The Role of Scpp Genes in the Loss and Replacement of Scales in Catfishes

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Abstract

Dollo's Law of Irreversibility states that a species cannot regain a structure that it has previously lost. The function of a lost structure may be replaced by a similar one, however. An example of the latter phenomenon is found in catfishes (Siluriformes). Scales were lost in the common ancestor of this group and replaced in some lineages by bony plates known as scutes. Scutes are functionally similar to scales but are structurally distinct. I hypothesized that the genes involved in scale development were retained in "naked" catfish and redeployed in the origin of scutes. Members of the Secretory calcium-binding phosphoprotein (Scpp) gene family were examined because they are thought to have played an important role in the evolution of vertebrate hard tissues. The specific family members, *scpp1* and *scpp5*, were chosen due to their known expression in bone and dental tissues of other species. Such tissues are found in both scales and scutes. By using an *in situ* hybridization approach to analyze the expression of *scpp1* and *scpp5* in the skin of three fish species, I have found both *scpp1* and *scpp5* to be expressed in the scales of zebrafish (*Danio rerio* – representing the ancestral condition), neither to be expressed in the trunk skin of the "naked" channel catfish (*Ictalurus punctatus*), and only *scpp1* to be expressed in the scutes of an armored catfish (*Corydoras fulleri*). These results, along with the expression of the genes in other structures, such as teeth and fin rays, suggest that scale loss did not lead to loss of all the genes required for scale development. Retained genes, such as *scpp1*, are therefore available for redeployment in the development of replacement structures contributing to the evolvability of the integumentary (skin) skeleton.

Keywords: zebrafish, channel catfish, armored catfish, *scpp1*, *scpp5*, scales, scutes, evolvability

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1. Introduction

1.1 Study Objective

Since the early 20th century, the idea that a morphological feature cannot return in evolution once it is lost has been considered a "law" (Dollo's Law of Irreversibility and Arber's Law of Loss – Arber, 1919). Recent research has focused on documenting exceptions to this rule, such as wings in stick insects and mandibular teeth in frogs (Collin & Miglietta, 2008; Galis *et al.*, 2010; Recknagel *et al.*, 2018). A less well-studied addendum to Dollo's Law is Arber's Law of Loss, which states that a lost feature can be replaced by a functionally similar yet structurally distinct one (Arber, 1919).

There are multiple examples of this replacement of lost structures throughout evolution, especially in the vertebrate integumentary (skin) skeleton, since mineralized structures present in the skin can develop in an array of forms. A familiar example of the integumentary skeleton is provided by the scales of living and fossilized fish (Sire *et al.*, 2009). These scales have been lost in most living tetrapods (land vertebrates), but some lineages have met their need for protective structures with bony plates known as osteoderms (Vickaryous & Sire, 2009). Since tetrapods are such a diverse group, there are multiple examples, including turtles, ankylosaur and stegosaur dinosaurs, Gila monster lizards, and armadillos, which suggests that the replacement of lost scales with osteoderms has occurred on multiple occasions (Hill, 2005; Vickaryous & Sire, 2009; Williams *et al.*, 2022).

The objective of my thesis is to understand the genetic mechanisms behind loss and functional replacement of components of the integumentary skeleton. An ideal group of

organisms to investigate would include relatively closely related species that exhibit the three main phenotypic states: ancestral presence of scales, the absence of integumentary skeleton, and the reappearance of a protective layer. These requirements are not met in most of the tetrapod examples listed above, but they are in ray-finned fishes (Lemopoulos & Montoya-Burgos, 2021). I have chosen representatives from the Otophysi clade (minnows, catfishes, tetras, and South American knifefishes – Nelson *et al.,* 2016) which have published genomes and are easily obtained as embryos/larvae. The common ancestor of the Otophysi possessed scales (Lemopoulos & Montoya-Burgos, 2021), which is a trait that was retained in most members of the Cypriniformes (minnows) and Characiformes (tetras). Unlike these orders, the common ancestor of siluriforms (catfishes) lost its scales (Liu *et al*., 2016; Lemopoulos & Montoya-Burgos, 2021). While most catfish remain "naked", there are five lineages that have independently regained an integumentary skeleton in the form of scutes, one of which is shown in Figure 1 (Liu *et al.,* 2016; Lemopoulos & Montoya-Burgos, 2021). Scutes, also referred to as bony plates, armor, or trunk dermal plates, are functionally similar to scales since they are both protective structures, but they are morphologically distinct (Lemopoulos & Montoya-Burgos, 2021; Mori & Nakamura, 2022; Liu *et al.,* 2016).

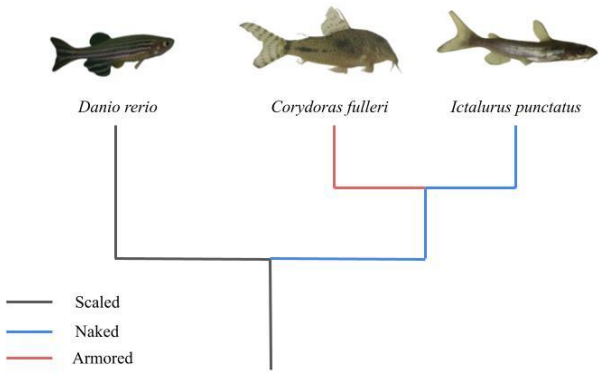

Figure 1. Simplified Phylogenetic Tree of *Danio rerio, Corydoras fulleri,* and *Ictalurus punctatus*.

The phylogenetic relationship of the three species investigated, as described by Liu *et al.* (2016), is illustrated. The black line leads to *D. rerio* (the zebrafish) and represents scaled species. The blue line (naked) leads to *I. punctatus* (the channel catfish) and is the most recent ancestral condition to the armored species (red) as represented by *C. fulleri*.

The three representative species I have chosen for analysis are *Danio rerio* (the cypriniform zebrafish, which possesses scales), *Ictalurus punctatus* (the siluriform channel catfish, which has a naked skin), and *Corydoras fulleri* (an armored catfish with scutes). *D. rerio* is an extremely popular model organism for biomedical research and has a published genome (Sprague *et al.*, 2003). These omnivores grow to a maximum length of 30 mm, spawn readily in the laboratory, and are therefore ideal for developmental and genetic studies (Spence *et al.* 2007). *I. punctatus* is an important species in aquaculture with embryos easily obtainable from breeders (Osage Catfisheries, Inc.), and it has a published genome (Liu *et al*., 2016). *C. fulleri*, a recently discovered species of armored catfish, also has a published genome, and laboratory breeding has been reported for this species (Bell *et al.*, 2022; Tencatt *et al.*, 2021).

The mechanism underlying the irreversibility of evolutionary loss is thought to be the accumulation of loss-of-function mutations in the genes needed to develop the structure (Marshall *et al.,* 1994). These mutations would accumulate unless the gene was required for another feature of the organism (*i.e.,* the gene is pleiotropic). In the case of these fishes, pleiotropy might explain the reappearance of similar structures, since the genes' roles in other features makes them more readily available to be used for the development of the replacement.

The genes I chose to examine for evidence of the above-mentioned evolutionary pattern of scale loss and replacement by scutes are members of the Secretory calcium-binding phosphoprotein (Scpp) gene family. Scpp genes control the concentration of calcium phosphate, which is the mineral component of the vertebrate skeleton (scales, teeth, and bones - Kawasaki & Weiss, 2003). The two specific genes I chose to investigate are associated with the development of an array of skeletal tissues. *scpp1* is involved in the development of bone and dentine (the main component of teeth), and *scpp5* is expressed in association with hypermineralized tissues (such as enamel), which are found on the outer surface of teeth and the scales of some fishes (Kawasaki, 2009; Kawasaki *et al.,* 2021; Rosa *et al.,* 2021). These data have been collected from RNA-seq or Real Time PCR techniques, which involve homogenization of tissues and lack the spatial resolution to identify clearly if the expression is in the skin or integumentary skeleton. In contrast, using a whole mount *in situ* hybridization, the method used in the present study, determines the location of expression in intact specimens. Another reason for choosing these two genes is that expression in the teeth and/or bone (Kawasaki, 2009) suggests a reason for retention in the genome following scale loss.

1.2 Background

Composition of the Integumentary Skeleton. The integumentary skeleton of vertebrates is extremely diverse in structure and composition. Sire *et al.* (2009) have interpreted this diversity by differentiating between the presence or absence of four mineralized tissue types. The first tissue type is the hypermineralized layer, which is composed of very little organic material and consists largely of calcium phosphate. Another tissue type is dentine, which is well-mineralized with an extracellular matrix rich in collagen proteins and is characteristic of teeth. Plywood-like tissues, the third tissue type, have collagen fibrils that stack upon one another in layers. The most common type of plywood-like tissue in fishes is elasmodine, which is the main component of elasmoid scales. The final type of mineralized tissue is bone. Sire *et al*. (2009) propose that the common ancestor of ray-finned fishes had a scale that consisted of all four tissue types, as found in the living genus *Polypterus* (bichirs). In this polypteroid scale type, the hypermineralized tissue is called ganoine, the plywood-like tissue is elasmodine, and both dentine and bone are also found. The next step in evolution, as interpreted by Sire *et al.* (2009), starts with the reduction of the polypteroid scale into the elasmoid scale, which can be found in *D. rerio*. As shown in Figure 2, most of the scale is made of elasmodine (derived from dentine), which is covered anteriorly with the dentine-like external layer and posteriorly with the hypermineralized limiting layer. This interpretation is not universally accepted, however. For example, Schultze (2018) concluded that the external layer is made of isopedine (a type of bone) rather than dentine. This view does not alter my hypothesis because *scpp1* is said to be expressed in both dentine and bone. Schultze (2018) provides another contradictory hypothesis that the limiting layer is not likely to be homologous to ganoine, which could also refute the idea of ganoine and hyaloine (the hypermineralized tissue of scutes) being related even though all three, including the limiting layer, are considered hypermineralized tissues.

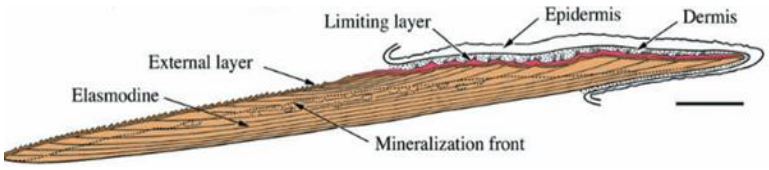

Figure 2. Cross-section of Scale Tissues

Diagram taken from Sire *et al.* (2009) showing the various tissues of which elasmoid scales are composed.

The scutes of armored catfish are not uniform in structure, but in the genus *Corydoras* (Fig. 3), which is the subject of my research, the scutes consist of bone covered by hypermineralized hyaloine (Lemopoulos & Montoya-Burgos, 2021; Sire *et al.*, 2009). The scutes also support tooth-like structures, known as odontodes or dermal denticles, that consist of dentine covered with hypermineralized enamel.

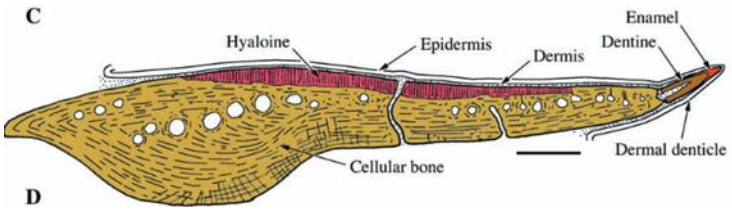

Figure 3. Cross-section of a Scute

Diagram taken from Sire *et al.* (2009) showing the tissues of which a scute is composed.

Details of Scpp Gene Expression and Predictions for Expression in Scales and Scutes. The expression of *scpp1* and *scpp5* has been most extensively studied in teeth. In *D.*

rerio, *in situ* hybridization showed *scpp1* expression in dentine and weakly in bone and enameloid (a hypermineralized tissue similar to enamel) and *scpp5* expression in enameloid and dentine (Kawasaki, 2009). Another study that used *in situ* hybridization found *scpp5* was expressed in the hypermineralized tissues of teeth of *D. rerio* and in the cells that produce ganoine in the teeth and scales of a gar, a ray-finned fish (Kawasaki *et al.*, 2021). Expression of *scpp1* and *scpp5* is less studied in the scales. Kawasaki *et al.* (2021) also used immunohistochemistry to demonstrate the Scpp5 protein in the ganoine of gar. Liu *et al.* (2016) used Real Time PCR to find the presence of both genes in various *D. rerio* tissues, including scales, but the exact location cannot be confirmed using this method. Finally, Bergen *et al.* (2022) collected RNA-Seq data from *D. rerio* to conclude that both *scpp1* and *scpp5* have increased expression while the scales are regenerating.

All the previous data were collected from *D. rerio* and a species of gar, and there has not been much research conducted on armored catfishes. RNA-Seq data from Liu *et al.* (2016) identified expression of both genes in the armored species *Platydoras armatulus*. No conclusions, to my knowledge, have been made about the genes in members of the armored catfish genus *Corydoras*. Contradictory results have been reported about *scpp1* and *scpp5* in the naked catfish *I. punctatus*. The paper that motivated my research into this topic, Liu *et al.* (2016), suggested the importance of *Scpp1* and *Scpp5* in the formation of scales and scutes due to the intact reading frames of both genes in *D. rerio* and armored catfishes but not in *I. punctatus*. Furthermore, they failed to detect *scpp1* and *scpp5* in the skin transcriptome of this species. In contrast, Thompson *et al.* (2021) provided evidence for apparently functional versions of both genes in *I. punctatus.*

1.3 Hypotheses and Predictions

From consideration of the above data, I hypothesize that both genes will be expressed in the scaled *D. rerio* and armored *C. fulleri* but not in the naked *I. punctatus*, due to the lack of protective tissues in the latter species, as illustrated in Figure 4. I predict that *in situ* hybridization in *D. rerio* will detect *scpp1* in association with elasmodine and the external layer, and *scpp5* will be expressed in association with the hypermineralized limiting layer. In *I. punctatus*, there should not be any expression of either gene in the trunk, but I expect *scpp1* to be found in bone-like and dental tissues and *scpp5* to be expressed in teeth. Such results would support the retention of these genes in the genome due to their pleiotropic function in other morphological features. Finally, I predict *scpp1* to be expressed in the bone-like tissues and *scpp5* to be expressed in the hypermineralized hyaloine in *C. fulleri*. Both genes are also expected to be found in the odontodes of this species due to their similarity to teeth.

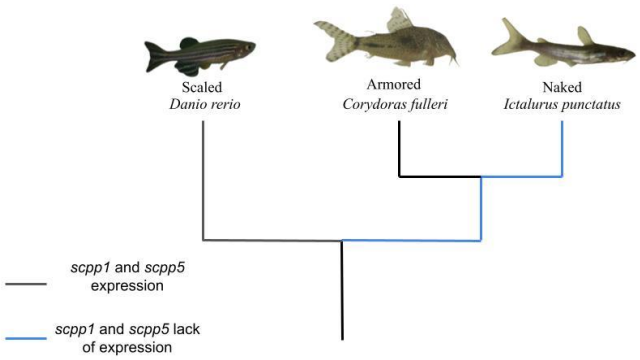

Figure 4. Predicted Expression of *scpp1* and *scpp5*.

Phylogenetic tree showing the predicted expression of *scpp1* and *scpp5* in trunk skin using the relationships depicted in Figure 1. Both genes are hypothesized to be found in *D. rerio* and *C. fulleri* but not in *I. punctatus.*

2. Methods

2.1 Animals

Larvae of *D. rerio* (Tübingen wild-type strain – Haffter *et al.*, 1996) and *C. fulleri* were obtained from breeding colonies in the Department of Ecology and Evolutionary Biology at the University of Colorado Boulder. Adult *D. rerio* were maintained in a flow-through aquarium system (https://aquaneering.com/) and adult *C. fulleri* in a 208-liter static aquarium. Embryos were collected from natural spawning of these adults and maintained until larval stages on the flow-through system (*D. rerio*) or in 38-liter static aquaria (*C. fulleri*). Embryonic and larval *I. punctatus* were obtained from Osage Catfisheries, Inc. (http://www.osagecatfisheries.com/) and raised to the stages of interest in 450-liter aquaria. Larvae of all three species were sacrificed by overdose with buffered tricaine methanesulfonate.

2.2 RNA Extraction

Total cellular RNA was extracted from multiple 100-hour post-fertilization *D. rerio* larvae, the jaws of a single juvenile *I. punctatus*, and the skin of a single larval *C. fulleri* using the Qiagen RNeasy Mini Kit (https://www.qiagen.com/) according to the manufacturer's instructions.

2.3 Reverse Transcription

RNA was reverse-transcribed to produce cDNA using the ThermoFisher Scientific Superscript IV reverse transcriptase protocol (https://www.thermofisher.com/). A 13 µl solution containing 3.8 µM random hexamer primers, 0.77 mM each dNTP, and 5.0 µg of total RNA was heated at 65 °C for 5 minutes and placed on ice. A 7 µl mixture containing 2.8X SSIV buffer, 14.3 mM Dithiothreitol (DTT), 40 units of Promega rRNasin RNase inhibitor

(https://www.promega.com/), and 200 units of Superscript IV reverse transcriptase was then added, followed by incubation at 23 °C, 52 °C, and 80 °C for 10 min each.

2.4 Primer Design

Primer-BLAST (Johnson *et al.*, 2008) from the National Center for Biotechnology Information (NCBI) was used to design primers for PCR amplification of coding exons of the following genes: *D. rerio scpp1* (NM_001145240), *D. rerio scpp5* (XM_678269), *I. punctatus scpp1* (XM_017463183), and *I. punctatus scpp5* (XM_017460912).

Because the genome assembly of *C. fulleri* (GCA_019802505.1) is not annotated, we used tBLASTn to search for *scpp1* using the *I. punctatus* ortholog as a query. No significant match was obtained, so we searched a genome assembly from a closely related species, *Corydoras maculifer* (GCA_019802435.1), using the same process. Multiple significant matches were obtained to scaffold 113 of this genome. We selected the longest of these matches along with 50kb on either end and searched this sequence for potential protein-coding genes with GENSCAN (Burge & Karlin, 1997). BLASTp searches using each of the predicted proteins as queries identified one as a significant match to *I. punctatus* Scpp1. We then used Clustal Omega (https://www.ebi.ac.uk/) to align the predicted *C. maculifer* Scpp1 protein with orthologs from the additional catfish species, *Pangasianodon hypopthalmus*, *Ictalurus punctatus*, and *Tachysurus fulvidraco* as shown in Figure 8. Absolutely conserved blocks of seven amino acids were reverse translated to design degenerate primers for amplification of *scpp1* from *C. fulleri*.

We used BLASTn to search for *scpp5* in the *C. fulleri* genome assembly. This search resulted in two short matches with contig 32. We selected 50,000 bases on either side of the longest match and searched the sequence using GENSCAN for putative protein-coding

sequences. One of these proteins matched *I. punctatus scpp5* using BLASTp. We aligned this *C. fulleri* protein with Scpp5 proteins from the additional catfish species, *Silurus meridionalis, Ictalurus punctatus,* and *Pangasianodon hypopthalmus* as shown in Figure 9. Because the *C. fulleri* sequence did not match the others well outside of the original BLAST hit, we manually revised the protein-coding sequence by searching for matches with reverse-translated short, highly-conserved sequences from other catfishes. We also found more BLASTn matches within contig 32 using *P. hypopthalmus scpp5* as a query. Matches to highly conserved amino acid sequences and the *P. hypopthalmus* mRNA were extended in length through consideration of likely splice donor and acceptor sites, as well as the observation by Kawasaki & Weiss (2006) that Scpp genes have phase 0 introns (occurring between rather than within codons). We assembled a predicted complete *C. fulleri scpp5* cDNA sequence using this approach and designed primers for amplification of a portion of this sequence using the APE program (https://jorgensen.biology.utah.edu/wayned/ape/). All primers used for Scpp gene amplification in the three species are shown in Table 1.

2.5 PCR Amplification

PCR amplification of fragments of Scpp cDNAs was carried out using the Phusion Hot Start Flex DNA polymerase kit from New England BioLabs (https://www.neb.com/) with the following conditions: 1x Phusion HF buffer, 0.2 mM each dNTP, 0.5 µM each primer, 1 µl of the 20 µl reverse transcription reaction, and 0.5 units of Phusion DNA polymerase. These mixes were placed in a thermocycler programmed for 98 °C for 30 seconds and followed by 35 cycles of 98 °C for 10 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds. These cycles were followed by a final extension at 72 °C for 5 minutes and storage at 4 °C. PCR product size was confirmed with agarose gel electrophoresis.

2.6 Purification, Ligation, and Transformation

PCR products were purified using the Qiagen MinElute PCR Purification Kit. The purified PCR product was then ligated to the pCR 4Blunt-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit from Invitrogen (https://www.thermofisher.com/). The ligation reaction was used to transform Invitrogen One Shot TOP10 chemically competent *E. coli* cells. Transformed cells were then spread on Double Yeast Tryptone (DYT) plates containing 100 µg/ml carbenicillin. Isolated colonies were used to inoculate DYT medium containing carbenicillin and incubated overnight. Plasmid DNA was extracted from the cultures using the Qiagen QIAprep Spin Miniprep Kit. Mixtures of this plasmid DNA and sequencing primers were submitted to Quintara Biosciences (https://www.quintarabio.com/) for Sanger sequencing. The identity of the insert sequences was confirmed by BLASTn searches.

2.7 Linearization of Plasmid DNA and Digoxigenin-labelled Riboprobe Synthesis

5.0 µg of plasmid DNA was linearized with either NotI or SpeI restriction enzymes. Digested DNA was purified using MinElute Spin columns. Gene-specific digoxigenin-labelled antisense riboprobes were synthesized in 20 µl reaction mixtures with 1 µg of linearized plasmid DNA, 1X Roche transcription buffer, 1X DIG RNA labeling mix (Roche), 40 units of either T3 or T7 RNA polymerase, and 20 units of rRNasin that were incubated for 2 hours at 37 °C. Riboprobes were purified from this mixture by ethanol precipitation and resuspended in 100 µl of tissue culture water containing 20 units of rRNasin.

2.8 Whole Mount in situ Hybridization

Larvae were staged by standard length according to Parichy *et al.* (2009) for *D. rerio*, Reyes (2010) for *I. punctatus*, and Sire (1993) for *C. fulleri*. Following sacrifice, larvae were fixed

overnight in 4% formaldehyde in phosphate-buffered saline (PBS) at 4 °C, rinsed with PBS containing 0.1% Tween-20 (PBST), and stored in 100% methanol at -20 °C. Fixed larvae were subjected to whole-mount *in situ* hybridization as described by Jackman *et al*. (2004). Briefly, larvae were rehydrated through a graded series of PBST in methanol. They were then incubated in proteinase K at concentrations of either 2.5 µg/ml or 25 µg/ml in PBST for 30 minutes before fixation in 4% formaldehyde in PBS. Following fixation, specimens were prehybridized at 60 °C in hybridization mix (50% formamide, 1.3X saline sodium citrate pH 5, 5 mM EDTA pH 8, 50 µg/ml yeast RNA, 0.2% Tween 20, 0.5% CHAPS detergent, 100 µg/ml heparin) for an hour. They were then transferred to 600 µl hybridization mix containing an estimated 300 ng of probe and incubated overnight at 60 °C. Excess probe was removed the next day with multiple washes of hybridization mix. Specimens were then rinsed with maleic acid buffer containing 0.1% Tween 20 (MABT) and blocked with heat-treated sheep serum and blocking reagent (Roche) in MABT before the addition of anti-digoxigenin-alkaline phosphate antibody (Roche). Following overnight incubation with the antibody at 4 °C, specimens were washed extensively with MABT. They were then washed with NTMT solution (50 mM $MgCl_2$, 100 mM Tris-Cl pH 9.5, 1% Tween 20, 1 mM levamisole), transferred to BM purple staining solution (Roche) and incubated overnight. Stained specimens were fixed in formaldehyde in PBS to inactivate the alkaline phosphatase and rinsed in PBST. Melanin pigments were removed by overnight bleaching in a solution containing 0.5% saline sodium citrate (SSC), 5% formamide, and 1% H_2O_2. Specimens were then transferred to PBST and imaged in agarose-coated tissue culture dishes with a Leica MZFLIII stereomicroscope equipped with a Leica DFC7000 T camera.

3. Results

3.1 Corydoras Scpp Sequences

The *C. maculifer* Scpp1 protein sequence we inferred from GenScan analysis of its genome assembly was 361 amino acids long (Figure 8) and encoded by eight exons. In comparison, the Scpp1 sequence of *I. punctatus* (XP_017318672.1) is 335 amino acids long and also encoded by eight exons. The cDNA fragment of *scpp1* that we amplified from *C. fulleri* encoded 333 amino acids, which were 96.3% identical (excluding gaps) to *C. maculifer* Scpp1 and 59.3-61.4% identical to the Scpp1 proteins of the other catfishes we examined.

cDNA sequencing and comparison to the genome assembly of *C. fulleri* allowed us to infer a total length of 170 amino acids for its Scpp5 protein that is encoded by ten exons. In comparison, the Scpp5 sequence of *I. punctatus* (XP_017316401.1) is 226 amino acids long and encoded by 13 exons. *C. fulleri* Scpp5 protein is 65.6%-68.9% identical (excluding gaps) to the Scpp5 proteins of the other catfishes we examined (Figure 9).

3.2 Danio rerio in situ Hybridization

Expression of *scpp1* during scale development of *D. rerio* was most apparent in the two larval individuals (9.4 mm and 10.4 mm standard length – SL) treated with the high proteinase K concentration (25 µg/ml). Both specimens (Fig. 5A-B) possessed scales at early and later stages of development. In early scale development, *scpp1* expression was strong in the margins and weaker in the center, while at later stages, expression remained strong but became restricted to the margins. *Scpp1* expression was also found in the fin rays (Fig. 5C), and it was most noticeable at the low proteinase K concentration. The soft rays of *D. rerio* are segmented (Parichy *et al.*, 2009), and *scpp1* was found to be expressed just outside of the boundaries of these segments. In the 72 hours post-fertilization (hpf) specimens, bone and tooth mineralization

is just starting to begin. *scpp1* was found to be expressed in the cleithrum (a dermal bone of the shoulder girdle) at this stage but not in the pharyngeal teeth (Fig. 5D).

scpp5 expression was weak at the early stages of *D. rerio* scale development (9.0 mm SL – Fig. 5E) and stronger at later stages (10.2 mm SL – Fig. 5F) where it exhibited a punctate pattern towards the posterior end of each scale. *scpp5* was strongly expressed in the 72 hpf larva (Fig. 5G) in the pharyngeal teeth as well as in the parasphenoid bone (a dermal bone of the palate).

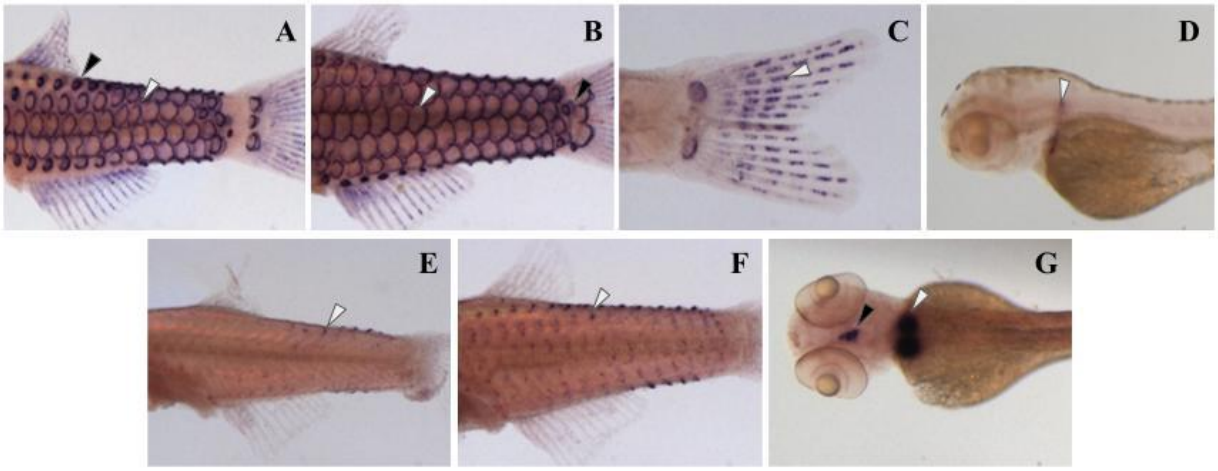

Figure 5. The Expression of Scpp Genes in *D. rerio*.

A, B) Expression of *scpp1* in early (black arrowhead) and late (white arrowhead) development of the 9.4 mm SL (A) and the 10.4 mm SL (B) larvae. C) *scpp1* expression in the caudal fin rays (arrowhead) of the 8.9 mm SL larva. D) *scpp1* expression in the cleithrum (arrowhead) of a 72 hpf specimen. E, F) Expression of *scpp5* in the posterior margins of scale development (arrowhead) of the 9.0 mm SL (E) and the 10.2 mm SL (F) specimens. G) At 72 hpf, *scpp5* is expressed in the pharyngeal teeth (black arrowhead) and the parasphenoid bone (white arrowhead). A-B, E-F) lateral views of the posterior trunk. C) lateral view of the caudal fin. D, G) lateral and dorsal views, respectively, of the head and anterior trunk.

3.3 Ictalurus punctatus in situ Hybridization

No expression of *scpp1* or *scpp5* was found in the trunk skin of the *I. punctatus* larvae examined (Fig. 6A and 6D respectively). *scpp1* was expressed in the fin rays of the anal and caudal fins (Fig. 6A and 6B, respectively) and weakly in the oral teeth (Fig. 6C). *scpp5* was strongly expressed in the teeth (Fig. 6F), but there was no expression in the fin rays (Fig. 6E). Expression in the fin rays and/or teeth suggests that the failure to detect trunk expression of either gene is not a result of the probes' quality.

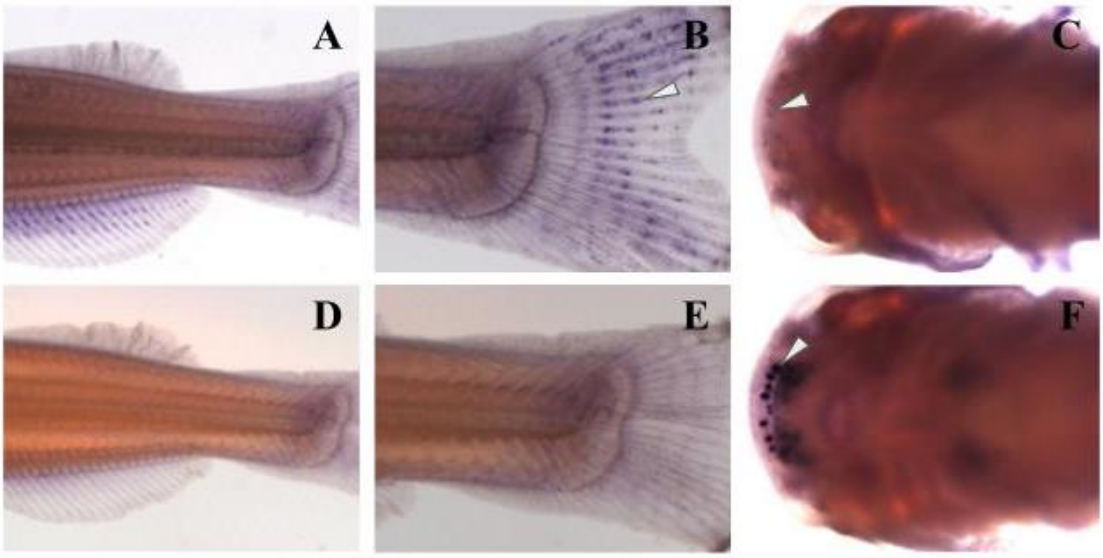

Figure 6. Expression of Scpp Genes in *I. punctatus*.

A, B) *scpp1* expression in the fin rays (arrowhead) but not trunk skin of an 18.5 mm SL specimen. C) Weak expression of *scpp1* in the oral teeth (arrowhead) of a 17.0 mm SL fish. D, E) *scpp5* expression was absent from the trunk skin and fin rays of the 17.0 mm specimen. F) strong expression of *scpp5* in the oral teeth (arrowhead) of a 17.0 mm SL fish. A-B, D-E) lateral views of fins and caudal peduncle. C, F) ventral views of head.

3.4 Corydoras fulleri in situ Hybridization

Scpp1 expression was found in association with the scutes of *C. fulleri* (Fig. 7A, B). Weak expression with a punctate pattern outlined the margins of the scutes, while stronger expression was exhibited found in a ring around the base of the odontodes attached to the scutes. This latter expression may represent the bone of attachment. In addition, the adipose fin spine, which is believed to be homologous to the lateral scutes on the trunk (Stewart *et al.*, 2019), showed strong expression of *scpp1* (Fig. 7C). *scpp1* was also strongly expressed in the fin rays (Fig. 7D).

The only expression of *scpp5* found was in the odontodes (Fig. 7E-H). *scpp5*-expressing odontodes developed on the lateral scutes (Fig. 7E, F), the adipose fin ray (Fig. 7G), and the caudal fin rays (Fig. 7H). This expression appeared to be localized to the tips of the odontodes, rather than the bases (Fig. 7F).

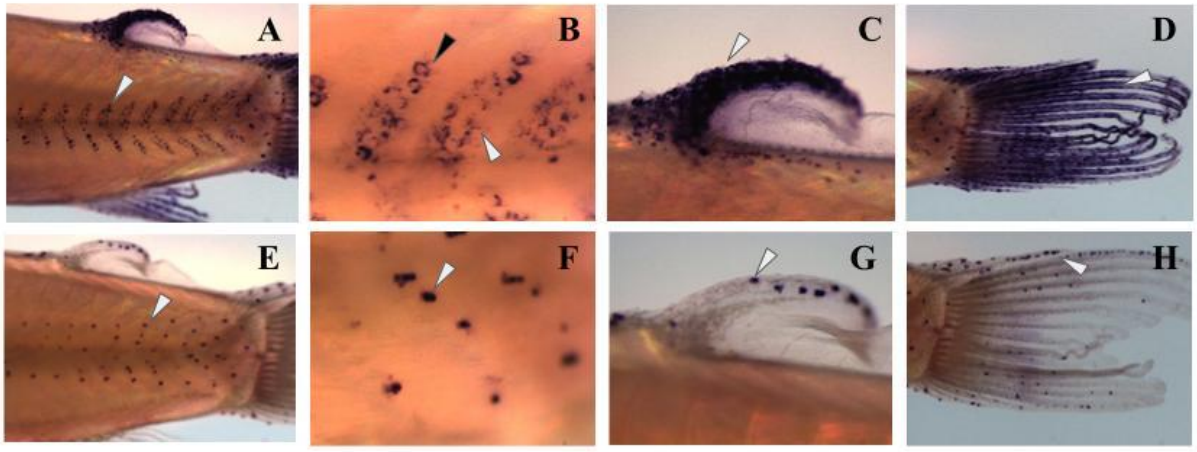

Figure 7. The Expression of Scpp Genes in *C. fulleri*.

A-D) *scpp1* expression in a 15.0 mm SL specimen. *scpp1* is expressed in scutes (white arrowheads in A and B) and the bases of the odontodes (black arrowhead in B). *scpp1* is also expressed in the adipose fin spine (arrowhead in C) and in the caudal fin rays (arrowhead in D). E-H) *scpp5* expression in odontodes (arrowheads) attached to the scutes (E, F), the adipose fin ray (G), and the caudal fin rays (H) of the 16.0 mm SL specimen. A, E) lateral views of the

caudal peduncle. B, F) higher magnifications of A and E. G) Lateral view of the adipose fin ray. D, H) lateral views of the caudal fin.

4. Discussion

Since *scpp1* is expressed in *D. rerio* and *C. fulleri* and not in the skin of *I. punctatus*, there is evidence in support of this gene's importance in the development of scales and scutes. This result also indicates that *scpp1* was retained in the naked lineages and redeployed in the armored species since it was found before scales were lost and after scutes appeared.

4.1 Scpp Gene Expression in D. rerio

Expression of *scpp1* and *scpp5* has been previously reported in the scales of *D. rerio* (Liu *et al*., 2016; Bergen *et al*., 2022), but this study provides the first spatial description of both genes' expression in elasmoid scales. Expression of *scpp1* in the early stages of scale development is consistent with expression in cells contributing to either the external layer or the elasmodine, as these are the first two mineralized tissues to form (Sire *et al*., 2009). Sire *et al.* (2009) proposed these two tissues are derived from dentine while Schultze (2018) interpreted the external layer as a bony isopedine layer and elasmodine as a novel tissue. Both conclusions would predict *scpp1* expression in scales, since bone and dentine show expression of *scpp1* in other contexts (Kawasaki, 2009).

The location of *scpp5* expression is consistent with its predicted presence in the limiting layer due to its stronger expression in the later stages and its expression in the posterior edges of each scale where the limiting layer resides. The limiting layer was suggested by Sire *et al.* (2009) to be homologous to the hypermineralized ganoine found in the *Polypterus* and gar scales. This

interpretation is consistent with the finding by Kawasaki *et al*. (2021) that *scpp5* expression is present in the ganoine-forming cells of the gar and the results from my study suggesting *scpp5* expression associated with the hypermineralized limiting layer. These data contrast with the conclusion of Schultze (2018) that the limiting layer and ganoine are not related.

In addition to *scpp1* and *scpp5* expression in the scales of *D. rerio*, I found expression of *scpp1* in the fin rays and *scpp5* in the teeth. The expression of *scpp1* in both the scales and fin rays supports the classical hypothesis that fin rays are derived from scales (Goodrich, 1904).

4.2 Scpp Gene Expression in I. punctatus

The lack of *scpp1* and *scpp5* expression in the trunk of *I. punctatus* supports the conclusions made by Liu *et al*. (2016), but my findings of *scpp1* in the fin rays and teeth and *scpp5* expression in the teeth contradict that study. Expression in those locations suggest that the genes are still functional. This result also indicates a potential mechanism for the retention of the genes even after scale loss. Interestingly, *scpp5* in the genomes of the common seadragon (*Phyllopteryx taeniolatus*) and the alligator pipefish (*Syngnathoides biaculeatus*) have degenerated into pseudogenes (inactive genes), likely due to the lack of teeth and scales in these species (Qu *et al.,* 2021). In addition, the bony plates that comprise the integumentary skeleton of these fishes lack a hypermineralized layer (Sire *et al.* 2009), for which *scpp5* would likely be required.

4.3 Scpp Gene Expression in C. fulleri

scpp1 was expressed weakly in the scutes, but it was strongly expressed in the adipose fin spine which is thought to be derived from scutes (Stewart *et al.,* 2019). The thinner membrane of the adipose fin might have allowed for more penetration of the probe as compared to the scutes

of the trunk, which might explain the difference of expression. *scpp1* expression in other structures has been reported in the bone-like tissues (Kawasaki, 2009) suggesting that its expression in the scute of *C. fulleri* is in the bony layer.

Expression of *scpp5* was not found in the scutes of *C. fulleri,* which is surprising given the presence of the hypermineralized hyaloine in these structures and the expression of *scpp5* in association with the ganoine of gar scales, the enameloid of *D. rerio* teeth (Kawasaki 2009; Kawasaki *et al.*, 2021), and the limiting layer of D. rerio (this study), all of which are hypermineralized. Because of this, I propose this result as a limitation of the study since the age at which the hyaloine forms may be later than that of the fish I examined (see below).

There was expression of both *scpp1* and *scpp5* in the trunk odontodes which is consistent with Sire's (2001) view that odontodes represent teeth outside of the mouth.

4.4 Scpp Genes and the Evolvability of the Integumentary Skeleton

Vertebrates have shown the ability to regain an integumentary skeleton even after losing this feature (Hill, 2005; Vickaryous & Sire, 2009; Williams *et al*., 2022; Lemopoulos & Montoya-Burgos, 2021). The ability to revert to an ancestral trait gives insight into the species' evolvability, which is the ability for the genome to generate heritable adaptive phenotypic variation (Payne & Wagner, 2019). With climate change increasingly relevant, understanding evolvability can help us predict a species' ability to adapt to the changing world. A possible component of the evolvability of the integumentary skeleton is the pleiotropy of the genes involved in its development, which makes them available for deployment in a replacement structure. My study supports this idea since both *scpp1* and *scpp5* were retained in *I. punctatus* and *scpp1* was redeployed in *C. fulleri.*

4.5 Limitations of the Study

Further *in situ* hybridizations of *scpp1* using different concentrations of proteinase K might provide deeper penetration of the probe in *C. fulleri*. Trunk scutes are covered in more tissue than the adipose fin, so if I could get better penetration in the deeper layers of the trunk skin, I would likely detect more robust expression.

Another limitation of my study is the age at which the *C. fulleri* larvae were sacrificed. Sire (1993) indicated that hyaloine, which is where I predicted *scpp5* to be expressed, begins to form in larvae of *C. arcuatus* at about 20 mm SL, but the *C. fulleri* larvae I examined were only 15-16 mm SL.

4.6 Future Directions

There are many ways to continue this research in addition to overcoming the limitations described above. Investigating the expression of other Scpp genes known to be associated with scale development like *scpp6, scpp7,* and *scpp8* (Zhang *et al.*, 2022), is a promising and straightforward direction to this research. In addition to other genes, other groups of fish could be examined. There are four other catfish lineages (Liu *et al.,* 2016) that had scales, lost them, and regained scutes, so examining such lineages would test the generality of my findings. Finally, an interesting future experiment would be to examine *scpp1* and *scpp5* expression in species of the genus *Astroblepus.* This genus belongs to a catfish lineage that evolved scutes and eventually lost them as well (Liu *et al.*, 2016).

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Supplementary Materials

D. rerio scpp1	Forward: AGAGAACATCCGTAGGGGCT
	Reverse: CTAGGGCTCAGCGACATCAG
D. rerio scpp5	Forward: CAAAGGTGACCACGCTGACT
	Reverse: GGGGGACATTTGGTTGATCCT
I. punctatus scpp1	Forward: TTAGCTAGCAGCGAGATCAGC
	Reverse: AATGGGTCGTTGGTTCCGTC
I. punctatus scpp5	Forward: GGGATCAGGTCCTTGTGGTG
	Reverse: GATCGTTGCCCTGTGAAGGA
C. fulleri scpp1	Forward: GCCGGGATCCGCNGCNGCNGCNAAYCCNAT
	Reverse: GCCGGAATTCTGRAANCCRTTRAANGGRTC
C. fulleri scpp5	Forward: GCTGCCAACTTCTGTGACCATG
	Reverse: CATTTTGTGGAGGAGCAGCCTG

Table 1. Primers

This table lists the primers used to produce the probes for *in situ* hybridizations. The underlined sections indicate restriction sites that were added to facilitate cloning.

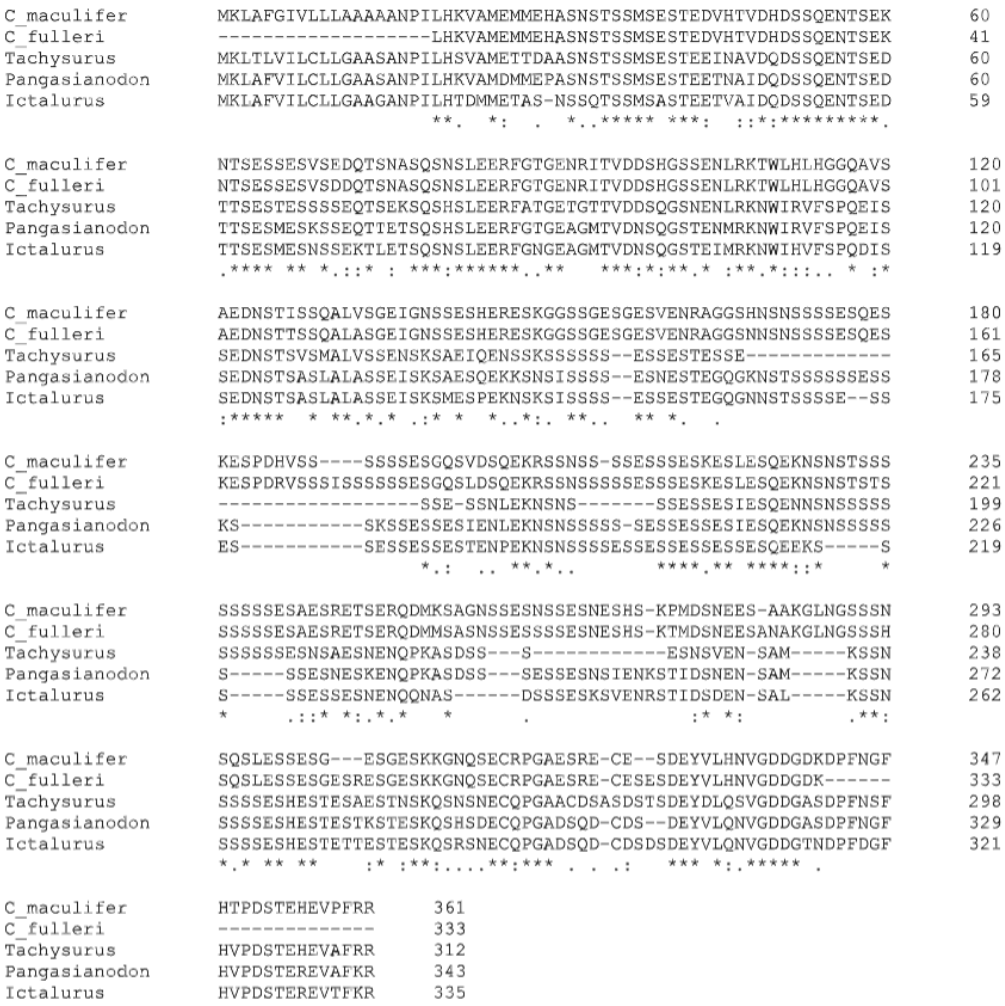

Figure 8. Alignment of Scpp1

This shows the Scpp1 alignment of *C. maculifer, C. fulleri, T. fulvidraco, P. hypopthalmus,* and *I. punctatus.* Asterisks indicate identical amino acids in all species and dots indicate chemically similar amino acids in all species. Dashes indicate alignment gaps in general, while the dashes at the beginning and end of the *C. fulleri* Scpp1 sequence represent missing data.

Figure 9. Alignment of Scpp5

This shows the Scpp5 alignment of *C. fulleri*, *S. meridionalis*, *Ictalurus punctatus*, and *Pangasianodon hypopthalmus*. Asterisks indicate identical amino acids in all species and dots indicate chemically similar amino acids in all species. Dashes indicate alignment gaps.