Evolution of Endothelin signaling and diversification of adult pigment pattern in *Danio* fishes

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Abstract

Fishes of the genus *Danio* exhibit diverse pigment patterns that serve as useful models for understanding the genes and cell behaviors underlying the evolution of adult form. Among these species, zebrafish *D. rerio* exhibit several dark stripes of melanophores with sparse iridophores that alternate with light interstripes of dense iridophores and xanthophores. By contrast, the closely related species *D. nigrofasciatus* has an attenuated pattern with fewer melanophores, stripes and interstripes. Here we demonstrate species differences in iridophore development that presage the fully formed patterns. Using genetic and transgenic approaches we identify the secreted peptide Endothelin-3 (Edn3)—a known melanogenic factor of tetrapods—as contributing to reduced iridophore proliferation and fewer stripes and interstripes in *D. nigrofasciatus*. We further show the locus encoding this factor is expressed at lower levels in *D. nigrofasciatus* owing to cis-regulatory differences between species.

Finally, we show that functions of two paralogous loci encoding Edn3 have been partitioned between skin and non-skin iridophores. Our findings reveal genetic and cellular mechanisms contributing to pattern differences between these species and suggest a model for evolutionary changes in Edn3 requirements for pigment patterning and its diversification across vertebrates.

Author summary

Neural crest derived pigment cells generate the spectacular variation in skin pigment patterns among vertebrates. Mammals and birds have just a single skin pigment cell, the melanocyte, whereas ectothermic vertebrates have several pigment cells including
melanophores, iridophores and xanthophores, that together organize into a diverse array of patterns. In the teleost zebrafish, *Danio rerio*, an adult pattern of stripes depends on interactions between pigment cell classes and between pigment cells and their tissue environment. The close relative *D. nigrofasciatus* has fewer stripes and prior analyses suggested a difference between these species that lies extrinsic to the pigment cells themselves. A candidate for mediating this difference is Endothelin-3 (Edn3), essential for melanocyte development in warm-blooded animals, and required by all three classes of pigment cells in an amphibian. We show that Edn3 specifically promotes iridophore development in *Danio*, and that differences in Edn3 expression contribute to differences in iridophore complements, and striping, between *D. rerio* and *D. nigrofasciatus*. Our study reveals a novel function for Edn3 and provides new insights into how changes in gene expression yield morphogenetic outcomes to effect diversification of adult form.

**Introduction**

Mechanisms underlying species differences in adult form remain poorly understood. Quantitative genetic analyses and association studies have made progress in identifying loci, and even specific nucleotides, that contribute to morphological differences between closely related species and strains. Yet it remains often mysterious how allelic effects are translated into specific cellular outcomes of differentiation and morphogenesis to influence phenotype. Elucidating not only the genes but also the cellular behaviors underlying adult morphology and its diversification remains a persistent challenge at the interface of evolutionary genetics and developmental biology.

To address genes and cellular outcomes in an evolutionary context requires a system amenable to modern methods of developmental genetic analysis and rich in phenotypic variation. Ideally the trait of interest would have behavioral or ecological implications, and its phenotype would be observable at a cellular level during development. In this context, adult pigment patterns of fishes in the genus *Danio* provide a valuable opportunity to interrogate genetic differences and the phenotypic consequences of these differences. *Danio* fishes exhibit adult pigment patterns that include horizontal stripes, vertical bars, dark spots, light spots, uniform patterns and irregularly mottled patterns [1]. Pattern variation affects shoaling and might plausibly impact mate recognition, mate choice, and susceptibility to predation [2–5]. Phylogenetic relationships among species and subspecies are increasingly well understood, as is their biogeography, and some progress has been made towards elucidating their natural history [1,6–9]. Importantly in a developmental genetic context, one of these species, zebrafish *D. rerio*, is a well-established biomedical model organism with the genetic, genomic and cell biological tools that accompany this status. Such tools can be deployed in other danios to understand phenotypic diversification.

Adult pigment pattern formation in *D. rerio* is becoming well described in part because cellular behaviors can be observed directly in both wild-type and genetically manipulated backgrounds. The adult pigment pattern comprises three major classes of pigment cells—black melanophores, iridescent iridophores and yellow–orange xanthophores—all of which are derived directly or indirectly from embryonic neural crest cells [10,11]. The fully formed pattern consists of dark stripes of melanophores and sparse iridophores that alternate with light “interstripes” of xanthophores and dense iridophores (Fig 1, top). During a larva-to-adult transformation, precursors to adult iridophores and melanophores migrate to the skin from locations in the peripheral nervous system [10,12,13]. Once they reach the skin hypodermis,
between the epidermis and the underlying myotome, the cells differentiate. Iridophores arrive first and establish a “primary” interstripe near the horizontal myoseptum [14–16]. Differentiating melanophores then form primary stripes dorsal and ventral to the interstripe, with their positions determined in part by interactions with iridophores. Later, xanthophores differentiate within the interstripe and these cells, as well as undifferentiated xanthophores, interact with melanophores to fully consolidate the stripe pattern [11,17–22]. As the fish grows, the pattern is reiterated: loosely arranged iridophores appear within stripes and expand into “secondary” interstripes where they increase in number and establish boundaries for the next forming secondary stripe [13,23]. Stripe development in *D. rerio* thus depends on serially repeated interactions among pigment cell classes. It also depends on factors in the tissue environment that are essential to regulating when and where pigment cells of each class appear [11,16].

Analyses of pattern development in other *Danio* are beginning to illuminate how pigment-cell “intrinsic” and “extrinsic” factors have influenced pattern evolution and the genetic bases for such differences [11,21,23–25]. Here, we extend these studies by examining pattern formation in *D. nigrofasciatus* (Fig 1, bottom). *D. rerio* and *D. nigrofasciatus* are closely related and occur within the “*D. rerio* species group” [9]. The essential elements of their patterns—stripes and interstripes—and the cell types comprising these patterns are the same. Nevertheless, *D. nigrofasciatus* has a smaller complement of adult melanophores than *D. rerio* and its stripes are fewer in number, with only residual spots where a secondary ventral stripe would form in *D. rerio* [26]. Given the broader distribution of patterns and melanophore complements across *Danio*, the *D. nigrofasciatus* pattern of attenuated stripes is likely derived relative to that of *D. rerio* and other danios [26,27]. Cell transplantation analyses revealed that species differences in pattern result at least in part from evolutionary alterations residing in the extracellular environment that melanophores experience, rather than factors autonomous to the melanophores themselves [26].

In this study, we show that *D. rerio* and *D. nigrofasciatus* differ not only in melanophore complements but also iridophore behaviors. We show that iridophore development is curtailed...
in *D. nigrofasciatus*, with a corresponding loss of pattern reiteration. Building on prior inferences [26] and using genetic and transgenic manipulations, we identify the endothelin pathway, and specifically the skin-secreted factor, Endothelin-3 (Edn3), as a candidate for mediating a species difference in iridophore proliferation. We find that *Danio* has two Edn3-encoding loci, arisen from an ancient genome duplication in the ancestor of teleost fishes [28,29], that have diverged in function to promote the development of different iridophore subclasses. One of these, *edn3b*, is required by hypodermal iridophores and has undergone cis-regulatory alteration resulting in diminished Edn3 expression in *D. nigrofasciatus*. Endothelin signaling is required directly by melanocytes in birds and mammals [30–33] but our findings indicate a specific role for Edn3b in promoting iridophore development, with only indirect effects on melanophores. These results suggest a model for the evolution of Edn3 function across vertebrates and implicate changes at a specific locus, *edn3b*, in altering cellular behavior that determines the numbers of stripes comprising adult pattern.

**Results**

**Different iridophore complements of *D. nigrofasciatus* and *D. rerio***

Iridophores are essential to stripe reiteration of *D. rerio* [23] and iridophore-deficient mutants have fewer melanophores [15,16,34]. Given the fewer stripes and melanophores of *D. nigrofasciatus* (Fig 2A) [26], we asked whether iridophore development differs in this species from *D. rerio*. Fig 2B (upper) illustrates ventral pattern development of *D. rerio*. Iridophores were confined initially to the primary interstripe but subsequently occurred as dispersed cells further ventrally [13,16,23]. Additional melanophores developed ventrally to form the ventral primary stripe. Dispersed iridophores were found amongst these melanophores and, subsequently, additional iridophores developed further ventrally as the ventral secondary interstripe. In *D. nigrofasciatus*, however, very few dispersed iridophores developed ventral to the primary interstripe (Fig 2B, lower). Melanophores of the prospective ventral primary stripe initially occurred further ventrally than in *D. rerio* (also see [26]), similar to mutants of *D. rerio* having iridophore defects [16]. Few iridophores were evident either within the prospective ventral primary stripe or further ventrally.

Iridophores arise from progenitors that are established in association with the peripheral nervous system. These cells migrate to the hypodermis where they differentiate [12]. Individual progenitors can generate large hypodermal clones that expand during pattern formation [13]. To assess initial iridophore clone size and subsequent expansion we injected *D. rerio* and *D. nigrofasciatus* with limiting amounts of *pnp4a:palmEGFP* to drive membrane-targeted EGFP in iridophores [21]. At transgene concentrations used, ~1% of injected embryos exhibited a single small patch of EGFP+ iridophores, consistent with labeling of individual progenitors [35,36]. Iridophore morphologies and initial clone sizes were similar between species, but subsequent expansion was significantly greater in *D. rerio* than *D. nigrofasciatus* (Fig 2C; S1 Fig).

These observations indicate that adult pattern differences between *D. rerio* and *D. nigrofasciatus* are presaged not only by differences in melanophore development [26] but changes in iridophore behavior as well. This raises the possibility that evolutionary modifications to iridophore morphogenesis or differentiation have contributed to overall pattern differences between species.

**Endothelin pathway mutants identify a candidate gene for the reduced melanophore complement of *D. nigrofasciatus***

Shared phenotypes of laboratory variants and other species identify candidate genes that may have contributed to morphological diversification [25,37–44]. *endothelin b1a receptor*
ednrb1a mutant zebrafish resemble *D. nigrofasciatus* with deficiencies in iridophores and melanophores compared to wild-type *D. rerio*, and a pattern of stripes dorsally with broken stripes or spots ventrally [16,34,45]. Prior genetic analyses failed to identify an obvious role for *ednrb1a* alleles in contributing to these species differences [38]. Ednrb1a is also expressed by pigment cells [34], whereas interspecific cell transplants suggested that pattern differences between *D. rerio* and *D. nigrofasciatus* likely result from differences in the tissue environment encountered by pigment cells [26]. Given that mutants for Ednrb1a ligand, Endothelin-3 (Edn3), cause pigment cell deficiencies in other vertebrates [46–48], and that Edn3 is likely expressed in the tissue environment of adult pigment cells in *Danio*, we hypothesized that...
differences in Edn3 expression contribute to the pigment pattern differences between *D. rerio* and *D. nigrofasciatus*. To first ascertain the phenotype of Edn3 mutants of *D. rerio* we induced mutations in each of two Edn3-encoding loci of zebrafish, *edn3a* (chromosome 11) and *edn3b* (chromosome 23) (S2 Fig).

Fish homozygous mutant for an inactivating allele of *edn3a* exhibited relatively normal stripes and interstripes, but were deficient for iridophores that normally line the peritoneum, resulting in a rosy cast to the ventrum (Fig 3). By contrast, each of three *edn3b* presumptive null alleles exhibited severe deficiencies of hypodermal iridophores and melanophores and patterns of stripes breaking into spots; similar to *D. nigrofasciatus*, none had defects in peritoneal iridophores (S3 Fig).

*ednrb1a* mutants are defective for both hypodermal and peritoneal iridophores [34], suggesting that Edn3 signaling may have been partitioned evolutionarily between the two paralogous, ligand-encoding loci. Consistent with this idea, fish doubly mutant for *edn3a* and *edn3b* were deficient for both types of iridophores and resembled mutants for *ednrb1a* (Fig 3). These observations also suggest that Ednrb1 need only interact with Edn3a and Edn3b ligands to fulfill requirements for adult pigmentation, though Ednrb1 receptors of other vertebrate lineages are capable of transducing signals via other endothelins [29].

**Genetic analyses implicate *edn3b* in pattern difference between *D. rerio* and *D. nigrofasciatus***

The similarity of *edn3b* mutant *D. rerio* and *D. nigrofasciatus*—with fewer hypodermal melanophores and iridophores than wild-type *D. rerio*, but persisting peritoneal iridophores—identified *edn3b* as a particularly good candidate for contributing to the species difference in pigmentation. To assess this possibility further we used an interspecific complementation test [38,42,49]. If a loss-of-function *edn3b* allele contributes to the reduced iridophores and melanophores of *D. nigrofasciatus* compared to *D. rerio*, we would expect that in hybrids of *D. rerio* and *D. nigrofasciatus*, substitution of a *D. rerio* mutant *edn3b* (*edn3b<sup>rerio</sup>*<sup>−</sup>) allele for a *D. rerio* wild-type *edn3b* (*edn3b<sup>rerio</sup>*<sup>+</sup>) allele should expose the "weaker" *D. nigrofasciatus* allele, reducing the complement of iridophores and melanophores. Such an effect should be of greater magnitude than substituting a mutant for wild-type allele in *D. rerio*, and should be detectable as an allele x genetic background interaction. We therefore generated crosses of *edn3b/+* *D. rerio* x *D. nigrofasciatus* as well as *edn3b/+ x edn3b/+* *D. rerio*. We grew offspring until juvenile pigment patterns had formed, then genotyped individuals of hybrid (h) or *D. rerio* (r) backgrounds for the presence of either *edn3b<sup>rerio</sup>*<sup>+</sup> or *edn3b<sup>rerio</sup>*<sup>−</sup>.

Hybrids between *D. rerio* and *D. nigrofasciatus* have patterns intermediate between the two species [38]. Fig 4A illustrates reduced coverage of iridophores and somewhat narrower stripes in fish carrying *edn3b<sup>rerio</sup>* as compared to siblings carrying *edn3b<sup>rerio</sup>*<sup>+</sup>. Total areas covered by interstripe iridophores were significantly reduced in hybrids compared to *D. rerio*, overall, and in both backgrounds by substitution of *edn3b<sup>rerio</sup>* for *edn3b<sup>rerio</sup> +* (Fig 4B). Moreover, hybrids were more severely affected by this substitution than were *D. rerio*, resulting in a significant allele x genetic background interaction. Melanophore numbers were also reduced by substitution of *edn3b<sup>rerio</sup>* for *edn3b<sup>rerio</sup> +* but hybrids were not significantly more affected than *D. rerio* (Fig 4C). These analyses suggest that the wild-type *D. nigrofasciatus* *edn3b* allele is hypomorphic to the wild-type *D. rerio* allele of *edn3b*, and support a model in which evolutionary changes at *edn3b* have affected iridophore coverage between species.

Two other genes, *augmentor-a1a* and *augmentor-a1b*, encoding secreted ligands for Leukocyte tyrosine kinase (Ltk), promote iridophore development in *D. rerio* and together have a mutant phenotype resembling *D. nigrofasciatus* [50,51]. Iridophore coverage in hybrids
Reduced edn3b expression in skin of D. nigrofasciatus compared to D. rerio owing to cis-regulatory differences

A hypomorphic allele of edn3b in D. nigrofasciatus could result from changes in protein sequence conferring diminished activity, or changes in regulation causing reduced Edn3b abundance. The inferred protein sequence of D. nigrofasciatus Edn3b did not have obvious lesions (e.g., premature stop codon, deletions or insertions), and the 21 amino acid mature peptide was identical between species.

We therefore asked whether D. nigrofasciatus edn3b might be expressed differently than the D. rerio allele. Presumably owing to low overall levels of expression, edn3b transcripts were not detectable by in situ hybridization, and transgenic reporters utilizing presumptive regulatory regions amplified by PCR (~5 kb) or contained within bacterial artificial chromosomes (~190
kb containing ~105 kb upstream to the transcriptional start) failed to yield detectable fluorescence, precluding the assessment of spatial variation in gene expression. Nevertheless, quantitative RT-PCR on isolated skins of post-embryonic larvae indicated edn3b expression in D. nigrofasciatus at levels approximately one-quarter that of D. rerio (Fig 5A). Expression of edn3b was similarly reduced in the sister species of D. nigrofasciatus, D. tinwini, which has fewer melanophores and iridophores than D. rerio, and a spotted rather than striped pattern (S4 Fig) [1,9].

This difference in edn3b expression raised the possibility that cis-regulatory factors (e.g., transcription factor binding sites, chromatin accessibility at edn3b) have been altered between D. rerio and D. nigrofasciatus. To test this idea, we compared expression of D. rerio and D. nigrofasciatus edn3b alleles in the common trans-regulatory background of D. rerio x D. nigrofasciatus hybrids. Allele-specific quantitative RT-PCR revealed approximately one-quarter the abundance of D. nigrofasciatus edn3b transcript compared to D. rerio edn3b transcript (Fig 5B). These observations suggest that species differences in edn3b result at least in part from cis-
regulatory variation that drives lower levels of edn3b transcription in Danio nigrofasciatus compared to Danio rerio.

Edn3b promotes increased iridophore coverage and secondarily affects melanophore pattern in Danio nigrofasciatus

If lower expression of edn3b contributes to the difference in pigment pattern between Danio nigrofasciatus and Danio rerio, then expressing edn3b at higher levels in Danio nigrofasciatus should generate a pattern converging on that of Danio rerio. To test this prediction, we constructed stable transgenic lines in both species to express Danio rerio Edn3b linked by viral 2A sequence to nuclear-localizing Venus, driven by the ubiquitously expressed heat-shock inducible promoter of Danio rerio hsp70l [16,23]. We then reared Danio rerio and Danio nigrofasciatus transgenic for hsp70l: edn3b-2a-nlsVenus, and their non-transgenic siblings, under conditions of repeated heat shock during adult pigment pattern formation.

Heat-shock enhanced expression of Edn3b increased iridophore coverage in Danio nigrofasciatus as compared to Danio rerio or non-transgenic siblings of either species (Fig 6A and 6E). Excess Edn3b failed to increase total numbers of melanophores in Danio nigrofasciatus (Fig 6B). Nevertheless melanophores were differentially distributed in these fish, as Danio nigrofasciatus overexpressing Edn3b had about twice as many cells localizing in a secondary ventral stripe (2V), and a correspondingly reduced number of cells in the primary ventral stripe (1V), as compared to control siblings (Fig 6D). In Danio rerio, total melanophore numbers were increased by Edn3b overexpression though melanophore distributions were not differentially affected between its normally complete stripes (Fig 6B, 6C and 6E).

The rearrangement of a constant number of melanophores in hsp70l:edn3b-2a-nlsVenus Danio nigrofasciatus, and a requirement for interactions between iridophores and melanophores during normal stripe formation in Danio rerio [15,16,23], raised the possibility that Edn3b effects on melanophores might be largely indirect, and mediated through iridophores. If so, we predicted that in a background entirely lacking iridophores, hsp70l:Edn3b should fail to affect melanophore numbers or distribution. We therefore generated fish transgenic for hsp70l:edn3b-2a-nlsVenus and homozygous for a mutant allele of leucocyte tyrosine kinase (ltk), which acts autonomously to promote iridophore development [15,50]. Consistent with iridophore-dependent Edn3b effects, neither melanophore numbers nor melanophore distributions differed between transgenic and non-transgenic siblings (Fig 6E, bottom panels).
These findings support a model in which lower expression of *edn3b* in *D. nigrofasciatus* results in diminished coverage by iridophores and a resulting failure of melanophores to more fully populate the secondary ventral stripe, as compared to *D. rerio*.

**Iridophore proliferation is curtailed in *D. nigrofasciatus* and *edn3b* mutant *D. rerio***

Finally, we sought to better understand the cellular bases for Edn3 effects on iridophore populations in *D. rerio* and *D. nigrofasciatus*. Given roles for Edn3 in promoting the proliferation of avian and mammalian neural crest cells and melanocytes [52–54], we hypothesized that *Danio* Edn3b normally promotes iridophore proliferation and we predicted that such proliferation would be curtailed in both *edn3b* mutant *D. rerio* and in *D. nigrofasciatus*.
To test these predictions, we examined iridophore behaviors by time-lapse imaging of larvalvae in which iridophores had been labeled mosaically with a pnp4apalm-mCherry transgene. We detected iridophore proliferation in stripe regions, where these cells are relatively few and dispersed, and also within interstripes, where iridophores are densely packed (Fig 7). Proliferation of stripe-region iridophores was ~10-fold greater than that of interstripe iridophores. But within each region, iridophores of wild-type (edn3b/+ D. rerio) were more likely to divide than were iridophores of edn3b mutants. Iridophores of D. nigrofasciatus had a proliferative phenotype intermediate to those of wild-type and edn3b mutant D. rerio. We did not observe gross differences in the survival or migration of iridophores across genetic backgrounds. These findings are consistent with Edn3b-dependent differences in iridophore proliferation affecting pattern formation both within D. rerio, and between D. rerio and D. nigrofasciatus.

**Discussion**

Towards a fuller understanding of pigment pattern diversification, we have analyzed cellular and genetic bases for differences in adult pattern between D. rerio and D. nigrofasciatus. Our study uncovers evolutionary changes in iridophore behavior between these species, identifies endothelin signaling as a candidate pathway contributing to these changes, and provides new insights into the evolution of endothelin genes and functions.

**Evolution of iridophore behaviors and impact on pattern reiteration**

An important finding of our analyses is that evolutionary alterations in iridophore behavior can drive species differences in overall pattern. D. rerio and D. nigrofasciatus have relatively
similar complements of iridophores during early stages of adult pattern formation, but the two species subsequently diverge from one another. In *D. rerio*, iridophore clone sizes expanded markedly as the fish grew and secondary and tertiary interstripes were added, whereas this expansion—and pattern element reiteration—were curtailed in *D. nigrofasciatus*. The difference in clonal expansion reflected, at least in part, differences in iridophore proliferation asrevealed by time-lapse imaging.

Prior efforts documented the essential function of iridophores in promoting melanophore stripe reiteration [16,23]. Here, we showed that enhancing the iridophore complement of *D. nigrofasciatus* by Edn3b overexpression was sufficient to reallocate melanophores from a well-formed primary ventral stripe into an otherwise vestigial secondary ventral stripe, resulting in a pattern more like that of *D. rerio*. This effect was probably mediated by interactions between iridophores and melanophores, as melanophores did not respond to the same transgene in the *ltk* mutant of *D. rerio*, which lacks iridophores. An indirect role for endothelin signaling in promoting melanophore stripe development has likewise been inferred from cell transplantation between wild-type and *ednrbg2a* mutant *D. rerio* [15], despite expression of *ednrbg1a* by newly differentiating melanophores [34] and a responsiveness of *D. rerio* melanoma cells to Edn3b in the absence of iridophores [35].

Our observations suggest that an early cessation of iridophore clonal expansion in *D. nigrofasciatus* has led to an earlier offset of interactions between iridophores and melanophores, and an attenuation of the stripe pattern in *D. nigrofasciatus*. In heterochronic terms, the *D. nigrofasciatus* pattern could thus be described as paedomorphic relative to an inferred ancestral state, and arising by progenesis, relative to overall somatic development [56]. That a temporal change in the availability of interactions with iridophores has cascading effects on pattern is reminiscent of observations for xanthophores: precocious widespread xanthophore development, and arising by progenesis, relative to overall somatic development [56]. That a temporal change in the availability of interactions with iridophores has cascading effects on pattern is reminiscent of observations for xanthophores: precocious widespread xanthophore development, and arising by progenesis, relative to overall somatic development [56]. That a temporal change in the availability of interactions with iridophores has cascading effects on pattern is reminiscent of observations for xanthophores: precocious widespread xanthophore development, and arising by progenesis, relative to overall somatic development [56].

A role for endothelin signaling in *Danio* pattern evolution

The numerous pigment mutants of *D. rerio* might be expected to include genes that have contributed to evolutionary diversification within *Danio*, particularly when patterns of mutants and species resemble one another. We found that *edn3b* mutants of *D. rerio* have fewer iridophores and pattern elements than wild-type *D. rerio*, similar to the naturally occurring pattern of *D. nigrofasciatus*. This similarity of final phenotype was presaged by similarity of developmental phenotype, as both *edn3b* mutant *D. rerio* and *D. nigrofasciatus* had reduced iridophore proliferation relative to wild-type *D. rerio*.

Our study provides several lines of evidence to support a model in which alterations affecting Edn3b have contributed to the species difference in pigmentation. First, hybrids of *D. rerio* and *D. nigrofasciatus* carrying a loss-of-function mutant *D. rerio* allele of *edn3b* had a more severe iridophore deficiency than heterozygous *D. rerio* carrying the same mutant allele, suggesting that the *D. nigrofasciatus* wild-type allele is weaker than the *D. rerio* wild-type allele. Second, *edn3b* overexpression was sufficient to increase iridophore coverage, and (indirectly) alter melanophore distributions in *D. nigrofasciatus* to a state more similar to that of *D. rerio*. Third, we found reduced expression of *edn3b* in skin of *D. nigrofasciatus* compared to *D. rerio* during adult pigment pattern formation. Fourth, species differences in expression of *edn3b* alleles were re-capitulated even in a shared hybrid genetic background, pointing to evolutionary change in *cis*-regulation of this locus. Both *D. nigrofasciatus* and *D. tinwini* exhibited lower
levels of edn3b expression compared to D. rerio so regulatory alteration(s) likely occurred prior to divergence of D. nigrofasciatus and D. tinwini, or within the lineage leading to D. rerio itself. cis-regulatory evolution affecting abundance of a secreted ligand that acts on pigment cells to affect pattern is similar to xanthogenic factor Csfla of Danio [23], melanogenic Kit ligand of stickleback [57], and some aspects of anti-melanogenic Agouti in deer mice [58].

Our findings support a role for edn3b in Danio pattern evolution yet they also point to roles for additional factors. For example, overexpression of Edn3b in D. nigrofasciatus increased the coverage of iridophores and allowed for some rearrangements of melanophores, but failed to entirely recapitulate the pattern of D. rerio. Indeed, melanophore numbers were unchanged in transgenic D. nigrofasciatus, in contrast to the larger overall numbers of melanophore in wild-type D. rerio and the still larger number of melanophores induced indirectly by Edn3b overexpression in D. rerio (Fig 6B). Thus, pigment pattern differences between these species are clearly polygenic, and it seems likely that additional loci, of the endothelial pathway or other pathways, will be identified as contributing to attenuated stripes and interstripes of D. nigrofasciatus compared to D. rerio.

The endothelin pathway has been implicated in naturally arising strain differences previously. Besides the spontaneous mutant alleles of mouse Edn3 and Ednrb that allowed the pathway to be first characterized molecularly [47, 59], endothelin pathway genes or differences in their expression have been associated with tabby coloration in domestic and wild cats [60], melanocyte deficiency in ducks [61], white and hyper-melanistic variants of chicken [62–64] and the white mutant axolotl [48]. It is tempting to speculate that mild alleles of endothelin pathway genes or alterations that affect their expression have relatively few pleiotropic effects, particularly in Danio, in which functions of Edn3 paralogues have become subdivided between distinct classes of iridophores. Pigmentary phenotypes associated with this pathway may be particularly accessible targets for natural or artificial selection.

**Evolution of endothelin genes and functions**

Finally, our investigation of Edn3b bears on our understanding of how the endothelin pathway and its functions have evolved. Endothelins were discovered for their roles in vasoconstriction and have since been identified to have a variety of functions [29]. In the context of pigmentation, endothelins and their receptors have been most extensively studied in mammals and birds, in which they regulate proliferation, migration, differentiation and survival at various points within the neural crest–melanocyte lineage [30, 31, 33]. In teleosts, our results in Danio suggest that Edn3 acts primarily to promote iridophore development, with only indirect effects on melanophores. By contrast, the salamander Ambystoma mexicanum requires edn3 for the development of melanophores, xanthophores and iridophores [48, 65, 66] and such effects are not plausibly mediated through iridophores, which develop long after the requirement by melanophores and xanthophores is first manifested.

In teleosts, an additional round of whole genome duplication has resulted in extra genes as compared to non-teleost vertebrates [67–69]. Though many duplicated genes have been lost, those having roles in pigmentation, including genes of the endothelin pathway have been differentially retained [28, 29, 70–72], presumably owing to the partitioning of ancestral functions and the acquisition of new functions. Our finding that edn3a and edn3b are required by complementary subsets of iridophores is consistent with subfunctionalization of an ancestral locus required by all iridophores.

Given requirements for Edn3 in other species—and our findings in Danio that edn3a and edn3b are required by iridophores, edn3b is required only indirectly by melanophores, and neither locus is required by xanthophores—we can propose a model for functional evolution in
which: (i) an ancestral vertebrate Edn3 locus promoted the development of all three classes of pigment cells in ectotherms (a situation currently represented by *A. mexicanum*); (ii) loss of iridophores and xanthophores in mammals and birds obviated an Edn3 role in these cell lineages; (iii) Edn3 functional requirements became limited to iridophores in the lineage leading to teleost fishes and then were further partitioned between iridophore populations, at least in *Danio*. Further testing of this scenario will benefit from analyses of additional anamniotes, including gar, which diverged from the teleost lineage prior to the teleost genome duplication [69,73] and might be expected to have an Edn3 requirement similar to that of *A. mexicanum*.

**Materials and methods**

**Ethics statement**

All animal research was conducted according to federal, state and institutional guidelines and in accordance with protocols approved by Institutional Animal Care and Use Committees at University of Washington, University of Virginia and University of Oregon. Anesthesia and euthanasia used MS-222.

**Fish stocks and rearing conditions**

Fish were reared under standard conditions (14L:10D at ~28˚C) and staging followed [14]. *Danio rerio* were inbred wild-type WT(ABb), a derivative of AB+. CRISPR/Cas9 mutants were induced in WT(ABb) (*edn3b*<sup>p.r30c1</sup>) or ABC x TU (*edn3a*<sup>b1282</sup>, *edn3b*<sup>b1283</sup>). *Danio nigrofasciatus* was field-collected in Myanmar in 1998 [38] and maintained in the laboratory since that time. *Danio tinwini* was obtained from the pet trade in 2014. Transgenic lines *hsps70*:edn3b-2a-nlsVenus<sup>p.r30</sup> and *hsps70*:edn3b-2a-nlsVenus<sup>p.r30</sup> were generated in WT(ABb) and *D. nigrofasciatus* backgrounds, respectively. *augmentor-a1a/+* and *augmentor-a1b/+* *D. rerio* [51] were generously provided by E. Mo and S. Nicoli (Yale School of Medicine). *ltk*<sup>9s1</sup> (*primrose*) is a spontaneous allele of *ltk* identified by S. Johnson, into which *hsps70*:edn3b-2a-nlsVenus<sup>p.r30</sup> was crossed.

Fish were fed marine rotifers, brine-shrimp and flake food. Fish were allowed to spawn naturally or gametes were stripped manually for *in vitro* fertilization. Interspecific hybrids were generated by *in vitro* fertilization in both directions using *D. rerio* heterozygous for wild-type and *edn3b*<sup>p.r30c1</sup> allele; progeny were reared through formation of juveniles patterns and then genotyped using primers to amplify *D. rerio* alleles by PCR from fin clips, followed by Sanger sequencing to identify carriers or WT(ABb) or *edn3b*<sup>p.r30c1</sup> alleles. For *hsps70*-inducible Edn3b transgenes, transgenic siblings and non-transgenic controls were reared from stages DR through J under conditions of repeated daily heat shock (38˚C, 1 h) [16,23].

**CRISPR/Cas9 mutagenesis, transgenesis and clonal analyses**

For CRISPR/Cas9 mutagenesis, 1-cell stage embryos were injected with T7 guide RNAs and Cas9 protein (PNA Bio) using standard procedures [74]. Guides were tested for mutagenicity by Sanger sequencing and injected fish were reared through adult stages at which time they were intercrossed to generate heteroallelic F1s from which single allele strains were recovered. CRISPR gRNA targets (excluding proto-spacer adjacent motif) were: *edn3a*<sup>b1282</sup>, GCCAGCTCCTGAAACCCCAC; *edn3b*<sup>p.r30c1</sup>, GAGGATAAATGTACTCACTG; *edn3b*<sup>b1283</sup>, GGATAAACTGTACTCACTGTG.

For transgenesis, constructs were generated using the Tol2Kit and Gateway cloning [75] and injected by standard methods with Tol2 transposase mRNA [76]. For Edn3b-containing transgenes, F0 mosaic adults were screened for germline transmission and progeny tested for
hsp70l-induction of linked fluorophore. Clonal analyses used mosaic F0 larvae and limiting amounts of pnp4a:palmEGFP transgene to insure that integrations were rare between and within individuals so that only single clones were likely to be labeled [35,36]. Sparsity of transgene+ embryos and similarity of starting clone sizes within such embryos between species suggests that labeling was indeed clonal. Transgene+ individuals were imaged at stages PR+ and J++. 

Sequences, genotyping, RT-PCR, and quantitative RT-PCR

Accession numbers for *D. rerio* and *D. nigrofasciatus edn3b* are NM_013112.13 and MH705096. For distinguishing *D. rerio* wild-type and mutant alleles we sequenced across induced lesions using primers edn3b+: F-TGCACCTCATCAGTCTTCTC, R-GTGTGACAGCGAAAGTAGTGAC. For assessing persistence of transcript in wild-type and mutant backgrounds of *D. rerio*, we amplified edn3b and control cDNAs using primer sets: edn3b.Dr-c238, F-TTGGACATCAGCAGAAGAAGC, R-CATAAGCAGCGAAGAAGAACC; actb2: F-ACGTGGATGACATGGAAGTAGAG, R-GTGTGAGGTCCTCGGACATGA.

For assessing edn3b transcript abundance quantitatively across species, skins were harvested from stage-matched *D. rerio*, *D. nigrofasciatus* and *D. tinwini* and total RNAs isolated by Trizol (ThermoFisher) extraction as previously described [23]. For RT-PCR, first strand cDNAs were synthesized with SuperScript III reverse transcriptase (ThermoFisher) and oligo-dT primed First strand cDNAs were synthesized with iScript and oligo-dT priming (BioRad) and analyzed on an ABI StepOne Plus real time PCR instrument using custom designed Taqman probes against target sequence shared by *D. rerio* and *D. nigrofasciatus* (identical to *D. tinwini*). edn3b expression was normalized to that of rpl13a; normalization to a conserved actb1 amplicon (ThermoFisher assay ID #Dr03432610_m1) yielded equivalent results in pilot analyses. Expression levels were assessed using the 2−ΔΔCt method [77] with *D. rerio* expression levels set to 1. Comparisons of species differences in expression were repeated 4 times (with 2–4 biological replicates each) using matched stages of fish between DR+ and J. We did not detect significant differences between replicates/stages, or species x replicate/stage interactions, and so present normalized values across all replicates in the text. For analyzing allele-specific expression in hybrids, custom Taqman probes were designed to amplify an edn3b target from both species alleles, or from only *D. rerio* (Dr) or *D. nigrofasciatus* (Dn). Amplifications of Dr and Dn probes were normalized to that of the Dr, Dn probe. Hybrid samples included a total of 4 biological replicates. Primers (F, R) and target probes (T) were: edn3b (AIWR326): F-CAGAGAATGTGTTTATTACTGTCACTTGGG, R-CCAAGGTGAACGTCCTCTCA, P-FAM-CTGGGATTACACCCACACG; edn3b (AI20TXP, Dr): F-TGGTGGTTCCAGCGTGTTG, R-GTGTGACAGCGAAAGAAGC, P-FAM-CAAGCTTCGTCTCTTC; edn3b (AI1RVHR, Dn): F-GCTCTTTTGTCTTAATTGAGGTTCTTG, R-ACCAGAGAGACTGGGAGATGAGT, P-FAM-CCTGACTGCTAAGAAC; rpl13a (Dr, Dn): F-CAGAGAATGTGTTTATTACTGTCACTTGGG, R-CCAAGGTGAACGTCCTCTCA, P-FAM-CTGGGATTACACCCACACG. Underlined bases are specific to the targeted species.

Imaging

Images were acquired on: Zeiss AxioObserver inverted microscopes equipped either with Axioacam HR or Axioacam 506 color cameras or a Yokogawa laser spinning disk with Evolve camera, and an AxioZoom v16 stereomicroscope with Axioacam 506 color camera, all running ZEN blue software. An Olympus SZX12 stereomicroscope with Axioacam HRc camera and Axiovision software was additionally used for some imaging. Images were corrected for color balance and adjusted for display levels as necessary with all treatments or species within
analyses treated identically. Images of swimming fish were captured with a Nikon D800 digital SLR equipped with Nikon AF-S VR Micro-Nikkor f2.8 IF/ED lens.

Counts of melanophores and coverage by iridophores used regions of interest defined dorsally and ventrally by the margins of the flank, anteriorly by the anterior insertion of the dorsal fin and posteriorly by the posterior insertion of the anal fin. Only hypodermal melanophores contributing to stripes were included in analyses; dorsal melanophores and melanophores on scales were not considered. All melanophore counts were performed on fish that had been treated with epinephrine, which contracts melanosomes towards cell centers and facilitates the identification of individual cells [16]. For assessing iridophore coverage, total areas covered by dense interstripe iridophores were estimated as these account for the majority of total hypodermal iridophores and areas covered by sparse iridophores within stripe regions could not be reliably estimated from brightfield images. Cell counts and area determinations were made using ImageJ. Time-lapse analyses of iridophore behaviors followed [21] and were performed for 15 h with 5 min frame intervals on *D. nigrofasciatus* as well as *D. rerio* siblings homozygous or heterozygous for *edn3b*vp*r30c1*. All iridophores initially within regions of interest were counted. Proliferating iridophores were evident as single cells that rounded-up and then divided to generate adjacent daughter cells. For statistical analyses, we compared the number of proliferating iridophores to the number of non-proliferating iridophores, calculated as total starting number less the number of cells that underwent division. Individual genotypes of larvae used for time-lapse imaging were assessed by Sanger sequencing across the induced lesion.

**Statistical analysis**

All statistical analyses were performed using JMP 14.0.0 statistical analysis software (SAS Institute, Cary NC) for Apple Macintosh. For linear models, residuals were examined for normality and homoscedasticity and variables transformed as necessary to meet model assumptions [78].

**Supporting information**

**S1 Fig. Expansion of iridophore clones differs between *D. rerio* and *D. nigrofasciatus***. Representative images for individuals of each species mosaic for iridophore reporter *pnp4a*pal-mEGFP at an early stage of pattern formation, and at a late stage, once patterns were complete. Dashed yellow lines indicate approximate regions of correspondence between early and late images and I1–I3 indicate primary through tertiary interstripes, if present; 1D, 1V, 2V indicate positions of stripes, if present. In each species, iridophores were present within interstripes, where they were densely packed, and within stripe, where they were loosely arranged. Inset 1, clonal derived early iridophores in primary interstripe of *D. rerio*. Inset 2, In some individuals, autofluorescent xanthophores (x) were apparent but were distinguishable from iridophores by differences in shape. Inset 3, early iridophores of *D. nigrofasciatus*. Inset 4, Examples of spindle-shaped “type-L” iridophores [79] present at low abundance in each species. (TIF)

**S2 Fig. Induced mutations in *D. rerio* Edn3 loci.** Panels show genomic structures of Edn3 loci with locations encoding the mature peptides (green) as well as local nucleotide and amino acid sequences. Untranslated regions are shown in brown. For *edn3a*, the *b1282* allele has a 43 bp deletion that removes 13 of 20 amino acids comprising the active Edn3a peptide, with the addition of 4 novel amino acids (red). For *edn3b*, two alleles were generated with deletions of existing nucleotides and insertion of new nucleotides (red) covering the splice donor site downstream of exon 2 (boxed), resulting in the addition of novel amino acids and premature stop codons (*). Both *vp.r30c1* and *b1283* are likely to be loss-of-function mutations as their
phenotypes were indistinguishable and also resembled independently derived edn3b alleles having similar lesions at the same target site [55]. Consistent with this inference, RT-PCR for edn3b transcript on skins of adult fish showed expression in wild-type (wt) but not edn3b\textsubscript{b1283} or edn3b\textsubscript{r30c1} mutants; no-templ, no template control. Open reading frames are in upper case and intronic sequence in lower case.

S3 Fig. Pigment pattern defects of edn3b mutants but not edn3a mutants resemble \textit{D. nigrofasciatus}. (A) Details of ventral patterns illustrating deficiency in peritoneal iridophores (arrowhead) in \textit{D. rerio} edn3a mutants but not edn3b mutants or \textit{D. nigrofasciatus}. (B) Defects in areas covered by iridophores and numbers of melanophores in heterozygous edn3b mutant \textit{D. rerio} (\(F_{2,48} = 292.6, F_{2,48} = 69.8\), respectively; both \(P<0.0001\)). Shown are least squares means±SE after controlling for variation in standard length (SL; both \(P<0.0001\)). Different letters above bars indicate means significantly different in Turkey-Kramer post hoc comparisons. Values above bars indicate samples sizes.

S4 Fig. Reduced edn3b expression in \textit{D. tinwini} compared to \textit{D. rerio}. (A) Pigment pattern of \textit{D. tinwini}. (B) Species differences in skin edn3b expression during adult pattern development (\(F_{2,7} = 48.2, P<0.0001\)). Shared letters indicate bars not significantly different in post hoc Turkey HSD comparisons of means (\(P>0.05\)). Numbers in bars indicate biological replicates.

S1 File. Supplementary Information File. Data Matrices. Numerical data used for quantitative analyses.

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Writing – original draft: Jessica E. Spiewak, David M. Parichy.
References


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