iridophores emerging along the horizontal myoseptum that serves as a morphological prepattern (*3*). At the margins of the first interstripe, iridophores switch shape and spread dorsally and ventrally as loose iridophores in the skin of the juvenile fish by proliferation and migration. They form a coherent net of cells covering the flank of the fish and generate additional light stripes at a distance from the horizontal myoseptum by patterned aggregation into dense iridophores (*5*). Melanoblasts emerge at their future position between these interstripes along peripheral neurons innervating the skin; they differentiate and expand in size to form the compact dark stripes (*5*–*7*). Thus, the dark and light stripes are formed by different cellular routes. Xanthophores are thinly distributed over the dark stripes and densely cover the iridophores in light stripes giving them a yellow tinge (*1*, *2*, *4*). Adult iridophores and melanophores originate from a small set of stem cells located at the segmentally reiterated dorsal root ganglia (*5*, *7*), but the origin of adult xanthophores has remained unknown.

The analysis of mutants lacking one or two of the three pigment cell types has revealed that interactions between all the three cell types are absolutely necessary for emergence of the final striped coloration (*3*, *8*). Iridophores play a leading role in stripe formation and mutants lacking iridophores do not add stripes to a basic pattern, whereas mutants lacking xanthophores (*pfeffer/fms/csl1a*) or melanophores *(nacre/mitfa)* exhibit a residual striped pattern, albeit irregular (fig. S1) (*3*, *8*–*10*). *pfeffer (pfe)* encodes *csf1a/fms* that is expressed and required specifically in xanthophores (*3*, *8*, *11*, *12*). In adult *pfe* mutants, a number of stripes and interstripes, although irregular, are discernible: the melanophore stripes break up into spots, dense iridophore regions invade the stripes, further, ectopic melanophores are observed in the interstripe regions. Introduction of xanthophore progenitor cells into *pfe* mutant embryos by blastomere transplantations restores a normal pattern indicating the importance of xanthophores in stripe sharpening and coloration (*8*, *11*). We examined xanthophore origin and behavior in vivo to establish their role in color pattern formation.

We used *Tg(fms:Gal4.VP16)* (*13*) in combination with appropriate UAS-reporter lines or *Tg(Pax7:GFP)* (see supplementary materials and methods) to label xanthophores from larval stages throughout metamorphosis (Fig. 1). Labeled cells were identified on the dorsal side of the embryo at ~24 hpf (movie S1). These cells migrate while dividing along the dorsolateral path of neural crest migration, differentiate into xanthophores and spread over the larval skin, ventrally decreasing in density (*1*) (Fig. 1, A and B). The larva at 5 dpf displays 6-8 xanthophores per segment. In the adult fish, xanthophores are distributed over the stripes and interstripes all along the skin (Fig. 1, C and D). The xanthophores associated with the interstripe region were densely packed, expanded, possessed small filopodial protrusions and displayed bright orange pigment (arrowheads in Fig. 1, D, E, and H; and fig. S2). In contrast, xanthophores in the stripe region displayed a netlike, loose organization; they were arborized, had long filopodial protrusions and displayed faint yellow pigment (arrows in Fig. 1, D, F, H; and fig. S2). Xanthophore density was ~2.5 times higher in the interstripe region as compared to the stripe region (Fig. 1I and fig. S2). This indicates two distinct types of xanthophores: compact and bright in the interstripes, and stellate and faint in the stripes.

Time-course analysis of xanthophores revealed that the larval xanthophores persisted, increased in number at the onset of metamorphosis and finally covered the entire dorsolateral skin (Fig. 1G). To obtain labeled clusters of xanthophores for lineage analysis, we transplanted cells of *Tg(pax7:GFP)* embryos into wild type hosts at the blastula stage (Fig. 2A). The xanthophore number remained constant until the onset of metamorphosis (~16-20 dpf) when xanthophores begin to divide and increase in number continuously with a doubling time of about one week (5 fish; 7 clusters of xanthophores; Fig. 2C). Second, we analyzed xanthophore clones induced in *Tg(sox10:ER^{T2}-Cre)* larvae in which tamoxifen-inducible Cre was expressed under the *sox10* promoter (*14*). DsRed-labeled larval xanthophores in such clones gave rise to metamorphic xanthophores (Fig. 2B; 6 clones). In contrast to previous studies (*10*, *15*), our in vivo analysis suggests that xanthophores, albeit barely visible by pigment content, are the first pigment cell type to cover the trunk skin before the appearance of metamorphic iridophores and melanophores (Figs. 1G and 2B, and figs. S3 and S4).

We further analyzed the behavior of clusters (coherent groups of clones) of labeled xanthophores in *Tg(sox10: Cre)* (*16*) individuals throughout metamorphosis (Fig. 3A and fig. S5). The xanthophore clus-

ters maintained their relative positions during the formation of the stripes and interstripes, whereas numbers of xanthophores in each cluster continuously increased throughout metamorphosis (Fig. 3C and fig. S6) by proliferation (fig. S7). Thus, there is no global reorganization of xanthophores during stripe morphogenesis (fig. S9). However, arrival of the iridophores during metamorphosis lead to a compaction of the overlaying xanthophores (fig. S4). Furthermore, upon arrival of melanophores in the stripe region, the xanthophores underwent local rearrangement to accommodate the increasing number of melanophores (Figs. 3B and 4). Over the period of metamorphosis, more melanophores appeared in the skin and expanded, forming a compact stripe (*5*). This resulted in cellular reorganization and separation of the xanthophores and a decrease in xanthophore density (Figs. 1, D to I, and 3B and fig. S6B). Xanthophores of the stripe region reorganize their filopodia to give way to melanophores that appear in their vicinity (Fig. 4A). The xanthophores spread out and become stellate with long filopodial extensions (arrows in Fig. 4, B and C) forming a netlike organization in the stripe region (figs. S8 and S9).

Xanthophores at the boundary of the developing stripes and interstripes often exhibited filopodial extensions directed toward the interstripe region leading to cell division followed by integration of the proximal xanthophore into the interstripe (arrow in Fig. 4B) or a short-scale movement of the xanthophore into the interstripe region (arrowheads in Fig. 4C, and fig. S10). Limited local proliferation and context-specific local reorganization was the most prominent behavior exhibited by xanthophores (Fig. 4C). This is in contrast to iridophores which exhibit extensive local proliferation combined with long-range dispersal along the dorsoventral axis and prefigure the stripe organization by patterned aggregation (*5*). Genetic analyses have indicated a role for xanthophores in sustaining melanophore numbers but also in sharpening the stripe-interstripe boundary by mutual repulsion (*3*, *8*, *11*). Xanthophores present in the first interstripe take part in elimination of the larval lateral stripe melanophores as well as metamorphic melanophores that get trapped in the interstripe. In contrast, the stellate xanthophores of the stripes might be required for survival of melanophores, and for compact stripe formation and maintenance. In summary, we found that the striped organization of pigment cells involves proliferation, local rearrangements and short-scale movements of xanthophores in the skin of the fish.

Our observations do not support Turing-type models in their current form, which assumes that stripe formation starts with a random distribution of melanophores and xanthophores that sort out by repulsion and attraction involving extensive cell movements (*15*, *17*–*19*). These models may hold for patterning in the fins [however see (*20*)], but would require significant modification for the body stripes to accommodate the cellular behaviors of xanthophores and melanophores observed in the present study, the *pfe* mutant phenotype and the involvement of iridophores (*3*, *5*, *8*). Long-term imaging indicates that stripe melanophores barely move whereas xanthophore movement is restricted to the boundary region between stripe and interstripe. The interactions between all the three kinds of pigment cells and their environment are crucial for stripe pattern formation (*3*, *8*, *10*). Xanthophores play a permissive role in the formation of the striped pattern by covering the skin of metamorphic fish, into which metamorphic iridophores and melanophores get integrated (schematic in fig. S11). The striped pattern is achieved by context-dependent cell shape changes occurring both in iridophores and xanthophores which switch between loose and dense shapes. In both cases the two forms are clonally related and depend on local cell-to-cell interactions. Melanophores in the stripes exist in only one shape. The precise superposition of the dense form of iridophores and xanthophores in the interstripe and the loose iridophores and xanthophores superimposed over the stripe melanophores cause the striking contrast between the golden and blue coloration of the pattern.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1254837/DC1
Materials and Methods
Figs. S1 to S11
References (*21–31*)
Movie S1

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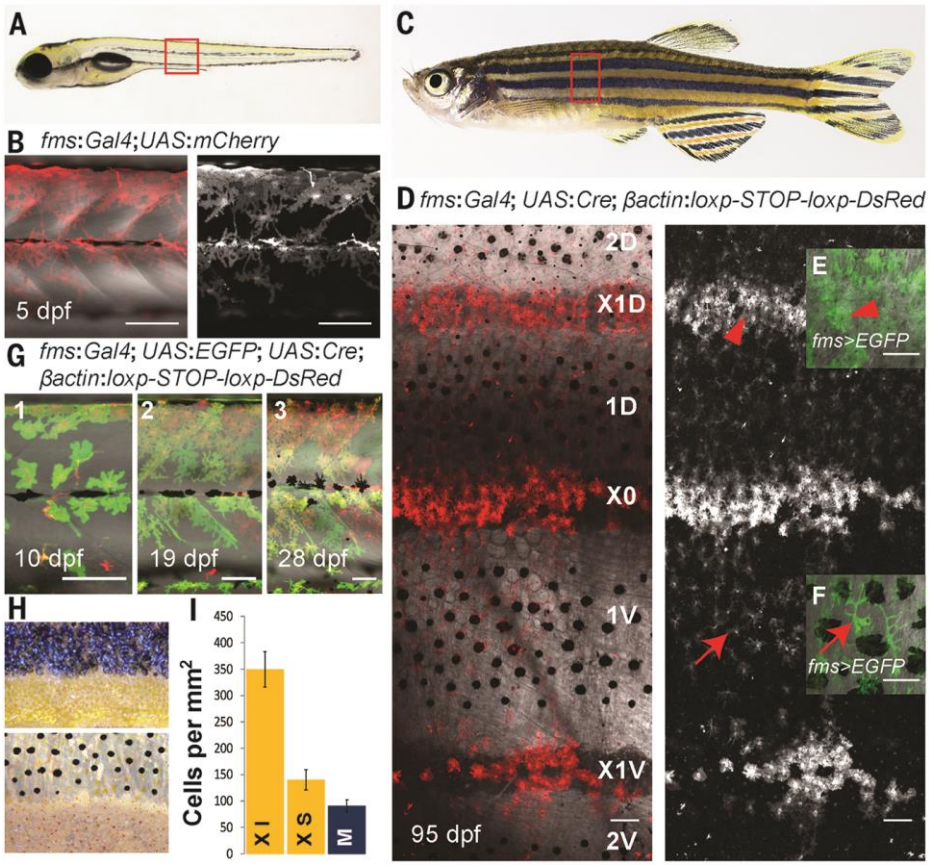

Fig. 1. Distribution of xanthophores in larval, metamorphic and adult zebrafish. Pigmentation pattern and xanthophores of the (**A** and **B**) larva and (**C** and **D**) adult. Arrowhead: dense interstripe xanthophores; arrow: loose stripe xanthophores. (**E** and **F**) xanthophores at higher magnifications. (**G**, 1-3) Continuous development of xanthophores from larval to postmetamorphic stages. (**H**) Normal (above) and epinephrine-treated (below) zebrafish adult skin. (**I**) Density of interstripe xanthophores (XI), stripe xanthophores (XS) and melanophores (M) in adult zebrafish. Scale bars: 100 μm.

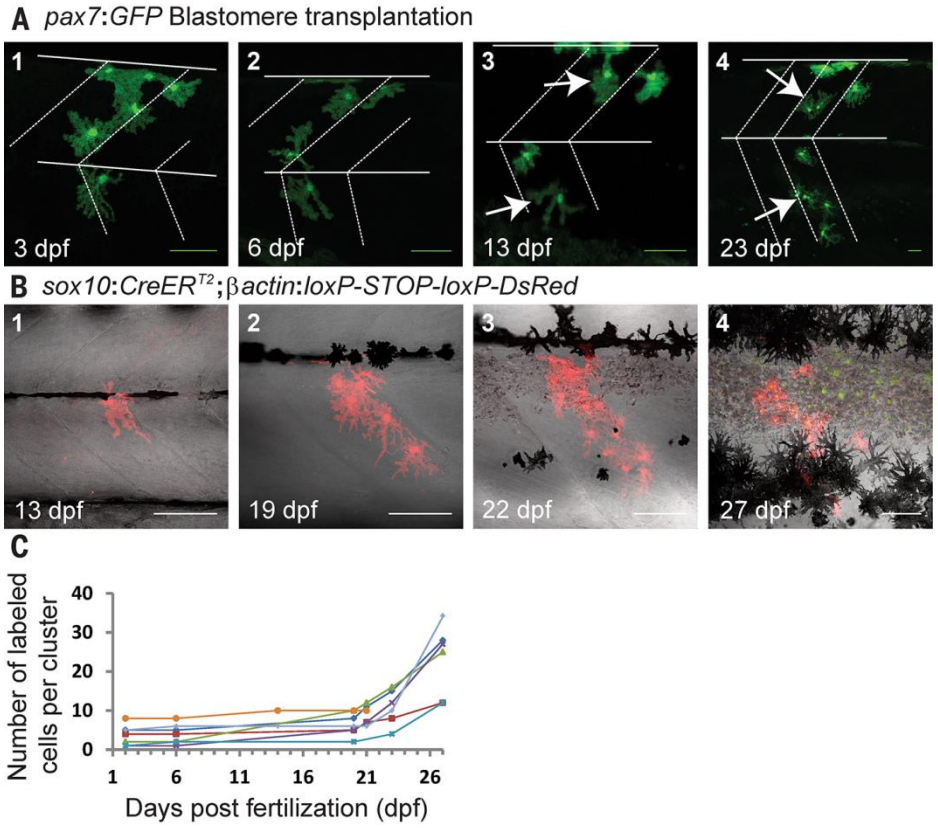

Fig. 2. The larval pattern develops continuously into the adult pattern. (**A**, 1-4) A *Tg(pax7:GFP)* labeled cluster of 6 xanthophores obtained by blastomere transplantation, White dotted lines: myosepta. Arrows in (A, 3-4): mitotic cells at 23 dpf. (**B**) Xanthophore clone induced at 5 dpf. (**C**) Increase in numbers of xanthophores per individual *Tg(Pax7:GFP)* cluster; each line represents a single identified cluster. Scale bars: 100 µm

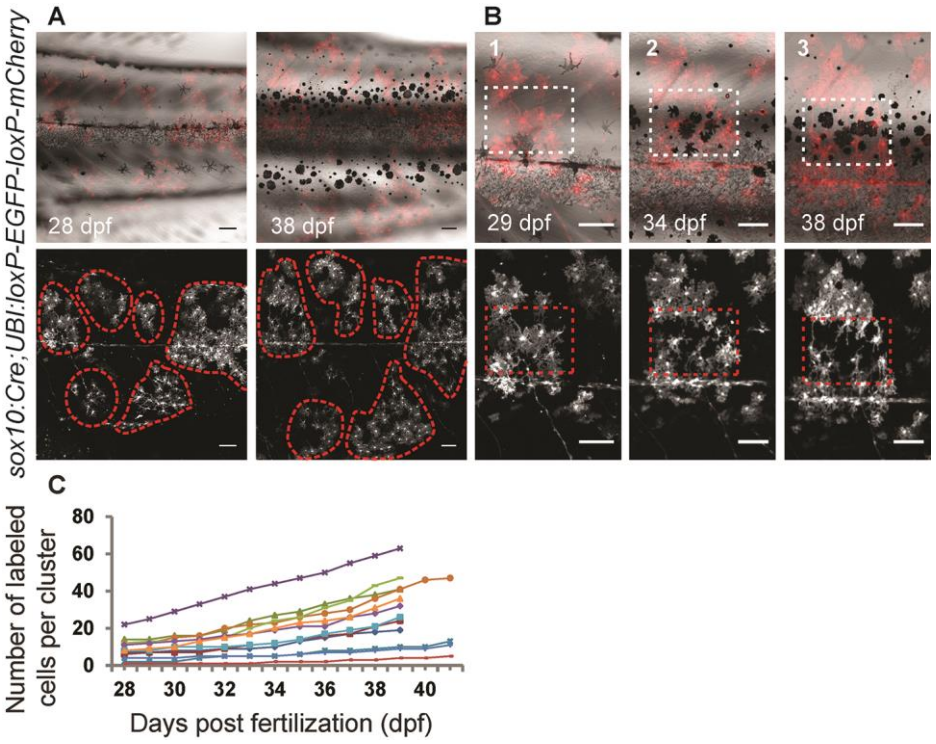

Fig. 3. Xanthophores exhibit limited global reorganization. (**A** and **B**) Clusters of *Tg(sox10:Cre)*-labeled xanthophores. Dashed lines encircle individual clusters that are identifiable throughout stripe morphogenesis. (B) Arrival of melanophores in the stripe region leads to separation of xanthophores in a cluster. Dashed square indicates presumptive stripe region where melanophores appear. (**C**) Increase in numbers of xanthophores per individual *Tg(sox10:Cre)*-labeled cluster; each line represents a single identified cluster. Scale bars: 100 μm

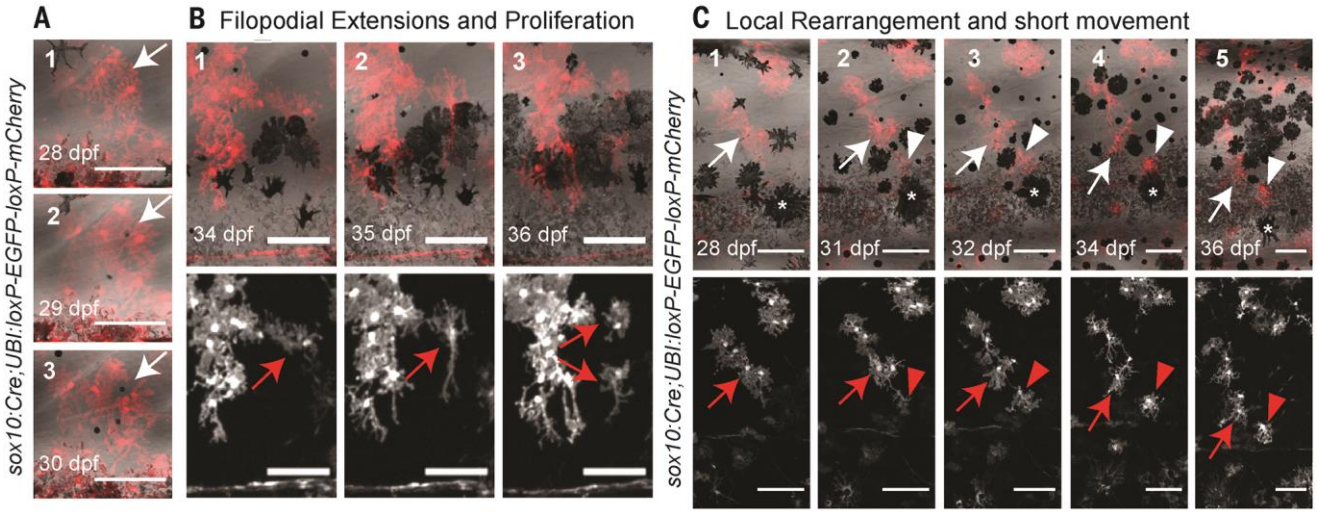

Fig. 4. Context-dependent changes in xanthophore behavior during stripe pattern formation. Time-lapse imaging of labeled xanthophores during metamorphosis. (**A**, 1-3) Xanthophores present in the presumptive stripe region give space (arrow) to arriving melanophore. (**B**, 1-3) a xanthophore near the stripe/interstripe boundary extends filopodial protrusions (arrows) directed toward the interstripe and undergoes cell-division. (**C**, 1-5) xanthophore (arrow) locally reorganizes upon arrival of melanophores in the stripe region. Short movement of xanthophore into interstripe region (arrowhead). Asterisk indicates a melanophore that moves away from the interstripe upon encountering a xanthophore (arrowhead). Scale bars: 100 μm