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Limb regeneration in salamanders: the plethodontid tale

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ABSTRACT Salamanders are the only vertebrates that can regenerate limbs as adults. This makes them ideal models to investigate the cellular and molecular mechanisms of tissue regeneration. *Ambystoma mexicanum* and *Nothopthalmus viridescens* have long served as primary salamander models of limb regeneration, and the recent sequencing of the axolotl genome now provides a blueprint to mine regeneration insights from other salamander species. In particular, there is a need to study South American plethodontid salamanders that present different patterns of limb development and regeneration. A broader sampling of species using next-generation sequencing approaches is needed to reveal shared and unique mechanisms of regeneration, and more generally, the evolutionary history of salamander limb regeneration.

KEY WORDS: *limb regeneration, salamander, plethodontid, tissue regeneration, skin*

Introduction

Salamanders are amphibians that belong to the order Caudata, approximately 739 species have been reported and are distributed among ten families (Table 1), representing 9% of amphibians reported so far (8,111 species of amphibians) (AmphibiaWeb, 2019). Salamanders are mainly distributed in the northern hemisphere (Wake, 2009). Of these, the Plethodontidae family also inhabits the tropics (Elmer *et al.*, 2013), while the Hynobiidae family is found only in Asia and the Salamandridae is distributed in the old world (Wake, 2009).

Several species of salamanders have served as experimental models for more than 100 years to understand different biological processes, such as development and regeneration (Joven *et al.*, 2019). One of the main authors referred to in preliminary studies on limb regeneration was Lazaro Spallanzani (Spallanzani,1768) who worked with aquatic salamander (probably a newt) (Simon and Odelberg, 2015,Tsonis and Fox, 2009). The first reports on limb regeneration staging were in 1973 for *N. viridescens* (Iten and Bryant, 1973) and for *A. mexicanum* in 1976 (Tank *et al.*, 1976).

N. viridescens and *A. mexicanum* are the main research organisms used as models to understand the cellular and molecular mechanisms of limb regeneration. However, both species present differences during their development (e.g axolotls are paedomorphic) and the environment they inhabit (terrestrial and/or aquatic) (Farkas and Monaghan, 2015,Simon and Odelberg, 2015). Salamanders have a genome ten times larger than the human genome (13-120 Gb), different labs around the world (Smith *et al.*, 2019,Keinath *et al.*, 2015,Nowoshilow *et al.*, 2018) have been contributing to collect enough genetic information to improve the quality of the genome, which was published in 2018 (Nowoshilow *et al.*, 2018). This preliminary version of the genome is going to facilitate the genetic and molecular analysis of different biological processes in these organisms such as tissue regeneration. Also, the genome of *Pleurodel waltl*, which belongs to the Salamandridae family, was reported (Elewa *et al.*, 2017).

Recently reviews have described the importance of salamanders as research organisms to understand tissue regeneration (Joven *et al.*, 2019,Dwaraka and Voss, 2019). However, in this review we discuss the efforts that have been done using salamanders from the Andes region such as *Bolitoglossa ramosi* and *Bolitoglossa vallecula*, to understand how conserved is limb regeneration between salamander families.

Biological features of *Ambystoma mexicanum* and *Notophthalmus viridescens*

Salamander families diverged from a common ancestor approximately 200 million years ago (Fig. 1), which has allowed each

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Abbreviations used in this paper: BC, basale commune; CAMP cathelicidin; dpa, days post amputation; ECM, extracellular matrix; Gb, giga base pairs; GO, gene ontology; MMP, matrix metalloproteinase; MYA million years ago; NGS, Next Generation Sequencing data.

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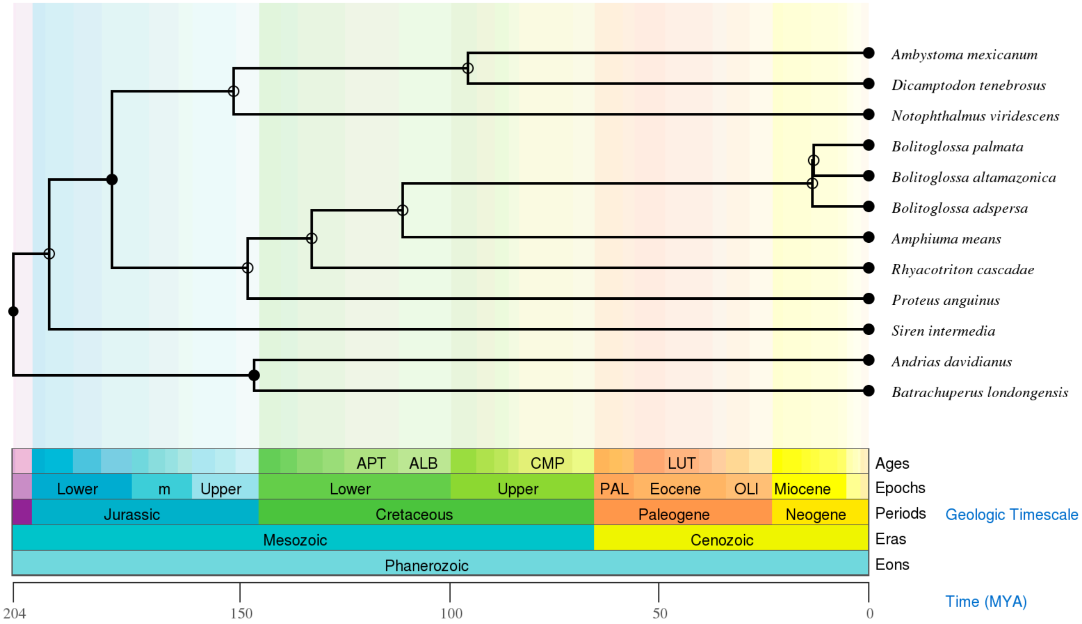


Fig. 1. Estimated divergence times between different species of salamanders representative of each family. *Ambistomatidae (*Ambystoma mexicanum*), Dicamptodontidae (*Dicamptodon tenebrosus*), Salamandridae (*Notophthalmus viridescens*), Plethodontidae (*Bolitoglossa sp*), Amphiumidae (*Amphiuma means*), Rhyacotritonidae (*Rhyacotriton cascadae*), Proteidae (*Proteus anguinus*), Sirenidae (*Siren intermedia*), Cryptobranchidae (*Andrias davidianus*), Hynobiidae (*Batrachuperus londongensis*).* Bolitoglossa *spp are estimated to have appeared in the Cenozoic period (65 MYA). Phylogenetic reconstruction was performed in TimeTree (Hedges* et al.*, 2015).*

TABLE 1

SALAMANDER SPECIES WITH NGS DATA ON LIMB REGENERATION

Family	Number of species reported	Geographic Distribution	Species with NGS data	NGS data related to limb regeneration
Ambystomatidae	32	North America	*Ambystoma andersoni* [a] *Ambystoma laterale* [b] *Ambystoma maculatum* [a,c] *Ambystoma mexicanum* [d] *Ambystoma texanum* [b] *Ambystoma tigrinum* [e]	Yes No Yes Yes No No
Cryptobranchidae	4	Andrias occurs in central China and Japan. Cryptobranchus in eastern North America.	*Andrias davidianus* [f]	No
Dicamptodontidae	4	Northwestern North America	No reported	-
Hynobiidae	82	Asia with one species distributed in Russia (European)	*Batrachuperus yenyuanensis*[g] *Hynobius chinensis*[h]	No No
Plethodontidae	**479**	North America, Central America, South America, southern Europe, Sardinia, Korea	*Bolitoglossa ramosi*[i] *Bolitoglossa vallecula*[j]	Yes No
Rhyacotritonidae	4	Western North America (California, Oregon and Washington)	No reported	-
Sirenidae	5	North America	No reported	-
Salamandridae	120	Europe, Asia, North America Middle East northwest Africa	*Cynops pyrrhogaster*[k] *Notophthalmus viridescens*[l] *Pleurodeles waltl*[m]	Yes Yes Yes

[a] Dwaraka *et al*., 2019. [b] McElroy *et al*., 2017. [c] Burns *et al*., 2017. [d] Bryant *et al*., 2017; Caballero-Pérez *et al*., 2018; Dwaraka *et al*., 2019; Tobias Gerber *et al*., 2018; Leigh *et al*., 2018; Nowoshilow *et al*., 2018; Rao *et al*., 2009; Smith *et al*., 2019; Stewart *et al*., 2013; Wu *et al*., 2013. [e] Eo *et al*., 2012. [f] Che *et al*., 2014; Fan *et al*., 2017; Geng *et al*., 2015; Huang *et al*., 2017; Jiang *et al*., 2016; Li *et al*., 2015. [g] Xiong *et al.,* 2019. [h] Che *et al*., 2014. [i]renas Gómez *et al*., 2018. [j]Arenas Gómez *et al.,* 2020.[k] Nakamura *et al*., 2014; Tang *et al*., 2017. [l]Abdullayev *et al.,* 2013; Looso *et al*., 2013. [m] Elewa *et al*., 2017.

The order Caudata is conformed by ten families of salamander which are distributed around the world, with the advances in the Next Generation Sequencing data (NGS) some species have NGS data that have been allowed to perform gene expression profile during limb regeneration.

of them to present different biological strategies of reproduction, development, regeneration, among others (Voss *et al.*, 2009; Wake, 2009). Newts (e.g. *N. viridescens*) and axolotls (*A. mexicanum*) diverged approximately 145 million years ago (Hedges *et al.*, 2015) (Fig. 1) and are found in different habitats and exhibit biological behaviors characteristic of each species (Simon and Odelberg, 2015,Farkas and Monaghan, 2015,Voss *et al.*, 2009).

The Ambystomatidae family has at least 32 species (Williams *et al*., 2013,McKnight and Shaffer, 1997) that are grouped into different family complexes. *A. mexicanum* (Fig. 2A) is part of the Ambystoma Tigrinum complex (Shaffer and Mcknight, 1996), which can be found in both aquatic and terrestrial environments and lives mainly in North America, from southern Mexico to southern Alaska. These species have indirect and paedomorphic development, wherein sexually mature adults phenotypically present youthful features such as gills (Voss *et al*., 2009). *A. mexicanum* have been consider as a good research organism to understand limb regeneration because the husbandry is straightforward, can be bred in captivity and different transgenic lines have been established (Fei *et al*., 2017; Fei *et al*., 2018; Flowers *et al*., 2017; Khattak *et al*., 2013; Kragl *et al*., 2009).

Other species of salamanders that have been used in regeneration studies belong to the Salamandridae family. These include the first newt model established in regeneration, *N. viridescens* (Eastern Triton, native to North America) (Simon and Odelberg, 2015) (Fig. 2B) well known for their remarkable capacity to regenerate eye lens throughout their entire life span (Sousounis *et al*., 2014), contrary to what occurs in axolotls (Grogg *et al*., 2006).

N. viridescens has been the most used model among the Salamandridae family. However, other models such as *Cynops pyrrhogaster* (Japanese fire-belly newt), which was used recently to understand the reintegration of joints during limb regeneration (Tsutsumi *et al*., 2015) and has been used to understand lenses regeneration (Eguchi *et al*., 2011).

Finally, one of the most promised salamanders of this family to be used in regeneration studies is *Pleurodeles waltl* (Iberian

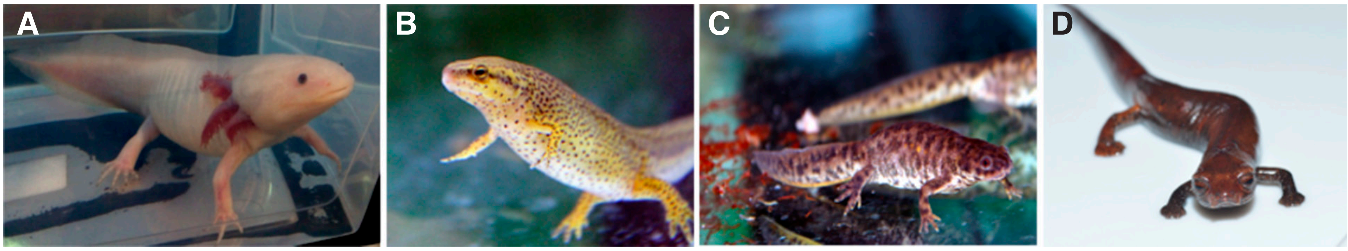


Fig. 2. Species of salamander in which limb regeneration has been widely described. (A) Ambystoma mexicanum *(Ambystomatidae),* **(B)** Notophthalmus viridescens *(Salamandridae),* **(C)** Pleurodeles waltl, *species used in regeneration biology, both have indirect development and during their adulthood they mainly inhabit aquatic environments,* A. mexicanum *conserves gills and the newts (*N. viridescens and P. waltl) *presents complete metamorphosis,* **(D)** Bolitoglossa ramosi *(Plethodontidae) species of interest in this review, presents direct development and is completely terrestrial throughout its life cycle.*

ribbed newt) (Fig. 2C), which has been used to establish transgenic lines, has a faster development than *N. viridescens* (Hayashi *et al*., 2013) and a partial genome assembly has been reported (Elewa *et al*., 2017).

Unlike *A. mexicanum*, newts generally have indirect development (presence of larval stages) with complete metamorphosis, part of their life cycle is terrestrial and in most cases, they return to an aquatic environment to reproduce (Simon and Odelberg, 2015,Joven *et al*., 2019). The biological cycle to reach adulthood takes approximately three years, while in axolotls it takes 10-18 months. In the field of regenerative biology, the newt *N. viridescens* (Fig. 2B) has been used in limb regeneration studies to understand an alternative mechanism of muscle regeneration which is through cell dedifferentiation, contrary to the axolotl, where the activation of the resident stem cell population (satellite cells) are the main source of muscle regeneration (Sandoval-Guzmán *et al*., 2014,Wang and Simon, 2016).

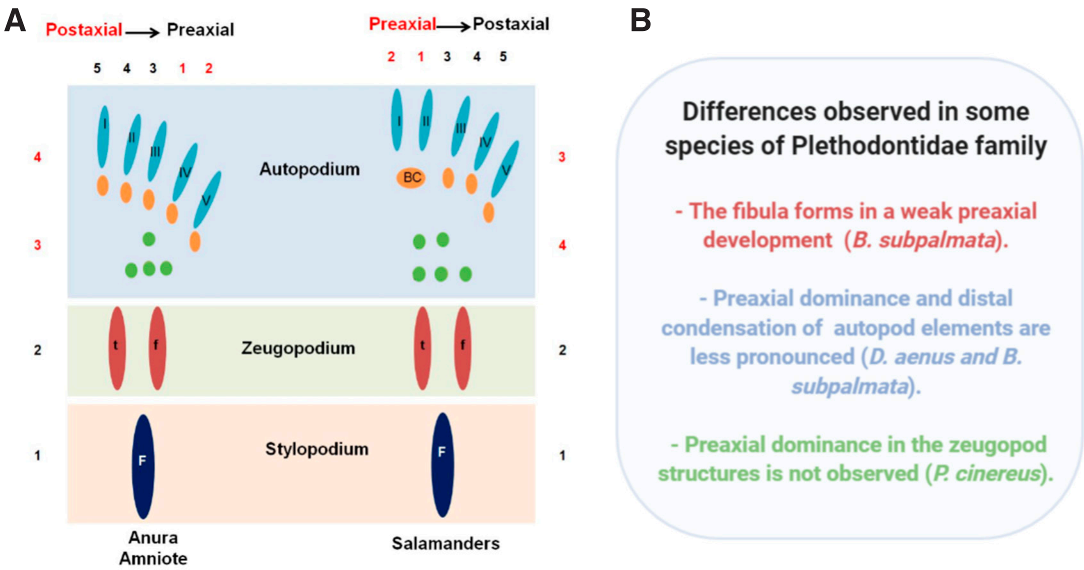
Biological features of plethodontids

This family of salamanders is distributed in the United States, Central, and South America, and also in southern Europe, Sardinia (Italy) and Korea. There are two subfamilies reported; Hemidactylinae and Plethodontinae and in the latter are three tribes; the Bolitoglossini, Hemidactyliini, and Plethodontini (Wake, 2012). Species richness is greatest in Colombia and rapidly decreases as one moves into the adjacent countries. Twenty-three species, belonging to the genus *Bolitoglossa* spp (Fig. 2D), have been reported (Elmer *et al*., 2013, Acosta Galvis, 2017), of these 19 are endemic to the country. It is estimated that species of this genus diverged approximately 65 MYA (Fig. 1) (AmphibiaWeb, 2019).

Salamanders of the Plethodontidae family are characterized by having some biological differences compared to other families such as presence of enucleated red blood cells, projectile tongue, absence of lungs (Wake, 2009), caudal autotomy (Mueller *et al*., 2004) and direct development (Chippindale *et al*., 2004).

Additionally, in species of the family Plethodontidae, the anterior and posterior extremities develop at approximately the same time. In salamanders with indirect development, the forelimbs may appear before the hind limbs or vice versa (Wake and Hanken, 1996). On the other hand, the morphogenetic pattern of the digits in salamanders is in a preaxial order, which suggests that the appearance of the digits occurs in the order II-I-III -IV (-V) (Fig. 3A) (Fröbisch and Shubin, 2011). However, in species of plethodontids, there are some changes (Fig. 3B), such as in *B. subpalmata*, where the condensation of the ulna/fibula is continuous and forms in a weak preaxial development. Also, in *Desmognathus aenus* and *B. subpalmata* the preaxial dominance and distal condensation of the

Fig. 3. Development pattern of limbs in salamander vs. amniotes. (A) *Salamanders have preaxial development of the digits (II-I-III-IV (-V),* **(B)** *Some differences have been reported in species of the Plethodontidae family during limb development. In the figure each digit is indicated with Roman numerals and the order of appearance of each digit during development with Arabic numerals (1-5). BC: basale commune, t:tibial, f:fibula, F:femur. Figure 3A modified from Fröbisch and Shubin 2011.*



skeletal autopod elements are less pronounced compared with the metamorphosing salamanders (Kerney *et al.*, 2018). Additionally, in *Plethodon cinereus* there is no preaxial dominance in the zeugopod structures (radius/tibia and ulna/fibula), the chondrocytes differentiation is nearly synchronous in this structures; different from the indirect developer salamanders where the radius/tibia differentiated first (Kerney *et al.*, 2018). Finally, in *A. mexicanum* during limb development cell death is absent (Cameron and Fallon, 1977), however in *D. aeneus* in the interdigital spaces of the developing limbs cell death was detected, which is important to allow digital condensation (Franssen *et al.*, 2005). Cell death during limb development has been an important cellular mechanism in tetrapods such as birds, mammals (Fernández-Terán *et al.*, 2006), and reptiles (Fallon and Cameron, 1977), but not in salamanders (Cameron and Fallon, 1977). These differences raise a number of questions on the evolution of limb development in these organisms. The study of other species of the Plethodontidae family could help understand the different adaptation that the limb undergoes during its regeneration and also if those mechanisms observed during limb development are recapitulated during limb regeneration in species with direct development.

To date, few studies have addressed tissue regeneration in plethodontids. The regeneration of the *Plethodon cinereus*, *Plethodon dorsalis*, *Desmognatus ochropaeus*, *Desmognatus fuscus,* and *Eurycea bislineata* species was described in 1981 (Scadding, 1981). In the case of species of the genus *Plethodon* sp., the regeneration time was found to be longer (50-71 dpa), in comparison with *A. mexicanum*. Additionally, in 1987 Session *et al.,* (1987) made a correlation between limb regeneration and the genome size of 23 different species of plethodontids; among them, *Bolitoglossa rufescens* showed that there is an indirect relationship between regeneration rate and genomic size, but descriptions of regeneration in these species were not made.

In Colombia and in other countries where plethodontids are found, studies have been mainly focused on phylogenetic and biogeographic studies (Elmer *et al.*, 2013), and molecular taxonomy studies for the discrimination of cryptic species present in this genus of salamanders. Additionally, morphometry analyzes have been performed to discriminate between species of this genus (Silva-González *et al.*, 2011). Species of *Bolitoglossa* spp genus have been used to understand the morphological characteristics of the interdigital membrane of the extremities (Jaekel and Wake, 2007), mainly to understand adaptive mechanisms.

For those of us studying regeneration biology in salamanders, cutting-edge technologies, like Next-generation sequencing tools, are considered a breakthrough, since these organisms have a large genome (≈30 Gb depending on the species), composed of repetitive regions and transposable elements (Smith *et al.*, 2019,Keinath *et al.*, 2015). However, the first genome of reference is unraveling the complexity of the salamander genome. Also, in the last decade, different transcriptomic studies have been published and served to identify genes and signaling pathways involved in the different regenerative processes.

The transcriptomic data reported to date are mainly from *A. mexicanum* (Baddar *et al.*, 2015,Voss *et al.*, 2015,Bryant *et al.*, 2017) and *N. viridescens* (Looso and Braun, 2015) which include different tissues and structures in the process of regeneration (e.g limbs, nerve tissue, lens). Additionally, a first version of the sequenced genome of *A. mexicanum* is available (Smith *et al.*, 2019,Nowoshilow *et al.*, 2018). In the case of other species, transcriptomic data have been obtained from *Andrias davidianus* (family Cryptobranchidae) (Jiang *et al.*, 2016), and *Hynobius chinensis* (family Hynobiidae) (Che *et al.*, 2014), however, these data are not related with limb regeneration.

Overview of limb regeneration

Tissue regeneration is a dynamic process that presents specific variations in very narrow periods of time. This includes gene expression changes that occur within and among cell populations at specific times. To capture the dynamics of gene expression change it is important to obtain tissue samples from time points that span the entire regenerative process (Voss *et al.*, 2015).

During limb regeneration in salamanders, a sequence of events is observed that eventually leads to the formation of a new limb. The three key events are wound closure, cell dedifferentiation

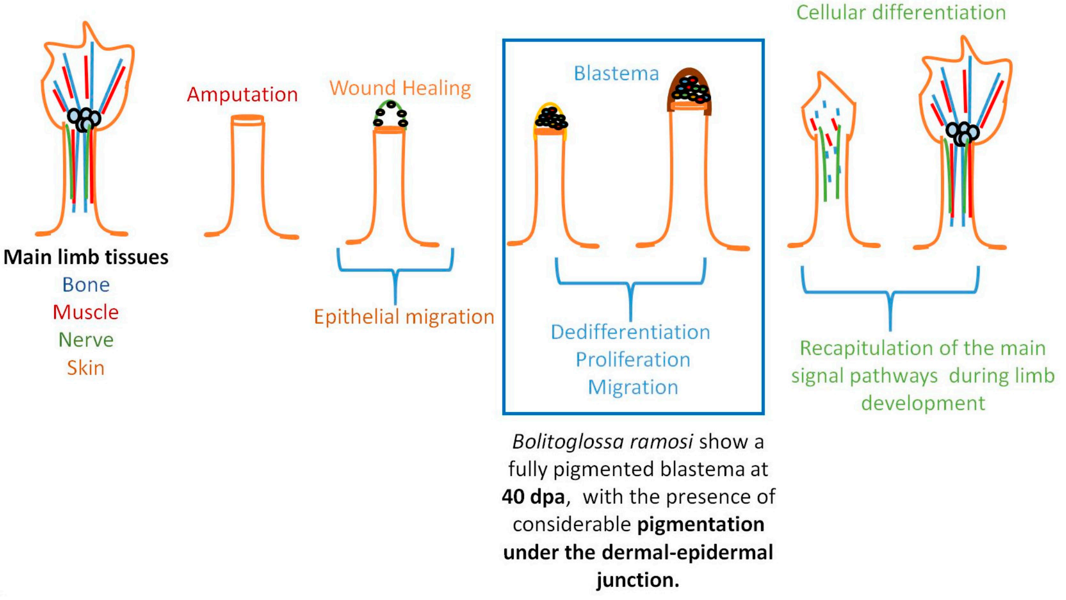


Fig. 4. General limb regeneration stages reported in salamanders. *The main difference observed during limb regeneration in* B. ramosi *was the time to obtain the digital outgrowth is longer than the other salamanders (95 dpa) and the intense pigmentation and collagen deposition of the blastema during the 40 dpa.* (*Arenas Gómez* et al.*, 2017).*

processes that lead to the formation of a structure called blastema, and finally, cell differentiation mechanisms (Stocum, 2017)(Fig. 4).

The wound closure in salamanders begins with the migration of keratinocytes, which migrate on the fibrin support that has been generated in response to the coagulation cascade (Lévesque *et al*., 2010). At the moment when both ends are connected, the regenerative epithelium is formed (Campbell, 2011), releasing important molecules that will participate in the dedifferentiation of the mesenchymal tissue that will ultimately form the blastema. In the regeneration of axolotl and newt limbs, this stage begins in the first 24 hours and goes until approximately 5 days post amputation (dpa) (Tank *et al*., 1976,L. Iten and Bryant, 1973), however, this timing varies depending on the influence of different factors (e.g size) (Seifert *et al*., 2012,Monaghan *et al*., 2014).

During this stage, one of the key points that trigger the formation of a scar and not a regenerative epithelium is the type of immune response (James W Godwin and Brockes, 2006). Several reports have described the direct relationship between the maturity of the immune system and the regenerative capacity of the organism, that is, as a more sophisticated immune response is presented, the regenerative capacity decreases (King *et al*., 2012,Mescher *et al*., 2013,J. Godwin, 2014). For example during mammalian embryonic development, the immune response is immature, therefore the embryo is able to perform regenerative processes in the absence of scarring (Godwin and Brockes, 2006; Leung *et al.,* 2012). Another clear example is during the process of metamorphosis in *Xenopus* sp., wherein individuals lose the capacity to regenerate limbs as the immune system matures (King *et al*., 2012,Mescher *et al*., 2013).

Salamanders have an archaic immune system that is governed primarily by an innate immune response since the adaptive response is almost null (Rollins-Smith, 1998). However, it has been reported that during the first 5 dpa the immune response is crucial to promote limb regeneration (Godwin *et al*., 2013). Godwin *et al.,* evaluated the immune response during wound closure by monitoring the activation of cells such as macrophages. Ablation of macrophages inhibited regeneration, thus establishing their essential role in modulating limb regeneration (Godwin *et al*., 2013).

After the regenerative epithelium is established, the second most important stage is blastema conformation. This occurs mainly by histolysis processes that are carried out by enzymes called extracellular matrix metalloproteinases (MMPs), which create an extracellular matrix permissive for the migration of mesenchymal cells that will dedifferentiate to give rise to a heterogeneous structure of progenitor cells called the blastema (Santosh *et al*., 2011). Cell populations that make up the blastema originate from the different tissues that are present in the limb, and this occurs mainly through cell dedifferentiation processes (Kragl *et al*., 2009; Nacu *et al*., 2013).

Additionally, it has been reported that the cells present in the wound bed have limited potency, for example, a muscle cell progenitor will only give rise to muscle cells (Kragl *et al*., 2009). Muscle regeneration in the limb is an event of great importance since it has been found that the mechanisms that lead to its regeneration are different between the axolotl (*A. mexicanum*) and the newt (*N. Viridescens*). In axolotls, muscle regeneration is mainly due to the participation of a pool of stem cells that are present around muscle fibers, called satellite cells, while in newts muscle regeneration occurs by dedifferentiation of muscle fibers (Sandoval-Guzmán *et al*., 2014). The cells present in the dermis (fibroblasts) are the most plastic cells during the regenerative process because they give rise to fibroblasts as well as to cells that make up the tendons and connective tissue (Kragl *et al*., 2009).

After dedifferentiation, processes of migration and cell proliferation begin to occur, which will finally allow for the formation and establishment of the blastema (Gardiner *et al*., 1986,Stocum, 2017). It has been described that this step is highly dependent on nerve tissue since the nerve secretes different neurotrophic factors important for cell proliferation and migration (Farkas *et al*., 2016,Satoh *et al*., 2008). Additionally, it has been reported that from 10-12 dpa the secretion of the glands found in the regenerative epithelium is crucial to continue the proliferation of blastema cells (Kumar, Godwin, *et al*., 2007).

During the formation of the regenerative epithelium, the expression of transcription factors such as sp9 is crucial for innervation (Satoh *et al*., 2008). The expression of *sp9* occurs in the basal keratinocytes that make up the regenerative epithelium, which is an embryonic marker of dedifferentiated cells during normal limb development. The expression of sp9 is activated by neurotrophic factors such as *Kgf* and *Fgf2*. Additionally, it has been suggested that *sp9* may be related to the expression of the anterior gradient protein (nAG) in the epithelial glands (Satoh *et al*., 2008), which is of vital importance during the regenerative process (Kumar *et al*., 2007). Also, RNA-single cell data during limb regeneration in *A. mexicanum* has allowed to discriminate among the different cell populations present in the regenerative epithelium and has served to identify regeneration-specific genes in the basal cells that conform this epithelium (Leigh *et al*., 2018). Besides, Tsai *et al.,* (Tsai *et al*., 2020) described how the regenerative epithelium is a key regulator of inflammation, ECM remodeling, and tissue histolysis during the first stages of limb regeneration in *A. mexicanum*.

One of the final stages in the limb regenerative process is the recapitulation of development, at this stage, the main mechanism is cell differentiation which is controlled by signaling pathways that regulate embryonic development, including highly conserved pathways such as WNT, SHH, BMP and FGF signaling pathways (Ghosh *et al*., 2008,Guimond *et al*., 2010,Nacu and Tanaka, 2011). Some molecular mechanisms have been established as unique during regeneration in salamanders such as the expression of the protein PROD1, which is expressed by the blastema cells (Kumar, Gates, *et al*., 2007) and important for the establishment of the proximodistal identity during limb regeneration (Echeverri and Tanaka, 2005).

The Colombian experience: limb regeneration in *Bolitoglossa ramosi*

B*olitoglosa* spp inhabits primary and secondary forests of low mountains (from 1200 to 3000 meters above sea level) and are arboreal and nocturnal terrestrial animals (AmphibiaWeb, 2019). Some of the species, such as *B. ramosi,* have forelimbs with four webbed digits, which could be an adaptation for their arboreal life (Alberch and Alberch, 1981; Wake, 1966). Another adaptative strategy is the color of the skin which is highly pigmented (dark brown). Because they are lungless salamanders, the skin is highly vascularized to favor air gas exchange (Arenas Gomez *et al*., 2017; Laurie J. Vitt, 2008). To our knowledge, colonies of a plethodontid species have not been established for regenerative studies; studies of regeneration have primarily used wild-caught animals.

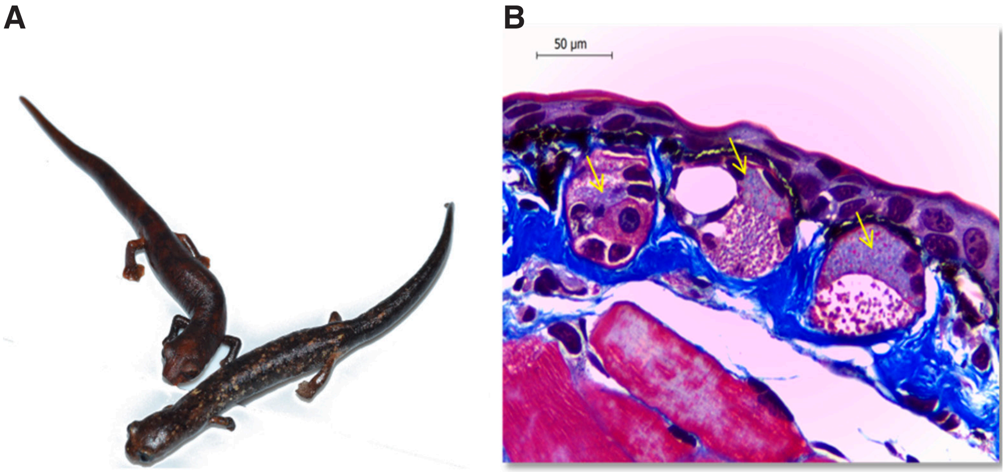


Fig. 5. Some biological characteristics of *Bolitolgossa* sp. (A) *The adult stage of* Bolitoglossa ramosi *(right) and* Bolitglossa vallecula *(left) were the species used to study limb regeneration.* **(B)** *This species has a skin with diverse and uncharacterized glands (yellow arrows) that may secret proteins that function in immunity and dehydration avoidance.*

In our lab, we developed a protocol to keep bolitoglossines in captivity during a short time period (Arenas Gómez *et al*., 2015). This will make them available to understand how limb regeneration works in species that belong to the Plethodontidae family. Observations of limb regeneration for two species of Bolitoglossa, *B. ramosi* and *B. vallecula* (Arenas Gómez *et al*., 2017), showed that digital outgrowth in this genus takes longer than that reported for aquatic, terrestrial and biphasic-lifestyle salamanders. For example in *B. ramosi*, digital outgrowth took 95 dpa whereas it takes 30-40 dpa in *A. mexicanum*. Also, differing from other salamander species, *B. ramosi* develops a fully pigmented blastema (40 dpa) with the presence of considerable pigmentation (eumelanin) under the dermal-epidermal junction (Fig. 4).

B. ramosi are terrestrial (Fig. 5A) and thus presumably use their amputated limbs to locomotion; a feat (no pun intended) that would seemingly, mechanically stress the blastema. We hypothesize that the pigmentation and morphological changes observed in *B. ramosi* confer support to blastema cells and stabilize the dermal-epidermal junction. Another morphological feature that we observed in *B. ramosi* was a high accumulation of mature collagen fibers under the wound epithelium (Arenas Gómez *et al*., 2017). This could be a response triggered by a mechanotransduction process to allow a stiff matrix that could give support and protect blastema cells during locomotion in a terrestrial environment.

Transcriptomic data from *B. ramosi* during limb regeneration showed that many of the differentially expressed genes were similarly to those differentially expressed in other salamanders (Arenas Gómez *et al*., 2018). These genes encoded proteins associated with the wound epidermis, extracellular matrix, basement membrane, blastema and differentiating chondrogenic precursor cells. Some genes showed a similar, correlated pattern of expression during limb regeneration.

Towards the search for other inputs in tissue regeneration

Another biological difference of *Bolitoglossa* spp in comparison to the axolotl and newts is skin architecture. The skin secretions of plethodontids are composed of different organic molecules such as acidic glycoproteins, carboxylated glycoproteins, and mannose sugars (von Byern *et al*., 2015) that are secreted by different types of glands (mucous, granular and mixed glands) (Fig. 5B). It seems likely that in addition to antipredator defenses, plethodontids skin secretions may function in innate immunity. In the case of *Bolitoglossa* spp, we reported that the skin of *B. ramosi* possesses both mucous and granular secretion glands (Arenas Gómez *et al*., 2017) which likely secrete distinct molecular cargos and affect different biological functions.

In general, it has been described that the amphibian skin secretes several defensive proteins such as cathelicidins (Hao *et al*., 2012; Yang *et al*., 2017), that contribute to amphibian's innate immunity against various microorganisms. Cathelicidins have multiple functions, some of which may be conserved across vertebrates (Avila, 2017), including activation of cell proliferation and migration during wound healing in humans (Ramos *et al*., 2011), and they are expressed during salamander regeneration (Voss *et al*., 2017). While extensive work has been done to characterize specific antipredator adhesive secretions in the skin of *Plethodon shermani* (Von Byern *et al*., 2017), thus far there have been no comprehensive descriptions of gene transcription associated with the production of defensive and wound healing compounds within the skin of plethodontids.

In salamanders, the identification of wound healing and immune system proteins present in the skin have been reported for the Chinese giant salamander (*Andrias davidianus*) (Geng *et al*., 2015). There is a need to explore molecules in the skin in other salamander families, and determine how secretions change in response to the environment and confer different biological functions.

In preliminary data (Arenas Gómez *et al*., 2020), we identified 4,007 orthologous genes expressed in the skin of *B. ramosi* and *B. vallecula*, showing Gene Ontology terms associated with immune system responses, including immunomodulation and skin barrier integrity. Genes associated with response to a stimulus (GO: 0050896) were also found. For example, TXLNA and TXLNG are antibacterial response proteins that belong to the GHK peptides family. These proteins have broad biological functions, including stem cell activation and regeneration of aged skin and wound healing (Pickart *et al*., 2015). Additionally, extracellular proteins such as LRIG1 and LRRN1, which are leucine-rich repeat proteins, may function during regeneration processes (Murad *et al*., 2015,Wolfe *et al*., 2004). Also, we identified a group of *B. vallecula* and *B. ramosi* sequences showing sequence identity to the cathelicidin (CAMP) protein family. Members of the CAMP protein family have variable biological functions as antimicrobial peptides providing protection against infection in most of the vertebrates (Kościuczuk *et al*., 2012). Additionally, cathelicidins promote proliferation and cell migration during wound healing (Ramos *et al*., 2011). This protein family exhibits great diversity in vertebrates, previous reports that have analyzed cathelicidins have only considered their antimicrobial potential in anurans (Hao *et al.*, 2012,Yu *et al.*, 2013), and one report described the presence of a cathelicidin-like peptide in salamanders (Yang *et al.*, 2017). Because salamanders have natural broad regenerative capacities, the identification of antimicrobial peptides with wound healing and immune response capacities is relevant and could be promising in the identification of peptides with therapeutic application in humans.

Conclusion

Limb regeneration is a complex process where different cell populations and signals have been reported, however, a lot of questions remain open about other important signals that allow this process and how this trait have evolved in this organisms. Our research provides an incentive to further explore limb regeneration in species of plethodontids; the largest family of salamanders, which present species with direct development and show different morphogenetic patterns during the development of the limb. The work that has been done in species of plethodontids such as *B. ramosi* has helped understand that some features of limb regeneration are conserved between salamanders. However, morphological and gene expression differences during limb regeneration, when compared with the well-established research organisms, such as *A. mexicanum*, leave open questions about how deeply conserved are the regenerative features of limb regeneration in other species of the family Plethodontidae and other families of urodeles. It also raises questions on how the habitat (e.g aquatic vs terrestrial), the type of development (e.g indirect vs direct development), physiological changes (e.g skin architecture), and other molecular inputs (e.g antimicrobial peptides) could affect limb regeneration.

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