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Morphogenesis of Striated Muscle: An Analysis of its Regeneration in Gill Filaments of *Salmo salar*

Morfogénesis del Músculo Estriado: Análisis de su Regeneración en los Filamentos Branquiales de *Salmo salar*

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SUMMARY: The objective of this study was to describe the formation of branchial striated muscle tissue during the Parr and Smolt ontogenetic stages of *Salmo salar* in response to natural environmental challenges. To achieve this, 15 Parr and 15 Smolt *S. salar* alevins were analyzed. Specimens were anesthetized and euthanized using 5 % benzocaine, following established animal welfare protocols. They were subsequently fixed in 10 % formalin and processed for histological analysis using Hematoxylin-Eosin/Alcian Blue double staining. The regeneration blastema area and the branchial region occupied by striated musculature were quantified. In *Parr* specimens, the gills were still under development, and striated musculature at the base of the filaments had not yet formed. In contrast, *Smolt* specimens exhibited a well-defined regenerative blastema, suggesting an adaptive response to environmental stressors such as hypoxia. Notably, striated muscle fibers were observed at the base of regenerating branchial filaments, fully differentiated from the blastema. Immunohistochemical analysis confirmed that these muscle fibers were positive for the anti-Shh antibody, indicating a role for Sonic Hedgehog signaling in their formation. These findings highlight the remarkable regenerative capacity of teleost fish, particularly in gill-associated musculature. Understanding the mechanisms underlying branchial muscle regeneration could provide valuable insights into fish physiology and aquaculture practices.

KEY WORDS: Atlantic salmon; *Salmo salar*; Branchial musculature; Striated muscle; Regenerative blastema; Branchial filaments.

INTRODUCTION

Salmonid muscle tissue exhibits remarkable plasticity in response to environmental changes occurring during the larval stages and early development (Johnston & Hall, 2004). Factors such as temperature fluctuations in the aquatic environment during the embryonic phase have long-lasting effects on skeletal muscle development and growth, influencing key processes such as the timing and extent of myotube formation, even persisting into adulthood (Johnston, 2006; Johnston *et al*., 2011).

Skeletal muscle formation involves a highly regulated process of cell fusion, essential for generating syncytial tissues (García-Orozco *et al*., 2024). During development, mononucleated myoblasts fuse sequentially to form multinucleated myotubes, which subsequently mature into functional skeletal muscle fibers. This differentiation process comprises several critical stages, including myogenic differentiation, cell adhesion, and membrane fusion, all of which are orchestrated by a complex network of genetic and molecular regulators (Aguilar *et al*., 2013; Kim *et al*., 2015; Hromowyk *et al*., 2020).

In this study, we focus on a specific type of striated muscle that forms exclusively at the base of branchial filaments during gill regeneration (Mierzwa *et al*., 2020). Fish gills are highly susceptible to a wide range of infectious and non-infectious diseases, including those caused by pathogens, zooplankton, and harmful algae (Ghanizadeh-

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Kazerouni *et al*., 2024). These stressors can induce physical damage, tissue loss due to necrosis and erosion, and ultimately impair gill function (Brauner & Richards, 2020; Emam *et al*., 2022; Esenkulova *et al*., 2022).

Striated Muscle Formation from a Regenerating Blastema. The presence of a regenerative blastema following experimental gill amputation in fish has been previously documented (Mierzwa *et al*., 2020; Ghanizadeh-Kazerouni *et al*., 2024). However, the specific origin of this blastema in salmonid gills, as well as its potential implications for industrial aquaculture, remain unexplored areas of research.

The regeneration blastema consists of a cluster of undifferentiated and/or progenitor cells that give rise to newly formed branchial filaments and their respective lamellae. Histologically, branchial filaments are lined by an epithelial covering and contain a central core composed of hyaline cartilage, blood vessels, and smooth muscle fibers (Peñailillo, 2011; Roa *et al*., 2011). Notably, in histological analyses, the presence of a regeneration blastema is frequently observed, along with conspicuous bundles of striated muscle at the base of developing gill filaments. Despite its frequent occurrence, the significance of this striated muscle in histopathological diagnoses has not yet been investigated.

The Sonic Hedgehog (Shh) protein is a key signaling molecule that functions as a morphogen—an organic molecule secreted by specific cells that diffuses to distant target cells, influencing their fate and differentiation (Gurdon & Bourillot, 2001; Tabata & Takei, 2004; Rojas *et al*., 2014). Shh expression is restricted to embryonic regions with organizing activity, where it establishes a concentration gradient that regulates the expression of various genes involved in cell and tissue specification (Scholpp *et al*., 2006; Wolpert, 2009). The cellular response to Shh is not solely dependent on signal intensity but also on the duration of exposure, highlighting the necessity of strict spatial and temporal regulation of morphogen activity (Yin *et al*., 2018).

Recent studies have demonstrated that salmonid branchial tissue is capable of regenerating following experimentally induced filament resection (Ghanizadeh-Kazerouni *et al*., 2024). However, these investigations did not provide a morphological characterization of the regenerative blastema, nor did they examine its differentiation into specific branchial tissue types.

In this study, we focus on two juvenile developmental stages of *Salmo salar*: Parr and Smolt. Parr individuals are characterized by the presence of distinct pigmentation patterns, known as "parr marks", on their sides and typically range from 10 to 15 cm in body length. As they transition to the Smolt stage, these markings disappear, and the fish develop a silvery coloration in preparation for their migration from freshwater to seawater, reaching an average length of 15 to 20 cm (Peñailillo, 2011).

The primary objective of this study is to investigate whether Shh is expressed in regenerating gill tissue in freshwater salmon alevins. Specifically, we aim to analyze the ability of *S. salar* at the Parr and Smolt stages to form a regenerative blastema and generate the branchial striated musculature required for the motility of newly developed gill filaments.

MATERIAL AND METHOD

In this work, a total of 30 clinically healthy fish from the freshwater fish farming industry in Southern Chile were studied. An assessment of the ontogenetic development of the specimens was carried out in order to ensure the health of gills and fins. Fish were kept in tanks filled with spring water in continuous flow at a temperature of 8–9 °C and 90–100 % oxygen saturation.

The age of the specimens was measured in Accumulated Thermal Units (ATUs), defined as the number of days of development multiplied by the water temperature. Thus, 950 ATUs correspond to a juvenile at the beginning of its first feeding, in the Parr stage, while at 1900 ATUs the individual is already in the smoltification phase.

The specimens were divided into two groups: i) Parr Stage (950 ATUs; n=15) and ii) Smolt Stage (1900 ATUs; n=15), which were subsequently euthanized using Benzocaine 5 % (BZ-20R; Veterquimica, Chile). The euthanasia procedure was carried out following the ethical regulations for the use of animals in experimental research, under the approval of the Ethics Committee in ACTA No. 14321 of Project No. 072/21 of the Universidad de La Frontera. Subsequently, all specimens were fixed by immersion at room temperature in 10 % buffered formalin for 48 hours.

The experimental paradigm contemplated the use of histological, histochemical and immunohistochemical techniques. All samples were scanned and digitized. The details of each procedure are described below:

Histochemical Techniques. The samples intended for histochemical analysis were dehydrated and embedded in paraffin. The branchial region of each fish was sectioned into serial sagittal sections of 5 µm thickness, using a Microm model HM 315R microtome.

Histological and Histochemical Stains. The Hematoxylin-Eosin (H-E)/Alcian Blue double stain (pH 2.5) allows us to observe the general arrangement of the tissues, as well as to identify the presence of glycosaminoglycans through their blue coloring, specifically for this purpose, which allows the identification of mucus-secreting cells.

Immunohistochemical Techniques. Specimens for this purpose were fixed in 10 % buffered formalin and subsequently embedded in paraffin. Serial histological sections of 5 µm each were obtained using a Microm microtome (HM315R) and were adhered to positively charged slides (Citoglas). Five sections per slide, considering a total of 2 slides per specimen were used. Antigen retrieval was performed by incubation in a steamer for 40 min, with the sections immersed in Antigen unmasking solution (Vector). Blockage of endogenous peroxidase was performed using hydrogen peroxide diluted in methanol and the blockage of the nonspecific proteins was performed by incubation in PBS + 3 % BSA. The histological sections were incubated overnight at 4 °C in the rabbit Shh primary antibody (Cat. H-160, Santa Cruz Biotechnology, USA) at 1/100 dilution in PBS.

Detection of the primary antibody was carried out by 15 min incubation in HRP-conjugated anti rabbit antibody. Diaminobenzidine (DAB, Vector Labs, USA) was used as substrate. Negative control was achieved by the complete development of the immunohistochemical procedure, excluding the incubations in the primary antibodies. The notorious immunological staining of the notochord was considered as a positive control. All the histological sections were analyzed and morphologically described consigning tissues and organs that were immunopositively labeled.

Digitization and morphological analysis. The histological sections were scanned using a NanoZoomer XR C12000 series tissue scanning microscope (Hamamatsu Photonics, Hamamatsu, Japan), generating data files corresponding to images in Whole Slide Image (WSI) format. The digital analysis was carried out with the NDP.view2 software, specific to the scanning equipment. The following parameters were digitally evaluated:

i) Total number of gill filaments and number of filaments involved in the regenerative blastema.
ii) Regenerative blastema area versus total branchial area.

RESULTS

In Parr specimens, the branchial filaments exhibited a central axis composed of hyaline cartilage, with blood vessels clearly observed at their base. The secondary lamellae, which emerge from these filaments, were in various stages of development, presenting either bilateral or unilateral arrangements. At this stage, it is also possible to detect the presence of some striated muscle fibers, located at the base of the filaments (Fig. 1).

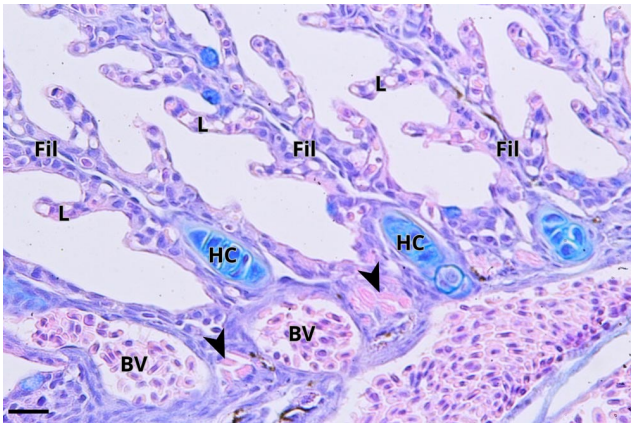

Fig. 1 Sagittal section of a gill from Parr-type salmon alevin. The branchial filaments (Fil) have lamellae (L) in formation. The hyaline cartilage (HC) tissue that constitutes the central axis of the structure is evident. At the base of the filaments, some blood vessels (BV) and incipient fascicles of striated muscles (arrowheads) can be observed. H-E/Alcian Blue staining. Calibration bar: 20 µm.

The proportion of neo-filaments formed from the regenerating blastema relative to the total number of branchial filaments are displayed in Table I. In Parr stage, the percentage of those neo-filaments, in relation to the total number of filaments present in the gills, reached a value of 46.01 %. On the other hand, this percentage decreased to 35.19 % for the Smolt stage.

Table I. Number of branchial filaments and neo-filaments that emerge from the regenerative blastema in Parr and Smolt alevins.

Developmental Stage	Total number of filaments (*)	Number of neo-filaments formed from the blastema	Percentage of neo-filaments (**)
Parr	55.2 ± 2.14	25.4 ± 9.77	46.01 %
Smolt	56.27 ± 4.97	19.8 ± 4.06	35.19 %

(*) All values expressed as average and their respective standard deviation; (**) Related to the total number of branchial filaments

In both Parr and Smolt individuals, well-formed branchial filaments were observed separated from each other (Figs. 2 and 3). Gills that presented areas of regenerative blastema were also evident, covering only a few filaments, while in other cases this blastema occupies up to half of the branchial area (Figs. 2B and 3B). It should be noted that the original filaments, that is, those that were formed immediately after hatching, involute until they become cysts, being replaced by neo-filaments originating from the regenerative blastema. In Smolt salmon, early stages are observed without a blastema and later stages in which that regenerative structure has already been formed (Fig. 2). In the absence of blastema, the gill filaments have a normal appearance, and the striated musculature is almost imperceptible or is still much reduced (Fig. 2A). When the blastema is formed, neo-filaments appear, and the striated muscles begin to differentiate inside the blastema (Fig. 2B).

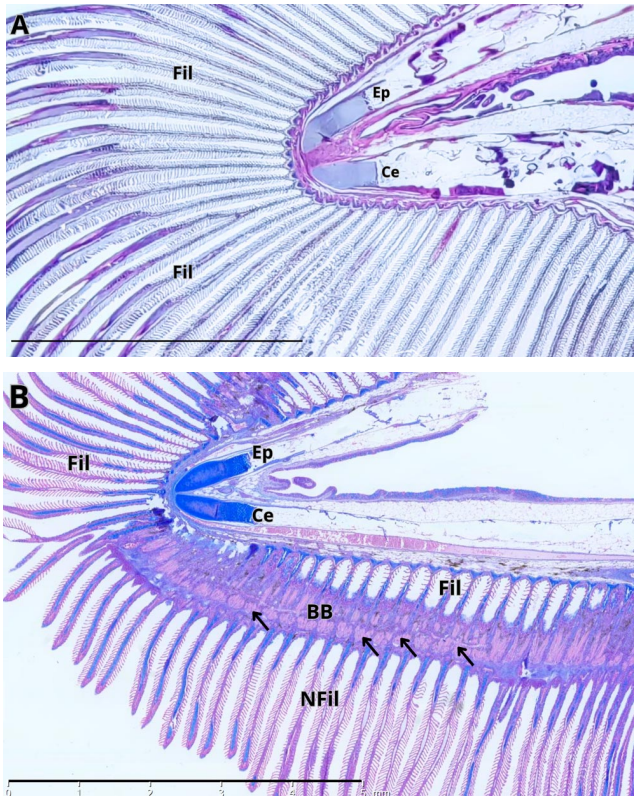

Fig. 2. Smolt-type salmon gills with and without blastema. A) Branchial arch formed by the Epibranchial (Ep) and the Ceratobranchial (Ce) bones. Branchial filaments (Fil) and their lamellae are observed. The presence of a regenerative blastema is not observed. B) Branchial blastema (BB), the initially originated filaments (Fil) and the neo-filaments (NFil) emerging from the blastema are observed. The differentiation of striated musculature (arrows) within the blastema is clear. H-E staining. Calibration bar 5 mm for A and B.

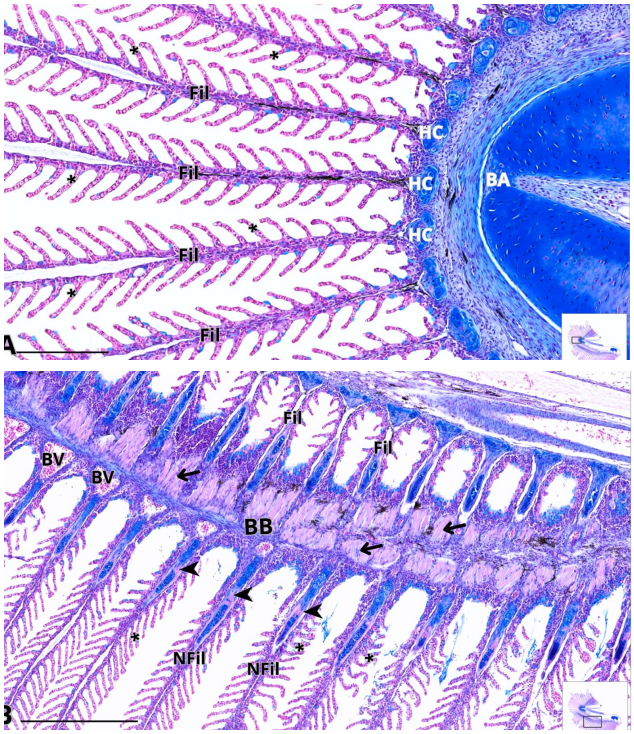

Fig. 3. Formation of striated muscles in gills of Smolt salmon in the recovery phase from an environmental nox. A) Partial view of a branchial arch (BA). In the region where there is no regenerating blastema, the striated musculature is absent. The filaments (Fil) and their lamellae (asterisks) are observed. There is presence of hyaline cartilage (HC) at the base of insertion of the branchial filaments. B) Blastema of regeneration zone. The filaments that were injured begin to involute below the blastema, forming true cysts. The neo-filaments have grown and formed new lamellae (*). Blood vessels are identified at the base of the new filaments (BV). Striated musculature is identified within the blastema. Hyaline cartilage of the neo-filaments is calcified (arrows). H-E/Alcian Blue. Calibrated grid: A=200 µm; B= 0.5 mm.

For the analysis of the total branchial area and the area corresponding to the regenerative blastema, the highest

Tabla II. Average branchial area of Parr and Smolt specimens *versus* regenerating blastema area.

Stage	Gill area (*)	Blastema area (*)	Percentage of branchial areas occupied by blastema (**)
Parr	26.14 ± 4.94.	2.13 ± 0.59.	1.74 % y 17.55 % Median: 8.15 %
Smolt	64.2 ± 11.57	3.62 ± 1.96	2.19 % y 10.60 % Median: 5.64 %

(*) Average area values and their respective standard deviation, expressed in mm^2. (**) Higher and lower percentage values are shown, including their central value (Median).

and lowest values of those parameters and the median value between both were taken into consideration (Table II). The morphometric analysis revealed that in Parr-type juveniles the average branchial area had a value of 26.14 ± 4.94 mm^2. The area of the regeneration blastema formed in these specimens reached a value of 2.13 ± 0.59 mm^2. Consequently, the percentage of regenerating blastema area relative to the average branchial area for the Parr salmon group ranged between 1.74 % and 17.55 %, with a central value (median) of 8.15 %.

For the Smolt salmon group, measurements indicated that the average branchial area reached a value of 64.2 ± 11.57 mm^2 in this case. The regeneration blastema showed an average value of 3.62 ± 1.96 mm^2. From these values the percentage of the area of the regenerating blastema in relation to the total area of the gills of Smolt salmon varied between 2.19 % and 10.60 %, with a central value (median) of 5.64 %.

Formation of striated muscle within the regenerating blastema. At early stages of development, the striated muscles present bundles of muscle fibers similar to myoblasts, with peripherally arranged nuclei. These fibers join together to give rise to multinucleated myotubes, which subsequently give rise to skeletal muscle fibers (Fig. 4A). Immunohistochemical analysis revealed that these muscle fibers were positive for the anti-Shh antibody, indicating the involvement of Sonic Hedgehog signaling in their differentiation and development (Fig. 4B).

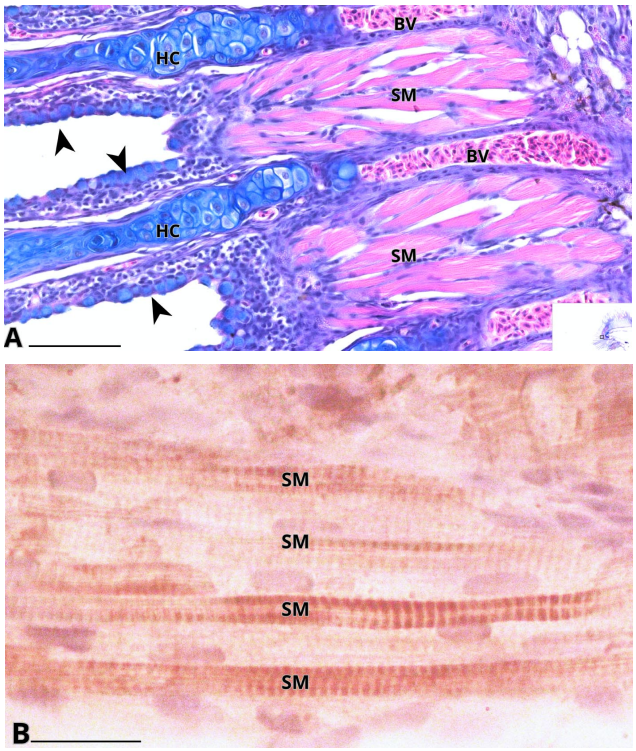

Fig. 4. Formation of striated muscles from the regenerative blastema in Smolt salmon. A) The regenerating blastema has differentiated into striated muscle fiber myoblasts (SM). In addition, new blood vessels (BV), hyaline cartilage (HC) and a lining epithelium with mucus-secreting cells (arrowheads) have been formed. H-E/Alcian Blue. B) Fully differentiated striated muscle fibers (SM) are clearly evidenced. Antibody anti-Shh counterstainned with H-E/Alcian Blue. Calibration bar: A=100 µm; B=50 µm.

The percentage of striated muscle formed in the regenerating blastema of Parr individuals was 0.84 ± 0.86 mm^2. On the other hand, for the Smolt-type salmon this value was 0.83 ± 0.47 mm^2, which indicates that there are no differences for this parameter between both stages of development.

DISCUSSION

Branchial diseases pose a significant threat to *Salmo salar* and can have a considerable impact on the salmon industry (Matthews *et al*., 2013; Herrero *et al*., 2018). In this study, we provide evidence that freshwater salmon alevins, particularly those at the smolt stage, exhibit gill lesions and deformations. These injuries trigger the formation of a regenerative blastema, from which branchial filaments develop, with a notable presence of newly formed striated muscle.

Our findings align with previous studies indicating that various stress factors, including pathogens, zooplankton, and harmful algae, can cause physical damage to the gills and compromise their function (Brauner & Richards, 2020; Esenkulova *et al*., 2022; Emam *et al*., 2022; Ghanizadeh-Kazerouni *et al*., 2024).

Additionally, environmental factors such as changes in water flow and algal blooms can induce hypoxic conditions, activating signaling pathways involved in the proliferation and differentiation of muscle progenitor cells (MPCs). These conditions also regulate protein synthesis and degradation, as well as gene expression patterns in fish (Johnston, 2006; Sánchez Roncancio *et al*., 2020).

Quantification of the total number of branchial filaments and the number of neo-filaments formed from the regenerating blastema. In our study, the proportion of neo-filaments formed from the regenerating blastema relative to the total number of branchial filaments ranged from 24.80 % to 54.60 %, with a median value of 38.97 %. These results align with recent studies highlighting the plasticity of salmonid gills (Ghanizadeh-Kazerouni *et al*., 2024, 2025).

Notably, previous research has also described a process of branchial filament calcification in these fish, which is believed to confer increased structural rigidity and facilitate gill recovery (Smok *et al.,* 2025). This suggests that both neo-filament formation and filament calcification may represent complementary adaptive mechanisms contributing to gill regeneration and functional restoration in salmonids.

Average branchial area of Parr and Smolt specimens versus regenerating blastema. In our study, the percentage of regenerating blastema area relative to the average branchial area of Parr salmon reached a median value of 8.15 %, whereas in Smolt salmon, this percentage was 5.64 %. These findings are consistent with previous studies describing the plasticity of various structures and organs in *Salmo salar*, including the spinal cord, fins, and eye bulb—regenerative processes that can be triggered by hypoxia (Rojas *et al*., 2024a,b).

There is a well-established consensus regarding the remarkable plasticity of myotomal muscles in teleost fish, which enables the continuous formation of new muscle fibers throughout their life cycle via secondary myogenesis (Johnston, 2006). This trait distinguishes teleosts from amniotes, where the dermomyotome loses its regenerative capacity at advanced developmental stages (García-Orozco *et al*., 2024). The continuous generation of muscle fibers may function as a compensatory mechanism against deformities, reinforcing the ability of teleost fish to adapt their musculature to environmental and genetic challenges throughout their development.

It is important to highlight that branchial musculature has a distinct embryonic origin compared to other muscle groups, as it is branchiomeric in nature (García-Orozco *et al*., 2024). In salmonids, this musculature is not typically noticeable in healthy gill filaments. Instead, it appears to form de novo from the regeneration blastema exclusively in response to tissue damage or injury and in the presence of newly formed filaments.

Our findings provide compelling evidence that the striated musculature of the branchial filaments originates within the regeneration blastema. We observed strong immunostaining for striated muscle fibers using the Anti-Shh antibody, consistent with previous observations in Zebrafish (Blagden *et al*., 1997; Yin *et al*., 2018). This suggests that the development of striated muscle fibers occurs post-hatching and continues throughout the lifespan of the fish. However, these aspects remain largely unexplored in the current state of the art. Further research is needed to elucidate their potential relevance in histopathological diagnoses and their possible applications in fish farming and aquaculture production processes.

Final Remarks. Unlike mammals, where muscle fiber formation ceases after birth, fish retain an exceptional capacity for muscle growth throughout their lives, enabling them to adapt to diverse environmental challenges.

Particularly noteworthy is the biological strategy by which fish generate new muscle fibers to provide motility to regenerating branchial filaments, effectively replacing those damaged by adverse environmental conditions.

The remarkable ability of teleost fish to adapt to environmental changes and regenerate complex structures such as gills and their associated musculature represents a topic of great interest, both from a biological perspective and in terms of its potential applications in aquaculture and fish health management.

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RESUMEN: El objetivo de este estudio fue describir la formación del tejido muscular estriado branquial durante las etapas ontogenéticas Parr y Smolt de *Salmo salar* en respuesta a desafíos ambientales naturales. Para ello, se analizaron 15 alevines de Parr y 15 de Smolt de *S. salar*. Los especímenes fueron anestesiados y eutanasiados con benzocaína al 5 %, siguiendo protocolos establecidos de bienestar animal. Posteriormente, fueron fijados en formalina al 10 % y procesados para análisis histológico mediante tinción doble de Hematoxilina-Eosina/Azul de Alcián. Se cuantificaron el área del blastema regenerativo y la región branquial ocupada por musculatura estriada. En los especímenes Parr, las branquias aún estaban en desarrollo y la musculatura estriada en la base de los filamentos no se había formado. En contraste, los Smolt presentaron un blastema regenerativo bien definido, lo que sugiere una respuesta adaptativa a estresores ambientales como la hipoxia. Se observaron fibras musculares estriadas en la base de los filamentos branquiales en regeneración, completamente diferenciadas a partir del blastema. El análisis inmuno-histoquímico confirmó que estas fibras musculares fueron positivas para el anticuerpo anti-Shh, lo que indica un papel de la señalización de Sonic Hedgehog en su formación. Estos hallazgos resaltan la notable capacidad regenerativa de los peces teleósteos, particularmente en la musculatura asociada a las branquias. Comprender los mecanismos subyacentes a la regeneración muscular branquial podría aportar conocimientos valiosos sobre la fisiología de los peces y su aplicación en la acuicultura.

PALABRAS CLAVE: Salmón del Atlántico; *Salmo salar*; Musculatura branquial; Músculo estriado; Blastema de regeneración; Filamentos branquiales.

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