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Evolution of pigment cells and patterns: recent insights from teleost fishes

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Skin pigment patterns of vertebrates are stunningly diverse, and nowhere more so than in teleost fishes. Several species, including relatives of zebrafish, recently evolved cichlid fishes of East Africa, clownfishes, deep sea fishes, and others are providing insights into pigment pattern evolution. This overview describes recent advances in understanding periodic patterns, like stripes and spots, the loss of patterns, and the role of cell-type diversification in generating pigmentation phenotypes. Advances in this area are being facilitated by the application of modern methods of gene editing, genomics, computational analysis, and other approaches to non-traditional model organisms having interesting pigmentary phenotypes. Several topics worthy of future attention are outlined as well.

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Introduction

Biological patterns are all around us: from regularly arranged termite mounds over hundreds of thousands of square kilometers, to rows of cilia in *Stentor* over just a few micrometers [1,2]. In between are patterns formed by cells in tissues, including those generated by pigment producing cells in the skin. Such patterns can be brilliantly colored, muted, or monochromatic, ordered or haphazard, variable or invariant between individuals, stable or transmutable over an organism's lifetime. The diversity of skin pigment patterns is matched by diversity in function: helping to avoid predators, facilitating social aggregation or evaluation of prospective mates, signaling to rivals, or protecting from UV [3,4]. Here, I focus on advances in understanding the evolution of pigment patterns in half of the world's vertebrates—the bony fishes.

Chromatophore origins and arrangements

Pigment patterns of mammals depend on a single type of pigment cell, the melanocyte, which produces melanin that can be transferred to keratinocytes for incorporation into hair. In teleosts and other ectotherms, however, colors and patterns depend on several classes of pigment cells, 'chromatophores,' that typically retain their pigments intracellularly [5]. The most well studied cells are black melanophores. Other chromatophores include yellow/orange xanthophores containing pteridines and carotenoids, and iridescent iridophores with flat, reflective guanine crystals that provide structural colors. Chromatophores are derived from the embryonic neural crest, either directly, or indirectly via latent progenitors in the peripheral nervous system or skin [6–9]. Pigment patterns of ectotherms thus depend on the types and arrangements of chromatophores, that is, when and where these cells differentiate, migrate, proliferate, or die.

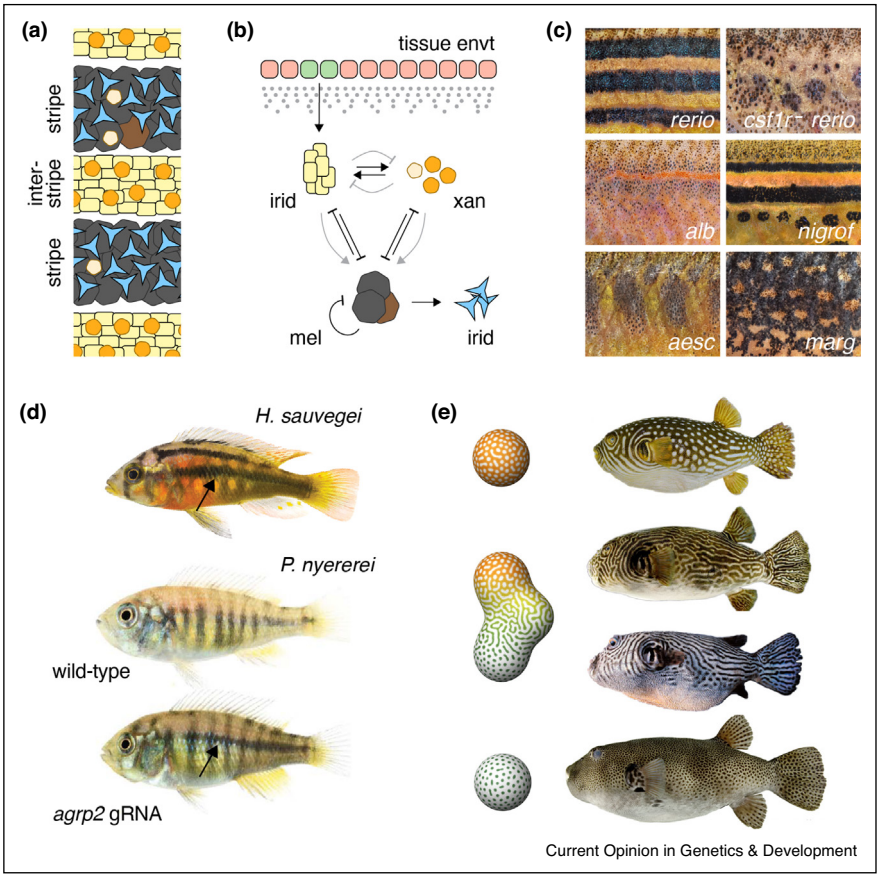
Much of what we know about chromatophore patterning comes from zebrafish, *Danio rerio*, with its blue–black stripes (melanophores with iridophores) and yellow–gold 'interstripes' (xanthophores with iridophores) (Figure 1a). This pattern depends on an early influence of the tissue environment, which provides positional information for initiating and orienting the pattern, as well as subsequent interactions within and between chromatophore classes that allow for a high degree of self-organization once patterning is underway (Figure 1b) [9–11,12•]. The critical roles of the environment and chromatophore interactions are evident in the defective patterns of mutants with perturbations of tissue architecture, missing chromatophore classes, or loss of specific signals required for cell–cell communication (Figure 1c, *top*).

Stripes, bars, and what lies in between

Inferences and approaches from zebrafish can provide a starting point to understand other patterns, especially of other *Danio* species (Figure 1c). For example, *Danio albolineatus* has a uniform pattern of intermingled chromatophores. Yet, a mutational approach revealed weak, underlying stripes [13], suggesting a striped pattern has been obscured evolutionarily.

Xanthophores of *D. albolineatus* are also especially abundant, maturing earlier and over a wider area than in zebrafish, associated with a *cis*-regulatory difference that drives earlier, broader expression of 'xanthogenic' Colony stimulating factor 1. These observations prompted the hypothesis that so many early xanthophores deprive

Figure 1



Periodic pattern development and evolution. **(a)** In zebrafish, *D. rerio*, dark stripes consist of melanophores (dark grey cells) and bluish iridophores (blue), whereas light interstripes have densely packed yellowish iridophores (yellow) and mature xanthophores (orange). Most melanophores derive from post-embryonic progenitors that differentiate during the larva-to-adult transition, but some develop directly from neural crest cells and persist from the embryonic pattern (brown). Some immature xanthophores occur within stripes (pale yellow). **(b)** During post-embryonic development, cues from the tissue environment (arrow, upper left) allow iridophores to differentiate in a first interstripe after which interactions among chromatotophores drive pattern implementation, refinement, and reiteration. Some interactions have positive effects on differentiation, survival or localization (arrows), others have negative effects (bars), some are at short range (black arrows/bars) whereas others are at long range (grey arrows/bars). Iridophores of interstripes and stripes are distinct subclasses (see text). During pattern formation and homeostasis, the tissue environment also provides supportive factors that regulate differentiation, survival, and proliferation (small grey circles). Summarized from Refs. [9–11,12•]. **(c)** Pattern variation in *Danio* showing interstripes and stripes of wild-type *D. rerio*, and defective pattern in a mutant for *colony stimulating factor 1 receptor a* that lacks xanthophores, and therefore, the chromatophore interactions in which xanthophores participate (top row). Also shown are naturally occurring patterns of other danios mentioned in main text, *D. albolineatus*, *D. nigrofasciatus*, *D. aesculapii*, and *D. margaritatus*. **(d)** In many African cichlids, horizonal stripes (arrow) are found on the flank and these species have lower levels of *agrp2* expression (top). In a species that normally lacks horizontal stripes (middle), inactivation of *agrp2* allows an ectopic stripe to develop (bottom, arrow; modified from Ref. [25••]). **(e)** Theory predicts a labyrinthine transitional state between light and dark spots (left). In actual pufferfishes, genomic analyses support the notion of ancestral hybridization giving rise to labyrinthine species (modified from Ref. [38••].

melanophores of directional cues needed for consolidating into stripes. Indeed, when Csf1 was expressed similarly in zebrafish, a pigment pattern resembling *D. albolineatus* developed, consistent with differential Csf1 expression contributing to the normal pattern difference between species [14].

Two recent studies further highlight how evolutionary changes in the timing or quality of chromatophore interactions can influence patterning. *Danio nigrofasciatus* has fewer stripes and interstripes than zebrafish (Figure 1c) and resembles zebrafish mutants with defects in signaling by Endothelins, a class of peptides secreted by skin cells and received by G-protein coupled receptors on pigment cells. Because hybrids of wild-type zebrafish with *D. nigrofascatus* and other *Danio* species resemble zebrafish [9,15], crosses of other danios to zebrafish mutants can help to screen candidate genes for differences between species, similar to complementation tests within a species. When *D. nigrofasciatus* were crossed to zebrafish

endothelin 3b mutants, progeny had fewer melanophores and iridophores than control hybrids, suggesting a hypomorphic allele in *D. nigrofasciatus* [16••]. Indeed, the *D. nigrofasciatus endothelin 3b* allele was expressed at lower levels than the wild-type zebrafish allele in a common hybrid background, consistent with a *cis*-regulatory change between species. A role for differential expression in generating the pattern difference was further indicated by transgenic supplementation of Endothelin 3b expression in *D. nigrofasciatus*, which resulted in additional iridophores and the organization of melanophores into an additional stripe, similar to zebrafish. Although Endothelin 3b might be assumed to act directly on melanophores—as Endothelin 3 acts directly on melanocytes in mammals—its primary effect here was on iridophores. This in turn suggests a model in which the pattern difference between species depends, at least in part, on reduced expression of Endothelin 3b in *D. nigrofasciatus*, which leads to fewer iridophores, fewer interstripes, and an earlier cessation of the iridophore–melanophore interactions necessary for reiterating stripes.

Another danio, *Danio aesculapii*, is the sister species of zebrafish [17] and has dark vertical bars and light 'interbars' (Figure 1c). To test if chromatophore interactions are conserved, despite the difference in pattern orientation, *D. aesculapii* mutants lacking each chromatophore class individually were generated and their phenotypes compared to those of corresponding mutants in zebrafish [18••]. Residual patterns in *D. aesculapii* suggested a more important role for interactions between melanophores and xanthophores, and a less important role for interactions with iridophores, as compared to zebrafish. To further test whether specific genes differ in their activities between species, additional mutants were generated in *D. aesculapii* for loci thought to mediate interactions between chromatophore classes. These were then used in reciprocal hemizygosity tests with wild-type and mutant zebrafish. One of these candidates, *kcnj13*, encodes a potassium channel needed to depolarize melanophore membranes upon contact with xanthophores; *kcnj13* mutant zebrafish have fewer, wider stripes [19,20]. Hybrid crosses suggested that *D. aesculapii* and two other danios have hypomorphic alleles of *kcnj13*, compared to wild-type zebrafish, and it will be interesting to learn how such differences have contributed to pattern diversification across the genus [18••].

Stripes and bars—and the investigations of them—extend beyond *Danio*. In East Africa, hundreds of cichlid species have originated over tens of thousands to a few million years with diverse behaviors and morphologies that include horizontal stripes, vertical bars and other patterns [21–24]. Stripes have evolved repeatedly, and genetic and developmental analyses have now identified a pivotal role for Agouti-related protein 2 (Agrp2) [25••]. Agouti represses melanin production in amniotes [26–28] and plays a similar role in zebrafish, leading to a pale ventrum [29,30]. In cichlids, *agrp2* is expressed broadly in the skin, but several striped species express less transcript because of independently evolved *cis*-regulatory variants [25••]. These observations suggested a model in which stripe evolution requires a loss of Agrp2-repression of melanophore differentiation, allowing these cells to develop in new locations. If true, then a species that normally lacks horizontal stripes might develop them upon inactivation of *agrp2*. Indeed, CRISPR/Cas9 targeting of *agrp2* led a vertically barred species, *Pundamilia nyererei*, to develop a horizontal stripe resembling that of its close relative, *Haplochromis sauvagei*, demonstrating a role for the locus and the power of applying modern developmental genetic methods to non-traditional models (Figure 1d).

Besides experiments on fish themselves, mathematical approaches have been helpful in understanding periodic patterns and transitions among them. For example, an agent-based model built on empirical results from zebrafish suggests that iridophores confer an overall robustness to zebrafish patterning, whereas differences in xanthophore effects on iridophores may contribute to stripe loss in *D. albolineatus* and spot acquisition in *Danio margaritatus* (Figure 1c) [31••]. This and a similar model [32•] help identify promising hypotheses to test, especially when pattern differences have a polygenic basis and when meiotic mapping is not feasible, as is true for many *Danio* and other species.

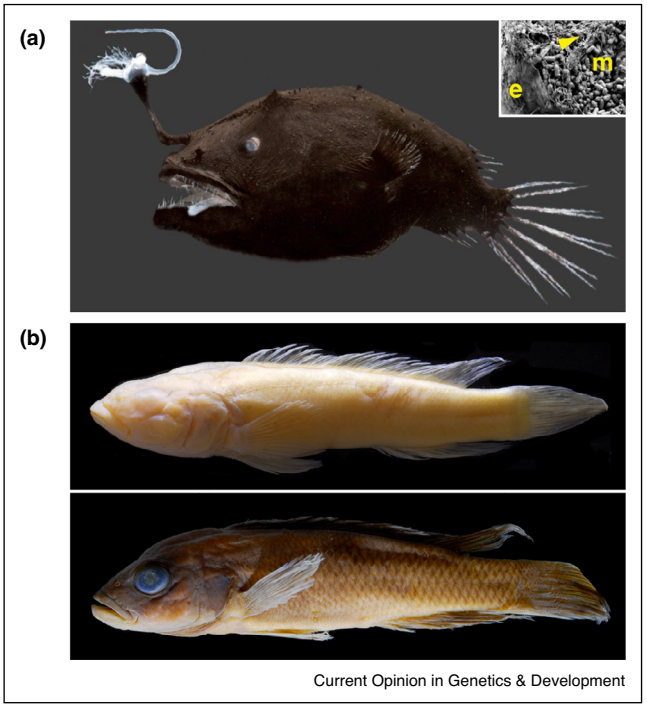
A different mathematical approach has considered chromatophore arrangements as Turing patterns, with molecular or cellular dynamics concordant with reaction-diffusion systems [10,33]. Recent advances have expanded the biological applicability of such models and place them within an increasingly rigorous framework for hypothesis testing [34•,35]. Two new studies hint at this potential. One of these studies focused on barred and spotted patterns of catfish, *Pseudoplatystoma*. Using a more elaborate Turing model than is typically applied, it was possible to account for different dynamics of processes occuring in different tissue compartments: a putative signal generating a pre-pattern in one layer of skin, and its impact on responding chromatophores in another layer of skin [36]. The second study built on an observation from simple Turing models that dark spots on a light background can transition to light spots on a dark background through a labyrinthine intermediate (Figure 1e). This mathematical property suggested the biological possibility that labyrinthine patterns might sometimes result from hybridization between species having reciprocal—dark versus light—spots [37]. To test this idea, phylogenetic distributions of 900 labyrinthine patterns (across >18,000 species) were assessed relative to spotted patterns and found to be concordant with repeated spot-to-labyrinthine transitions. Whole-genome sequencing of several labyrinthine and spotted *Arothron* pufferfish species further revealed extensive admixture

across phylogenetic lineages, with labyrinthine species having portions of spotted genomes, consistent with ancestral hybridization events leading to spotted–labyrinthine transitions [38••].

Fish in black and white

The preceding section emphasized studies of periodic patterns, but a different kind of pigmentation has evolved in deep sea fishes. Species across several orders are 'ultra-black,' reflecting as little light as the darkest of organisms or man-made materials (Figure 2a). This likely helps to avoid being seen by predators—or tipping off prey—even when the only light is from one's own bioluminescent lure. Ultra-black pigmentation has arisen convergently and depends on exceptionally large, rounded, and densely packed melanin-containing organelles— melanosomes—that meet theoretical predictions for minimizing reflectance [39••]. Remarkably, these melanosomes appear to be loose in the stroma, rather than contained within cells. It remains to be determined whether melanosomes are extruded from melanophores, similar to how melanocytes deliver melanin to keratinocytes for incorporation into hair or feathers, or melanosomes remain after melanophores die, or have another origin entirely. Deep sea fishes may never be amenable to experiments in the lab, but other approaches will hopefully provide clues to the cellular mechanisms that underlie this remarkable phenotype.

Figure 2



Uniform pigmentation when light is absent. **(a)** The ultra-black bathypelagic fish *Oneirodes eschrichtii*, illustrating dark integument and lure for catching prey. Inset, scanning electron micrograph showing melanosomes (m), amongst collagen fibrils (arrowhead) beneath the epidermis (e; modified from Ref. [39••]). **(b)** Preserved specimens of the albino cave cichlid *Lamprologus lethops* (top) and a closely related surface species *L. teugelsi* (bottom). Photo credits, © Danté Fenolio, DEEPEND project (a) and Melanie Stiassny (b).

The opposite phenotype occurs in another permanently dark environment, caves (Figure 2b). There are more than 200 species of cave-dwelling fishes, across which melanin has been lost independently. Whether this is because selection no longer maintains a pigmentary phenotype that cannot be seen, or because there are specific advantages to its elimination, remains unclear [40]. The most-studied case is Mexican tetra, *Astyanax mexicanus*, in which melanin has been lost owing to mutation in an orthologue of the human albinism gene *OCA2* [41,42]. An *oca2* mutation is likewise responsible for albinism in a cave-dwelling cichlid [43].

Studies of additional cave species should indicate whether changes at this locus or others [44] have contributed to albinism more generally, and would provide clues to how genetic architecture factors into regressive phenotypic evolution.

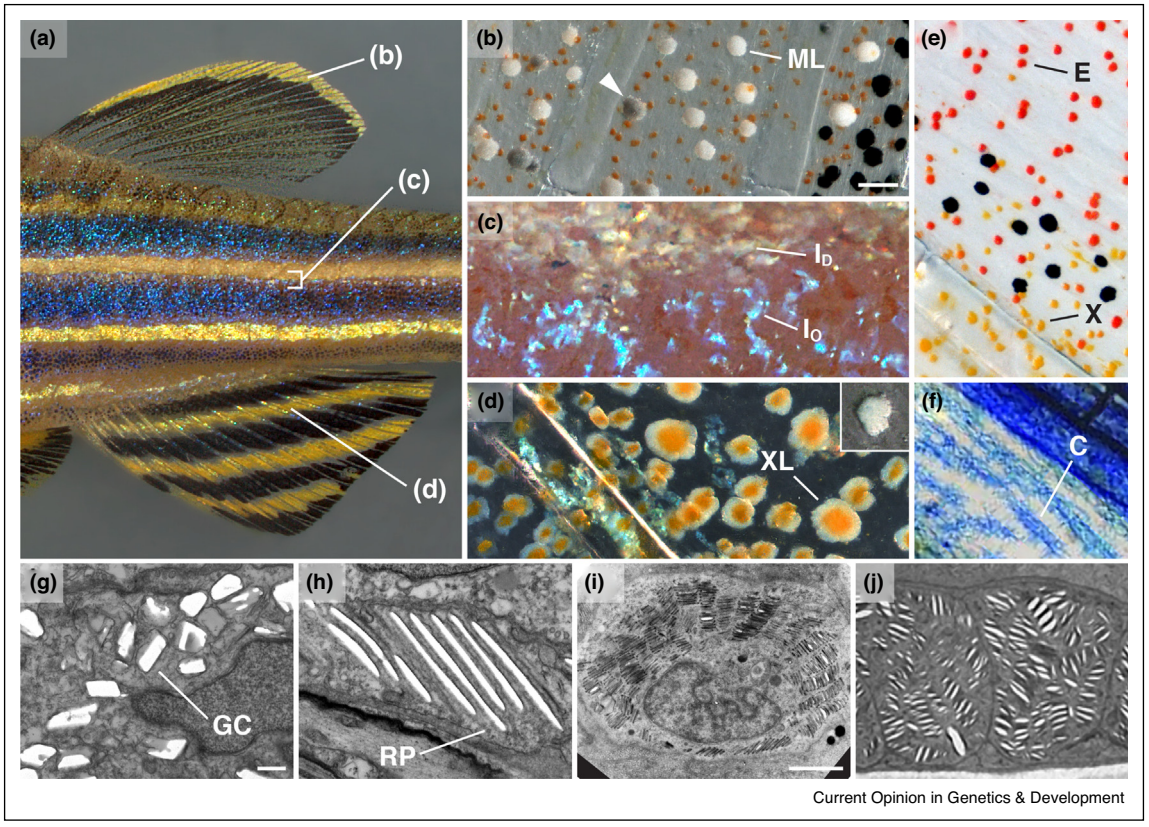
Diversification of chromatophore types

Cavefish can be white owing to loss of melanin, but even surface fish often have white pattern features. Their cellular bases point to roles for chromatophore-type diversification in generating pigmentary phenotypes. White chromatophores are referred to traditionally as 'leucophores' [5], and one example occurs in fin tips of zebrafish, where they may be especially visible during social interactions [45••] (Figure 3a,b). Surprisingly, these cells develop not from unpigmented stem cells, like other fin chromatophores [46], but from pigmented melanophores that lose their melanin while accumulating irregular crystals of guanine—the same material in iridophore-reflecting platelets [45••] (Figure 3g,h). The mechanisms underlying transformation from melanophore to 'melanoleucophore,' and the reasons why different danios have very different complements of these cells, remain to be determined.

A different type of white cell is responsible for white bars of clownfish *Amphiprion ocelaris* [47•] (Figure 4d, top left). Here, *bona fide* iridophores have centripetally arranged stacks of guanine-reflecting platelets, resulting in a matte white rather than iridescence (Figure 3i). These cells are just one of several iridophore subtypes, having distinct appearances and subcellular architectures, at least three of which occur in zebrafish alone [12•,48] (Figure 3c).

Other poorly known chromatophores include red erythrophores (Figure 3e), unusual blue cyanophores (Figure 3f), and even polychromatic cells with properties of more than one chromatophore class. For instance,

Figure 3



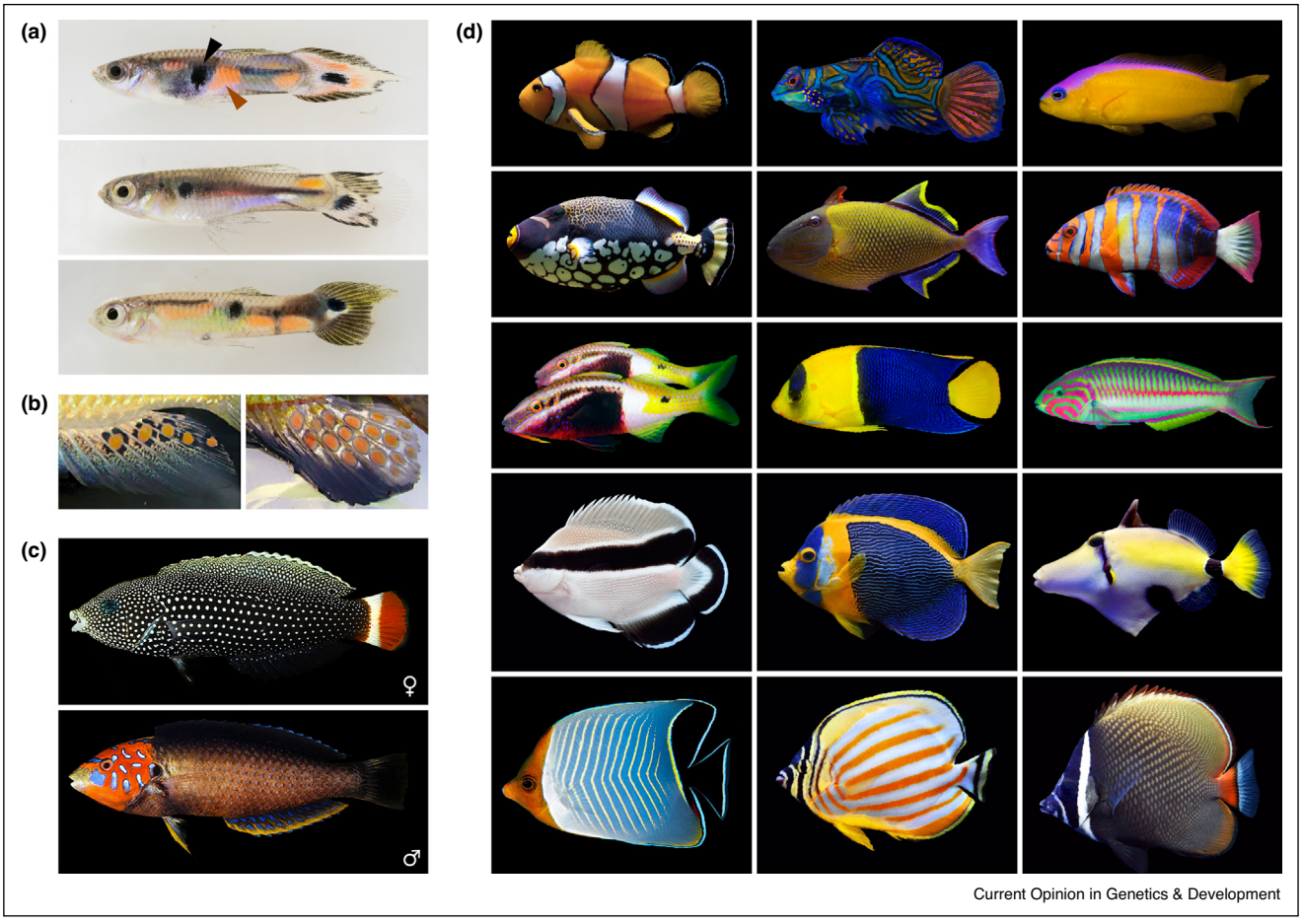
Chromatophore diversity. **(a)** The flank of a wild-type zebrafish, illustrating locations of cells shown in (b), (c), and (d). **(b)** Melanoleucophores (ML) with white deposits of crystalline guanine. Some of these cells, still in the process of losing melanin derived from their melanophore precursors, are also evident (arrowhead) [45••]. **(c)** Two of the three described classes of iridophores. I_D have disordered arrangements of reflecting platelets, occur densely packed in interstripes and have an intrinsically yellow hue. I_O have ordered arrangements of larger reflecting platelets, are sparsely arranged within stripes, and are able to change their hue physiologically from blue to yellow [12•]. Cells are shown are in a mutant that lacks melanin and carotenoids, making melanophores and xanthophores invisible. **(d)** Xantholeucophores (XL) in the anal fin with orange carotenoids centrally and white deposits peripherally. Some fin iridophores are evident as well. Inset shows loss of xantholeucophore orange coloration in a *scarb1* mutant defective in localizing carotenoid pigments [45••]. **(e)** Red erythrophores (E) and orange xanthophores (X) in the fin of *D. albolineatus*. Fish in (b), (d) and (e) were treated with epinephrine to mimic a natural physiological response in which pigment granules contract towards cell centers, allowing easier visualization. **(f)** Blue cyanophores (C) in the fin of Mandarin fish, *Synchiropus splendidus* (Figure 4d, top middle). **(g)–(i)** Transmission electron micrographs illustrating subcellular differences in membrane-bound guanine crystal arrangements. **(g)** When guanine crystals (GC) are shaped irregularly, as in this zebrafish melanoleucophore, they give a matte white appearance (g). When crystals take the form of flat reflecting platelets (RP) in stacks, they can lead to iridescence, as in a zebrafish fin iridophore (h), or a matte white appearance, as in clownfish (i). **(j)** In *Pseudochromis diadema*, irregularly oriented reflecting platelets are combined with carotenoids in the same cell to generate a matte violet (Figure 4d top right). Photo credits, [51] (f), [47•] (g), [52] (j). Scale bar in (b) for (b)–(f), 50 μm; in (g) for (g) and (h), 500 nm; in (i) for (i) and (j), 2 μm.

'xantholeucophores' of zebrafish have a white material that may be colorless pteridine, but also orange carotenoids, like xanthophores (Figure 3d) [45••]. Embryonic leucophores of medaka, *Oryzias latipes*, seem to use uric acid for their white component, but also yellow pteridine, similar to xanthophores with which they share a lineage [5,49,50]. Other fishes have reddish-violet 'erythro-iridophores' with reflecting platelets and carotenoids (Figure 3j), and blue-brown 'erythro-cyanophores' [51,52]. Additional cell types probably await discovery. The variety of cell types and lineage origins, which include both neural crest cells and latent stem cells, suggests the existence of exciting opportunities to understand phenotypic evolution through the gain (or loss) of mechanisms of specification and states of differentiation at the cellular level.

Fish pigment patterns: even more open questions

There are, literally, plenty of fish in the sea, and elsewhere, that raise additional questions about pattern evolution. For example, many species have distinctive 'ornaments' that are sometimes specific to males that use them as signals in courtship or spawning but can suffer

Figure 4



Pattern variation within and between species. **(a)** Different positions and numbers of ornaments in three male guppies (melanophores, black arrowhead; xanthophores, brown arrowhead). **(b)** Egg dummies in the fins of *Astatotilapia burtoni* and *Astatoreochromis straeleni* [55]. **(c)** In *Anampses chyrsocephalus* females (top) and males (bottom) are so different they were thought to be different species. **(d)** Reef fishes, from upper left: clownfish *A. ocelaris*, mandarinfish *Synchiropus splendidus*, *P. diadema*, *Balistoides conspicillum*, *Xanichthys mento*[T], *Choerodon fasciatus*[T], *Parupeneus barberinoides*[R], *Centropyge bicolor*[R], *Thalassoma rueppellii*[R], *Apolemichthys arcuatus*[T], *Chaetodontoplus duboulayi*[T], *Rhincanthus abyssus*[T], *Chaetodon* larvatus[R], *Chaetodon ornatissimus*[T], *Chaetodon collare*[T]. Images courtesy: Lengxob Yong (a); modified from Ref. [55] (b); John E. Randall (c); [T], Yi-Kai Tea, [R], Luiz Rocha (d).

increased predation because of them [3,53]. One famous example is the spots of guppy, *Poecilia reticulata*, which differ in number, color, and position between individuals (Figure 4a). Although such variation has long been ascribed to allelic differences on the Y chromosome, a recent analysis revealed sex-linkage of ornament size and color but not the presence or absence of the ornaments themselves [54•]. Autosomal control suggests new possibilities for meitoic mapping and identifying the relevant loci.

A second kind of ornament is the 'egg dummy' of cichlids, present in ~1500 species (Figure 4b). The presence of egg dummies in a species correlates with the presence of a transposable element insertion that drives higher expression of the transcription factor gene *fhl2b* in iridophores [55]. The developmental mechanisms responsible for these and other ornaments will be interesting to uncover, as they may offer a counterpoint to periodic patterns: one might expect especially important roles for positional information in the forms of cues provided by the tissue environment, and perhaps lesser roles for self-organizing interactions among chromatophores.

Male ornaments are just one type of sexual dichromatism, in which sexes differ in pigmentation (Figure 4c). How such very different patterns emerge, presumably in response to hormonal signaling, remains mysterious. Indeed, the evolution of endocrine control is itself a fascinating problem. For example, thyroid hormone is essential for abruptly remodeling tadpoles into frogs and

for chromatophore maturation over ~2 weeks in zebrafish [56,57•]. In reef fish, *Acanthurus triosegus*, however, a sudden maturation of adult vertical bars over ~4 hours (!) is independent of thyroid hormone, depending instead on a mechanism not yet identified [58].

Understanding how local chromatophore behavior is integrated with global endocrine control, and how behaviors evolve to be more or less responsive to hormonal influences will be important for understanding many types of patterns.

Finally, there is perhaps no group that better exemplifies diversity in pigmentation than reef fishes, which have pattern features, and probably cell types, not present in other models (Figure 4d). Advances in evolutionary genetic approaches, computational pattern description, gene editing, and captive husbandry are already making some of these patterns accessible to analysis [38••,59,60]. The prospect of identifying genes and cell behaviors underlying pattern diversification in these fishes is just one small reason, among many larger reasons, to preserve such extraordinary diversity and the environment it depends on.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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