# Evolution of gene regulation in association with the origin of armor in stickleback fishes

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**Abstract**

Whether the origin of novel structures requires extensive novelty at the genetic level remains an important question in Evolutionary Biology. The bony plates in the dermis (inner layer of the skin) of stickleback fishes (Order Gasterosteiformes) are novel structures that arose as a replacement for scales and whose genetic basis is becoming increasingly well-understood. Previous work has identified an enhancer (a short DNA sequence that regulates the expression of neighboring genes) that is necessary for the expression of the Ectodysplasin (*Eda*) gene in the bony dermal plates of the Threespine Stickleback (*Gasterosteus aculeatus*). This gene is necessary for the development of such plates, as well as the scales of other fishes which they replaced in evolution. In the present study, I first used phylogenetic character mapping to clarify the evolutionary origin of bony dermal plates, finding that it was equally likely that they arose directly from scales or through an intermediate condition of naked skin. To investigate the origin of the bony dermal plate enhancer of the *Eda* gene, I studied the corresponding gene in the Mexican Tetra (*Astyanax mexicanus*), a fish species possessing scales and a well-characterized genome. I first used *in situ* hybridization to confirm that *Eda* is expressed in scales of this species. I then used reporter transgenic analysis to search for a sequence corresponding to the stickleback bony dermal plate enhancer near the Mexican Tetra *Eda* gene. Specifically, I used genome sequence comparisons to identify a candidate enhancer, made a DNA construct in which this candidate enhancer was joined to a green fluorescent protein (*Gfp*) reporter gene, and injected this construct into the Zebrafish (*Danio rerio*), an easily manipulated model species that also possesses scales. I found that this enhancer was not capable of driving expression in scales, but did drive expression in the upper jaw and pelvic fin. The latter activity is also characteristic of the stickleback bony dermal plate enhancer. This result suggests that the stickleback enhancer arose through modification of an existing enhancer rather than *de-novo*. My work is therefore consistent with morphological novelty originating without the need for extensive genetic novelty.

## INTRODUCTION

The genetic basis of the origin of novel structures remains a fundamental question in evolutionary biology (Moczek 2008, Shubin *et al.* 2009, Wagner & Lynch 2010). For example, do novel structures arise by co-opting (adopting) existing genetic pathways and mechanisms or do the genetic networks regulating their development arise *de-novo* (with no obvious precursors)? It has been shown that existing genes may be co-opted in the origin of an evolutionary novelty, but the extent to which DNA-regulatory elements are co-opted remains poorly understood (Rebeiz & Tsiantis 2017). The current study focuses on an example of a novelty, the bony dermal plates in the skin of stickleback fishes (order Gasterosteiformes). These bony dermal plates apparently arose as a replacement for the thinner elasmoid scales characteristic of Teleost fishes, (modern ray-finned bony fishes) (Sire *et. al* 2009), but whether these plated fishes first appeared as naked forms after the loss of scales or whether scales transformed into bony plates remains unclear (Whitear 1986, Sire 1993). The starting point of my investigation was the Ectodysplasin (*Eda*) gene that is required for the development of both scales (Harris *et al*. 2008, lida *et al.* 2014, Aman *et al.* 2018) and gasterosteiform bony dermal plates (Colosimo *et al*. 2005, O'Brown *et al.* 2015.) *Eda* acts as a signaling molecule in the pathway responsible for development of appendages such as scales, hairs, and feathers, in the vertebrate skin (Sadier *et al.* 2014). In humans, a mutation in the *Eda* pathway results in a condition known as ectodermal dysplasia, distinguished by the abnormal development of the skin, teeth, hair, nails, sweat glands, and mucous membranes.

O'Brown *et al.* (2015) identified an enhancer (a non-coding DNA sequence regulating transcription) responsible for *Eda* expression in stickleback bony dermal plates. The main goal

of my study was to determine whether a similar enhancer, immediately downstream of the *Eda* gene, is also present in scaled fishes and responsible for *Eda* expression in scales.

I chose as model organisms to examine, the Mexican tetra (*Astyanax mexicanus*) and the zebrafish (*Danio rerio*), both of which are scaled and have fully-sequenced genomes (Howe *et al.* 2013, McGaugh *et al.* 2014). Additionally, both species, along with sticklebacks, are members of the Teleostei, a group of ray-finned bony fishes that underwent a genome duplication after diverging from holostean fishes such as gars (Braasch *et al.* 2016). Many gene pairs resulting from this duplication underwent loss of one of the members, and zebrafish and sticklebacks both lost one of the original gene pair of *Eda* and retained the respective opposite gene of the pair (Braasch *et al.* 2009). The latter authors termed the zebrafish version *Eda-b* and the stickleback version *Eda-a*, a terminology that will be followed in the present work. In contrast to the above two species, the Mexican tetra retained both copies of *Eda* in its genome (McGaugh *et al.* 2014, D Stock, personal communication). Because of this genome structure, one aspect of my study was to determine whether one or both of the *Eda* genes is expressed in the scales of this species by *in situ* hybridization. Figure 1 shows two *Eda* copies in the genome of the Mexican tetra, the loss of *Eda-a* in the zebrafish and the loss of *Eda-b* in the stickleback.

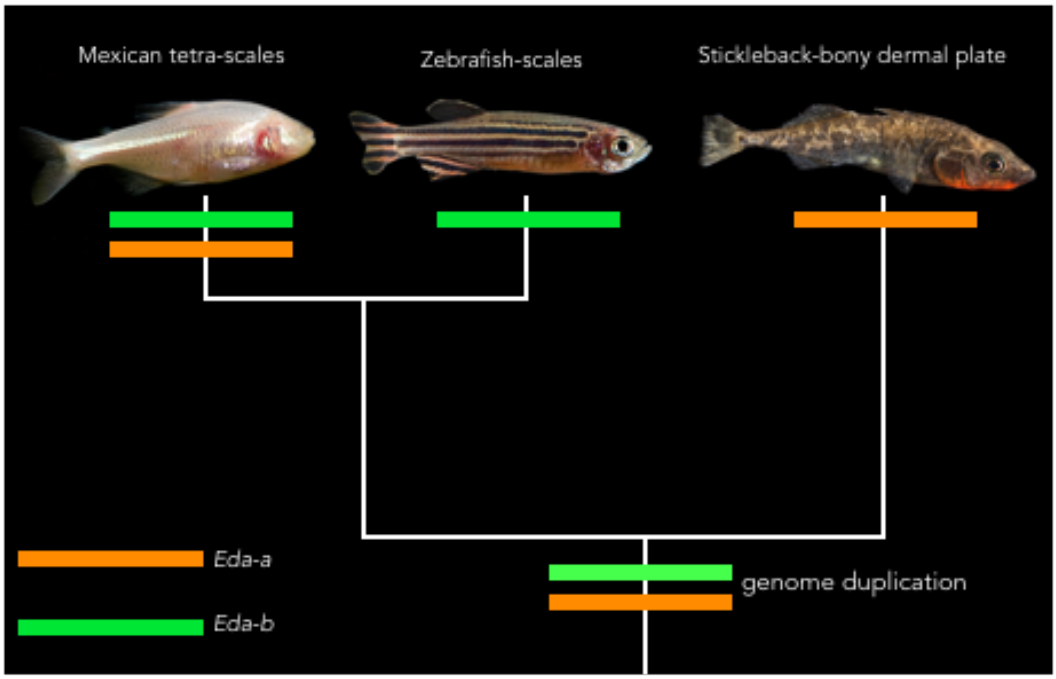


**Figure 1: *Eda* genes present in the Mexican Tetra, Zebrafish, and Stickleback.**
A genome duplication resulted in two copies of *Eda*: *Eda-a* and *Eda-b*. *Eda-a* was lost in the zebrafish and *Eda-b* was lost in the Stickleback, while the tetra retains both genes.

The method I used to assay enhancer activity is similar to that of O'Brown *et al.* (2015), who characterized the stickleback regulatory element. I cloned the region adjacent to the Mexican tetra *Eda-a* gene that corresponds to the enhancer identified by O'Brown *et al.* (2015) and assayed its ability to drive expression of a fluorescent reporter gene when injected into zebrafish embryos. I then compared the activity of putative regulatory elements from the stickleback and Mexican tetra to distinguish among possible origins of the dermal plate regulatory element. These origins include *de-novo* appearance, modification of a pre-existing element, and movement of an element within the genome. Due to the general similarities in the genes regulating the development of vertebrate skin appendages, I hypothesized that gasterosteiform dermal plates co-opted gene regulatory mechanisms that had been previously employed in the development of scales. More specifically, I hypothesized that bony dermal

plates arose in association with the modification of a pre-existing enhancer that drove expression in scales (ancestral condition to bony dermal plates).

## BACKGROUND

### The diversity of body coverings in fishes

The scales of fishes are skeletal structures, mineralized with calcium phosphate, that form in the inner skin layer (dermis) and include an extracellular matrix of collagen proteins (Harder 1976, Whitear 1986, Elliot 2000, Sire *et al*. 2009, Schultze 2016, 2018). These scales exhibit an enormous diversity of shapes, structures, and functions, ranging from heavy armor, to a thin flexible covering, to more than numerous prickles (Elliot 2000). This diversity is classified by the type of skeletal tissues present, including hyper-mineralized acellular tissues resembling tooth, with very little organic matter, 2) dentine, with higher organic matter content and organized like the inner layer of the mammalian tooth, 3) acellular plywood-like tissues that are poorly mineralized and consist of individual layers of parallel collagen fibers oriented perpendicularly to each other and 4) bone, which may be cellular or acellular (Sire *et al.* 2009).

The ancestral scale type of bony fishes is thought to resemble that of the modern-day African ray-finned fish family Polypteridae (bichirs and reedfishes) (Sire *et al.* 2009). Such scales include all four skeletal tissues in the order listed above from the outside in. In contrast, the typical scale of teleost (modern ray-finned) fishes, which make up 96% of all living fishes (Nelson *et al*. 2016), consists largely of the plywood-like tissue elasmodine and lacks bone altogether. It is controversial whether traces of hypermineralized and dentine-like tissues remain in elasmoid scales (Sire et. al 2009; Schultze 2016, 2018).

The zebrafish, Mexican tetra, and stickleback are members of the Teleostei (Nelson *et al*. 2016), a group of ray-finned bony fishes that underwent a genome duplication after diverging from holostean fishes such as gars (Braasch *et al.* 2016). Within the Teleostei, scales have frequently been lost and in a few instances, replaced by bony structures often referred to as scutes (Whitear 1986, Sire 1993, Elliot 2000, Sire *et al*. 2009). An example of the scute is seen in stickleback fishes and their relatives in the order Gasterosteiformes. The extensively studied model species of this order, *Gasterosteus aculeatus* (three-spine stickleback) has a series of lateral plates (bony dermal plates) consisting of acellular bone and no other skeletal tissues (Sire *et al.* 2009). The evolutionary origin of stickleback bony dermal plates is unclear because of uncertainty of the phylogenetic relationship of the group (Near *et al.* 2013; Nelson *et al.* 2016) and the absence of an obvious transitional state between elasmoid scales and bony dermal plates (Whitear 1986, Sire 1993). Traditionally, gasterosteiforms were thought to be related to seahorses and pipefishes (syngnathiforms), another group in which bony lateral plates are present, but recent molecular studies suggest that this is not the case (Near *et al*. 2013, Nelson *et al*. 2016).

**Regulation of transcription**

*cis*-regulatory elements are short stretches of DNA involved in the regulation of transcription. Transcription as a general process is controlled by two prominent types of *cis*-regulatory elements, promoters and enhancers. Both enhancers and promoters are non-coding sequences of DNA that bind transcription factors. The promoter provides a site for transcription to begin, and the enhancer acts as an activating sequence required to stimulate transcription. The binding of a regulatory transcription factor to the enhancer will increase the level of

transcription. Therefore, in the absence of enhancer sequences, most eukaryotic genes have very low levels of transcription.

Because of their functional importance, enhancers are expected to be under purifying selection and therefore conserved in evolution (Nelson & Wardle 2013), which is why identifying conserved DNA sequences outside of protein-coding regions is one method of identifying putative enhancers. The identity of these enhancers can then be confirmed by reporter-gene assays (Fisher *et al.* 2006). In this method, the conserved sequence is cloned next to a minimal promoter that is necessary but not sufficient to drive expression of a reporter gene such as *GFP* (green fluorescent protein). This DNA construct is then introduced into a developing organism; expression of *GFP* indicates that the DNA of interest functions as an enhancer.

**Defining a Novelty**

One criterion for designating a structure as an evolutionary novelty is absence of a homolog in other species (Moczek 2008, Wagner & Lynch 2010). Homology itself can be defined in various ways, but two general categories of homology have prevailed: historical homology and biological homology (Wake 2003). According to the historical homology concept, structures in two different species are homologous if they were derived from a common ancestral structure and have been continuously maintained in the lineages of both species. The absence of common tissues between elasmoid scales and scutes suggests that scales may have been lost before the appearance of bony dermal plates. If this is the case, the appearance of bony dermal plates as a dermal skeletal covering can be interpreted as a reversal, a form of homoplasy (similarity not due to common descent), rather than homology (Wake 2003). In contrast to the

historical homology concept, the biological homology concept suggests that structures in two different species (or two structures within the same species - serial homologs) are homologous if they develop by similar mechanisms. This is the case for elasmoid scales and bony dermal plates as suggested by the requirement for signaling through the *Wnt* and *Eda* pathways for the development of both scales and scutes (Harris *et al.* 2008; Iida *et al.* 2014, O'Brown *et al.* 2015; Aman *et al.* 2018).

## MATERIALS AND METHODS

### Phylogenetic character mapping

Figure 2 shows the skin covering (scales, scutes, or naked) of species in a monophyletic group including sticklebacks mapped onto the molecular phylogeny published by Near *et al*. (2013) using the parsimony option of the program Mesquite (Maddison & Maddison 2015). This monophyletic group corresponds to the suborders Zoarcoidei, Gasterosteoidei and Cottoidei within the teleost order Scorpaeniformes (Nelson *et al.* 2016). Species investigated and literature sources for their skin covering are as follows: *Anoplopoma fimbria, Hexagrammos otakii, Trichodon trichodon, Eumicrotremus orbis, Aspidophoroides monopterygius, Stellerina xyosterna, Bathymaster signatus, Cebichthys violaceus, Pholis ornatus,* and *Pholis crassispina* (Eschmeyer *et al.* 1983), *Cyclopterus lumpus, Paraliparis meganchus*, and *Liparis mucosus* (Knudsen *et al*. 2007), *Cottus carolinae* (Cross & Collins 1995), *Leptocottus armatus, Scorpaenichthys marmoratus*, and *Artediellus uncinatus* (Yabe 1985), *Hypoptychus dybowskii* and *Lycodes terraenovae* (Nelson *et al*. 2016), *Aulorhynchus flavidus* and *Aulichthys japonicus* (Nelson 1971), *Apeltes quadracus*, *Gasterosteus wheatlandi*, and *Gasterosteus aculeatus* (Bowne 1994), *Rathbunella hypoplecta* (Mecklenburg 2003), *Cryptacanthodes*

*maculatus* (Schnell & Hilton 2015), *Anarhichas lupus* (Collette & Kein-MacPhee 2002), *Allolepis hollandi* (Anderson *et al*. 2009), and *Lycodes diapterus* (Stevenson & Sheiko 2009).

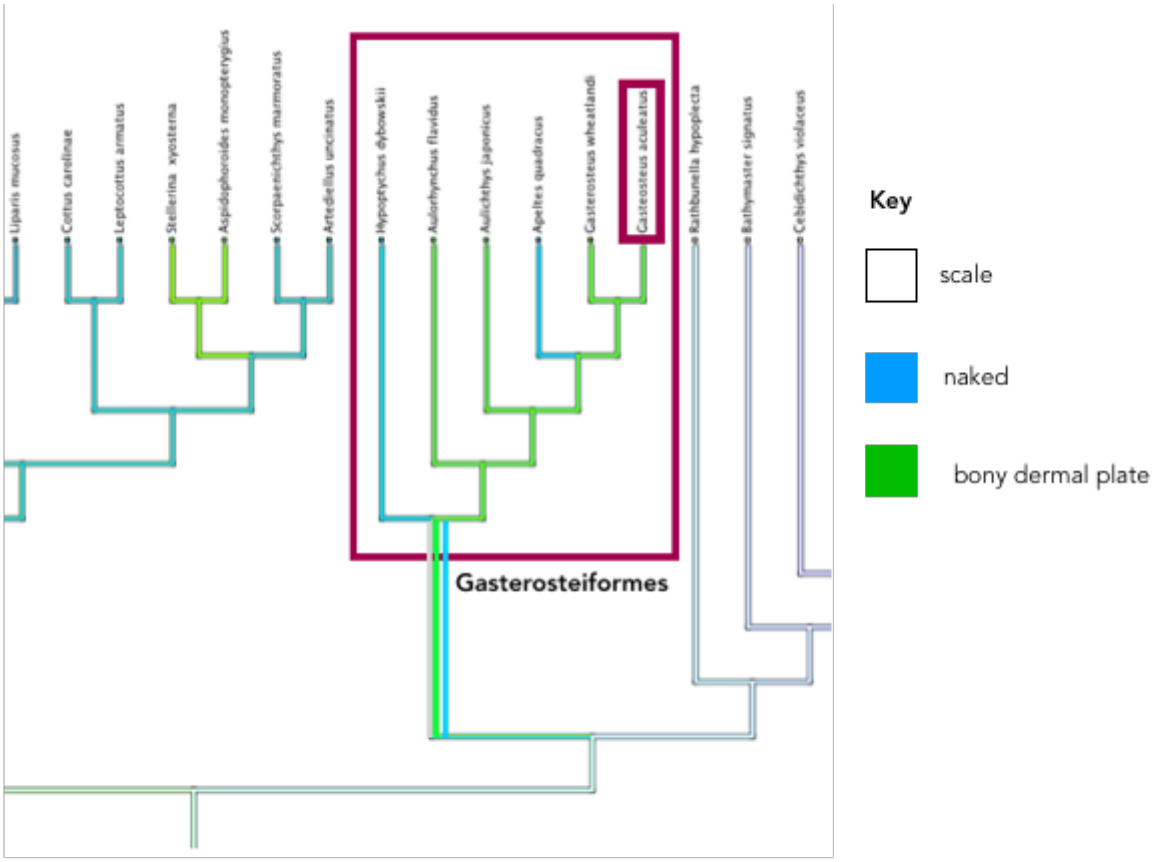


**Figure 2: Molecular Phylogenetic Tree of Gasterosteiformes with skin covering conditions mapped.** The model species of the order Gasterosteiformes, Gasterosteus aculeatus (Threespine Stickleback), is characterized by bony dermal plates. The ancestral condition to the order is scales (represented by white). The transitional condition from scales to bony dermal plates remains uncertain as depicted by three possible conditions: scale (white), bony dermal plate (green), or naked (blue).

**Analysis of gene expression by *in situ* hybridization**

Expression of the two *Eda* genes of the Mexican tetra (*Eda-a*, corresponding to stickleback *Eda*, and *Eda-b*, corresponding to Zebrafish *Eda* - Braasch *et al*. 2009) was examined by *in-situ* hybridization during the period of scale development following the protocol described by Jackman *et al.* (2014). Embryos of the blind cave form of this species were collected from natural spawnings in the laboratory and raised to 42 to 47 days post-fertilization before sacrifice. Specimens were fixed overnight at 4ºC in 4% formaldehyde, 1% DMSO in phosphate-

buffered saline (PBS), rinsed in PBST (PBS with 0.1% Tween 20), and stored in methanol at -20 ºC. Digoxigenin-labeled RNA probes were synthesized from cloned cDNA fragments of *A. mexicanus Eda-b* (Aigler *et al.* 2014) and *Eda-a* (D. Stock, unpublished) genes using T3 RNA polymerase.

Six larvae were rehydrated through a graded series of methanol in PBST and washed twice in 100% PBST. For each gene, larvae were digested with proteinase K at concentrations of 2.5, 10, and 25 µg/ml in PBST at room temperature for 30 minutes. The embryos were then washed with glycine in PBST and fixed in 4% formaldehyde for 20 minutes. Formaldehyde was removed by two PBST rinses. Specimens were then pre-hybridized in hybridization mix for several hours at 60ºC. *Eda-b* or *Eda-a* riboprobes were then added and hybridization was carried out overnight at 60 ºC.

Probes were removed the next day from the tissue, and the tissue was rinsed and washed with prewarmed hybridization mix, and then rinsed and washed with MABT (maleic acid buffer with 0.1% Tween-20) at room temperature. The larvae were next incubated in MAB + 2% blocking reagent for several consecutive hours, followed by addition of 20% heat-treated sheep serum. Alkaline phosphatase-coupled anti-digoxigenin antibody was then added at a 1:2000 dilution and the specimens rocked overnight at 4ºC.

Following incubation with the antibody, larvae were rinsed three times with MABT, and washed for five hours with MABT. The next day, the larvae were washed with NTMT, and transferred to 4-well microtiter plates. BM purple substrate was then added to the larvae and they were stored in the dark for several hours.

**Reporter transgenic analysis**

Figure 3 depicts an alignment of *Eda-a* sequences between the Mexican tetra and *Pygocentrus nattereri* (Red-bellied piranha). These sequences were obtained from GenBank and were aligned with the mVISTA program (Frazer *et al.* 2004) to identify regions of conserved non-coding DNA sequence.

This analysis identified 26 conserved non-coding elements (CNEs) 5' of the *Eda-a* transcription start site, 10 CNEs within introns and one CNE 3' of the gene. We tested the latter CNE (287 base pairs in length) that we designated *AmEdaLDR1* for enhancer activity because it corresponded to the stickleback enhancer identified by O'Brown *et. al* (2015).

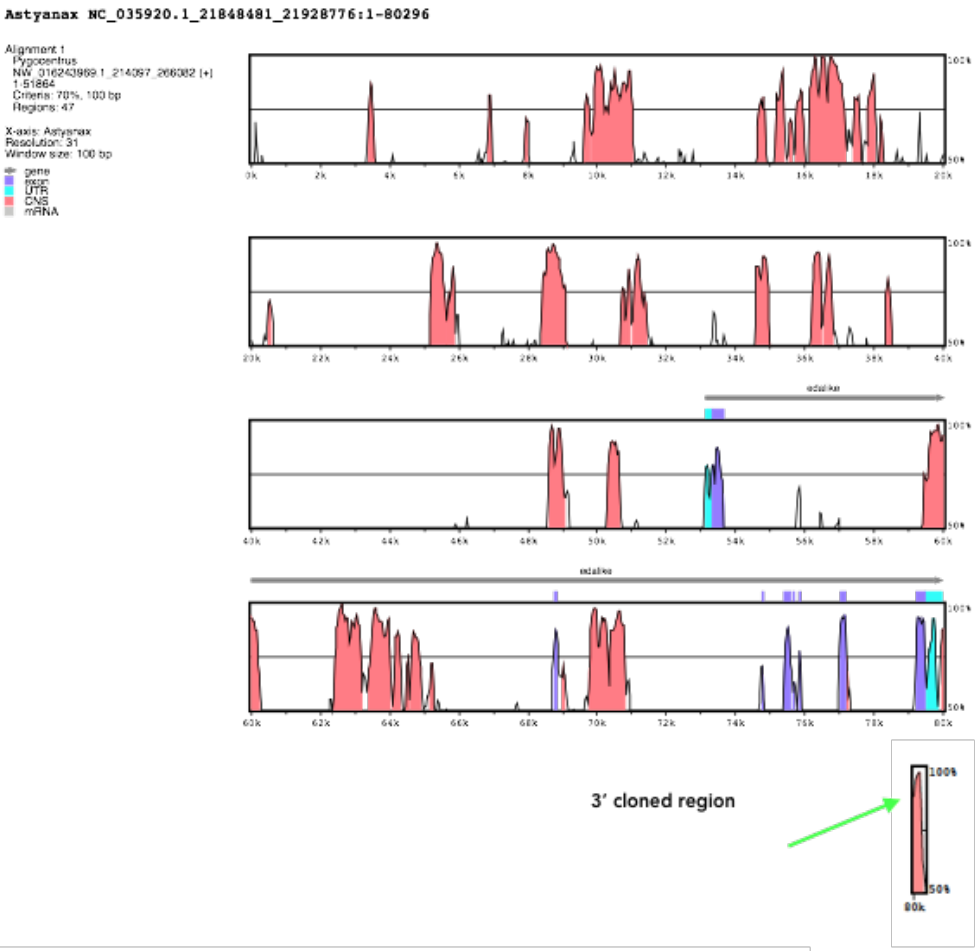


**Figure 3: Sequence conservation of *Eda-a* between Mexican Tetra and Red bellied piranha.** Peaks indicate significant sequence conservation (light blue=untranslated regions, dark blue=exons, and orange=noncoding regions.)

Primers were then synthesized and used to amplify this conserved stretch of intergenic DNA using the Polymerase Chain Reaction (PCR). After PCR had been performed on the target stretch of DNA, gel electrophoresis was used to identify a DNA band of the expected size. The PCR product was run over a column to isolate the DNA from the other components of the PCR reaction. The PCR product was then recovered in water and cut with two restriction enzymes, *Apa* I (which produces a sticky end cut) and *Sma* I (which produces a blunt end cut). These enzymes recognize sequences added to the PCR primers to facilitate cloning.

The digested PCR product was again subjected to agarose gel electrophoresis. A DNA band of the expected size was cut out of the gel with a razor blade. The digested PCR product was then purified by melting the agarose gel slice in a buffer, freezing the resulting solution, centrifuging the suspension to remove agarose fibers and ethanol precipitation. The PCR product (DNA) was then resuspended in a buffer. A plasmid vector (circular chromatin containing the accessory genes of a bacterium) cut by *Apa* I and *EcoR* V (a blunt cutter) was used to join the sticky end of the plasmid to the sticky end of the PCR product, and the blunt end of the plasmid to the blunt end of the PCR product. The digested PCR product was mixed with digested plasmid and DNA ligase (a repair protein), which covalently joined the strands together (a ligation reaction). The circular plasmid then contained the intended insert (the conserved intergenic region between *Eda-a* and *tnsf13 (= AmEdaLDR1).*

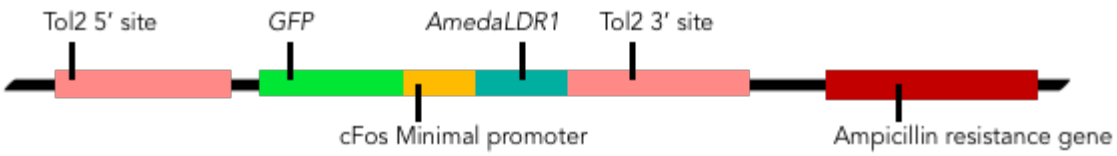


**Figure 4: pGreen E plasmid vector.**
This depicts the plasmid vector containing our intended insert (*AmedaLDR1*).

The plasmid, *pGreenE* (Figure 4), contains an ampicillin resistance gene, along with the left and right ends for the *Tol2* transposon (a transposable element). The plasmid also contains a promoter from the mouse *c-fos* gene, along with a GFP protein coding region. The potential enhancer (designated *AmEdaLDR1*) should exist next to the promoter between the two ends of the *Tol2* transposon. *E. coli* bacteria were then transformed with a ligation reaction, in which the bacteria took up the intended DNA. The transformed *E. coli* were then spread onto a nutrient agar plate containing ampicillin.

Because the vector possesses the ampicillin resistance gene, it can be assumed that all surviving colonies on the plates contain the ligated vector, which likely contains the DNA insert (enhancer) as well. Liquid cultures were then made and grown overnight. Making a liquid culture requires mixing a growth medium with ampicillin and inserting a sterile-pipet tip that had been touched to various isolated colonies. The next day, a cloudy mixture formed that contained the bacteria. A commercial kit (Qiaprep miniprep – Qiagen) was then used to purify the plasmid DNA from the liquid culture.

The presence of the *AmEdaLDR1* insert was tested for in the plasmid DNA by digesting it with two restriction enzymes. The resulting size of the digest was then measured via gel electrophoresis. The expected size of the digest should have been around 600 base pairs, and this size was achieved. A mixture of the plasmid DNA, along with mRNA for the *Tol2*-transposase gene, was then injected into 53 fertilized zebrafish embryos following the procedure of Fisher *et al*. (2006). The transposase mRNA was injected to allow translation into a protein that catalyzes insertion of all of the DNA between the two Tol2 ends into the zebrafish chromosomes. After the injections, all defective and dead embryos were removed, leaving 35

injected zebrafish embryos with normal morphology. Of the 35 embryos, seven zebrafish were isolated for highest Green Fluorescent Protein (*GFP*) expression.

The zebrafish embryos were then analyzed for *GFP* expression using fluorescence microscopy, and further analyzed for any deformities that may have resulted from an overdose of injected DNA. The embryos that fluoresced and lacked deformities were raised to adulthood (five of seven survived). Crossing these injected fish to wild-type zebrafish resulted in identification of a single transgenic founder capable of transmitting the reporter construct to its offspring. These offspring constitute a transgenic line. Because the development of scales in the zebrafish requires about a month, *GFP*-expressing transgenic embryos were examined weekly by fluorescence microscopy from one to five weeks' post-fertilization.

## RESULTS AND DISCUSSION

### Phylogenetic character mapping

The phylogenetic tree of selected scorpaeniform suborders with skin covering characters mapped on it is shown in Figure 2. This tree shows that the common ancestor of the Gasterosteoidei may have had bony dermal plates or scales or been naked with equal probability. Whether bony dermal plates arose directly from scales or from a naked condition after the loss of scales cannot be determined by this analysis. Because stickleback bony dermal plates do not share any skeletal tissue types with the elasmoid scales that they replaced (Sire *et al.* 2009), I tentatively conclude that bony dermal plates arose following scale loss.

**Analysis of gene expression by *in situ* hybridization**

Figure 5 shows the results of the *in situ* hybridization. Of the two probes, only the *Eda-a* gene showed specific expression in the scales of Mexican tetra larvae. The *Eda-b* gene was expressed in the skin of one embryo, but expression did not appear to be stronger in the scales. Additional analyses will be required to confirm these preliminary results, due to the relative damage caused by some of the proteinase-K concentrations.

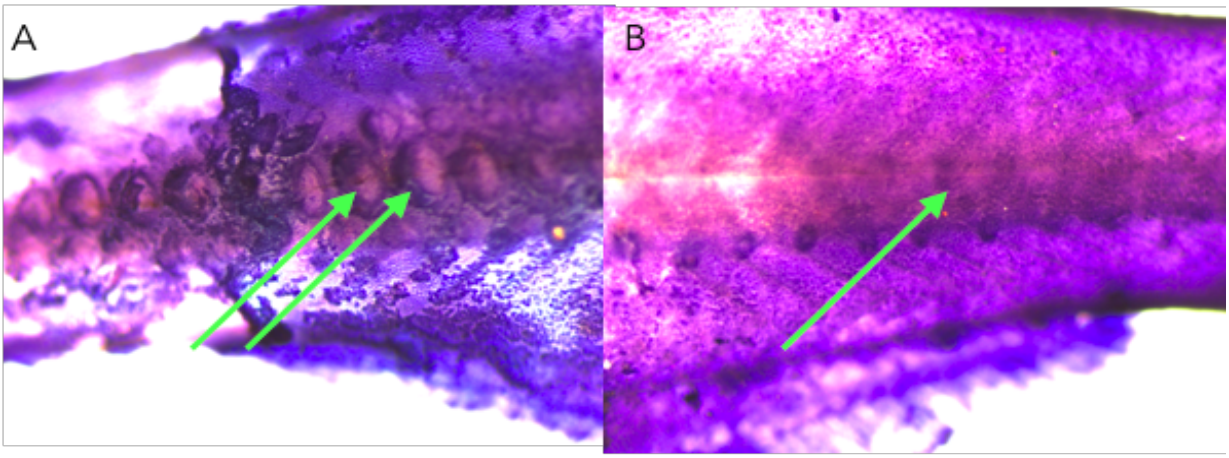


**Figure 5: *Eda* genes expressed in the Mexican Tetra.**
Figure A shows *Eda-a* expressed in the scales of the Mexican Tetra (the green arrows point to scale expression). Figure B show *Eda-b* expressed in the skin of the Mexican Tetra. The horizontal dots in Figure B are lateral line neuromasts (sensory organs).

If the results of *in-situ* hybridization can be confirmed, the evolution of scale expression in this species may have involved a gene duplication followed by subfunctionalization of the two genes with respect to scale expression.

**Reporter transgenic analysis**

Figures 6 and 7 show *GFP* expression in transgenic zebrafish. While analysis of *GFP* expression in *AmEdaLDR1* transgenic larvae revealed expression at the anterior ends of fins as well as in the premaxilla of the upper jaw, no expression was found in scales.

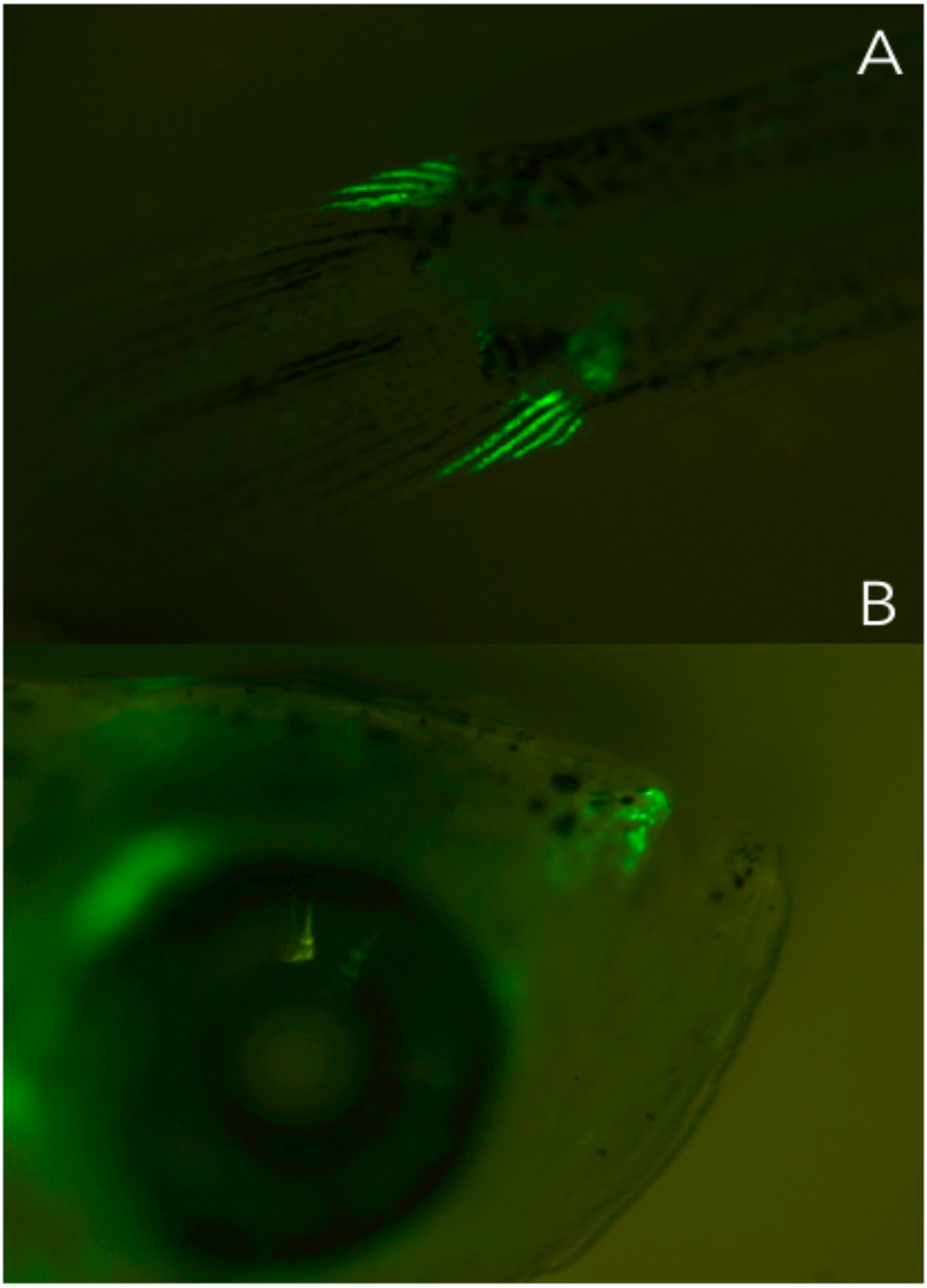


**Figure 6: *GFP* expression in F1 generation fish.**
*GFP* expression in the caudal fins of a fish from the F1 generation (A). *GFP* expression in premaxilla (upper jaw) of a fish from the F2 generation (B).

Because the corresponding stickleback *Eda-a* enhancer also drives expression in pelvic fins and the premaxillary, I conclude that a pre-existing enhancer that drove expression in the premaxilla, the pelvic fins, and the caudal fins acquired the ability to drive expression in the

bony dermal plates of stickleback fishes. Therefore, even though bony dermal plates likely constitute a morphological novelty, their genetic regulation did not arise *de-novo* but rather from pre-existing building blocks. Such acquisition of new regulatory activities by pre-existing enhancers has been documented in other systems (Rebeiz *et al.* 2011; Rebeiz and Tsiantis 2017) and may reflect a common pattern in evolution.

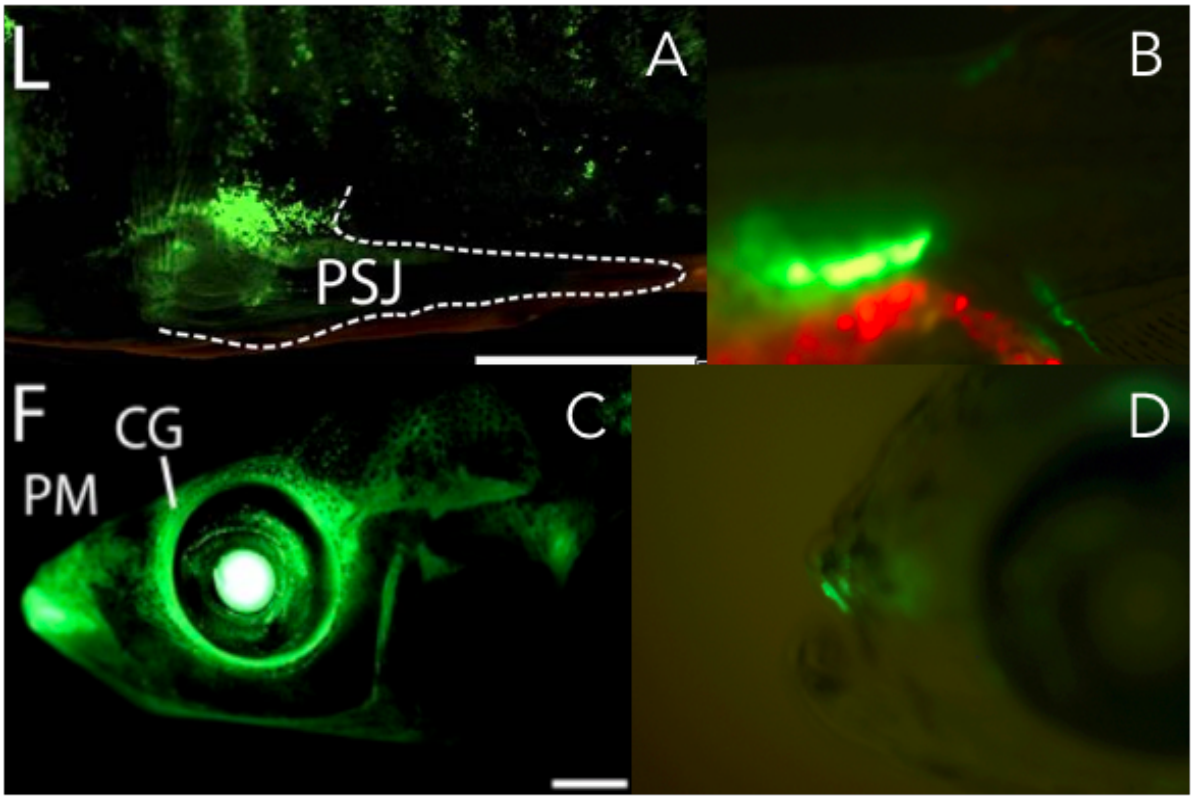


**Figure 7: *GFP expression in transgenic zebrafish.***
Imaging of *GFP* expression in the pelvic fins by O'Brown *et al.* 2015 (A), as compared to our imaging of *GFP* expression in the pelvic fins of a transgenic fish (B). Imaging of *GFP* expression in the premaxilla (upper jaw) by O'Brown *et al.* 2015 (C), as compared to our imaging of GFP expression in the premaxilla of a transgenic fish (D).

Figure 8 provides a summary of my hypothesis, in which the colored boxes represent enhancers corresponding to the colored structure on the fish. The first fish depicts a scaled ancestor of sticklebacks in which *Eda* is expressed in scales using the yellow enhancer, and an independent (green) enhancer drives expression in the premaxillary bone and pelvic fins.

Following the loss of scales, the premaxillary/pelvic enhancer retained its function and the scale enhancer may or may not have been lost, as depicted by the second fish. Expression of *Eda* in newly evolved dermal plates was made possible by modification of the premaxillary/pelvic fin enhancer to drive expression in the bony dermal plates.

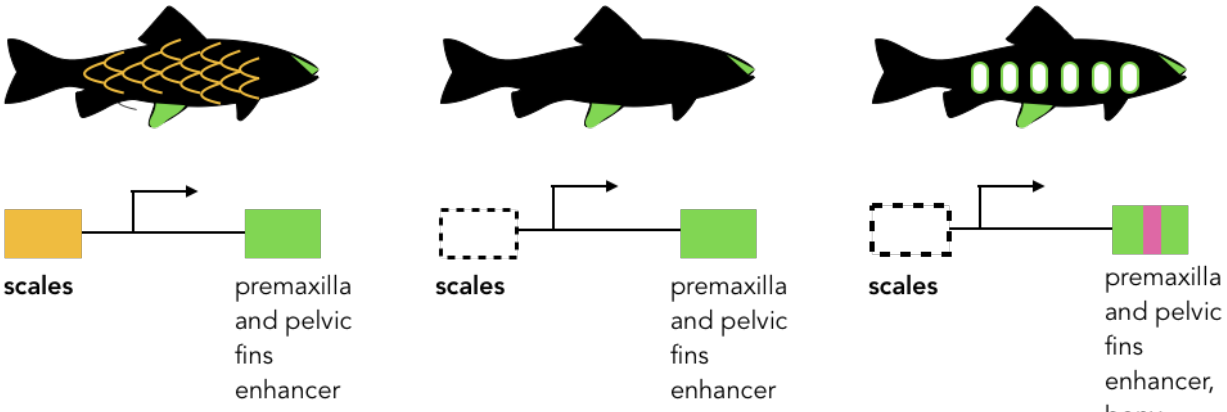


**Figure 8: Evolution of enhancer function in association with the replacement of scales by scutes in stickleback fishes.**
In the figure above, boxes represent enhancers responsible for *Eda* expression in specific locations. Coloring on the fish represents *Eda* expression driven by the enhancer of the same color.

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