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Is Salamander Limb Regeneration Really Perfect? Anatomical and Morphogenetic Analysis of Forelimb Muscle Regeneration in GFP-Transgenic Axolotls as a Basis for Regenerative, Developmental, and Evolutionary Studies

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ABSTRACT

The axolotl *Ambystoma mexicanum* is one of the most commonly used model organisms in developmental and regenerative studies because it can reconstitute what is believed to be a completely normal anatomical and functional forelimb/hindlimb after amputation. However, to date it has not been confirmed whether each regenerated forelimb muscle is really a “perfect” copy of the original muscle. This study describes the regeneration of the arm, forearm, hand, and some pectoral muscles (e.g., coracoradialis) in transgenic axolotls that express green fluorescent protein (GFP) in muscle fibers. The observations found that: (1) there were muscle anomalies in 43% of the regenerated forelimbs; (2) however, on average in each regenerated forelimb there are anomalies in only 2.5% of the total number of muscles examined, and there were no significant differences observed in the specific insertion and origin of the other muscles analyzed; (3) one of the most notable and common anomalies (seen in 35% of the regenerated forelimbs) was the presence of a fleshy coracoradialis at the level of the arm; this is a particularly outstanding configuration because in axolotls and in urodeles in general this muscle only has a thin tendon at the level of the arm, and the additional fleshy belly in the regenerated arms is strikingly similar to the fleshy biceps brachii of amniotes, suggesting a remarkable parallel between a regeneration defect and a major phenotypic change that occurred during tetrapod limb evolution; (4) during forelimb muscle regeneration there was a clear proximo-distal and radio-ulnar morphogenetic gradient, as seen in normal development, but also a ventro-dorsal gradient in the order of regeneration, which was not previously described in the literature. These results have broader implications for regenerative, evolutionary, developmental and morphogenetic studies. *Anat Rec*, 00:000–000, 2014. © 2014 Wiley Periodicals, Inc.

Key words: *Ambystoma*; anatomy; morphogenesis; muscles; regeneration; urodele amphibians; evolution

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The neotenuous axolotl *Ambystoma mexicanum* (Amphibia: Urodela) is a common model organism in evolutionary, developmental and regenerative studies. It is a particularly useful regenerative model because it is said that it can reconstitute a fully functional and complete forelimb/hindlimb (Kragl et al., 2009; see also the reviews of, e.g., Carlson, 2003, 2007; Nacu and Tanaka, 2011; Stocum and Cameron, 2011; Agata and Inoue, 2012). Amputation anywhere between the shoulder and the hand triggers the formation of a progenitor cell zone (blastema) that regenerates the epidermis, dermis, muscle, nerve, blood vessels and skeletal elements of the regenerated forelimb (e.g., Kragl et al., 2009). In a recent publication (Diogo and Tanaka, 2012), detailed descriptions of each pectoral and forelimb muscle of both wildtype and GFP-transgenic axolotls and an updated discussion and tables of the muscle homologies between these amphibians and other tetrapod taxa were presented. That publication is part of a long-term project focused on the development, homologies and evolution of the head, neck, pectoral and forelimb muscles of all major vertebrate clades (e.g., Diogo, 2007; Diogo and Abdala, 2007, 2010; Diogo et al. 2009; Abdala and Diogo, 2010). This long term project provided the anatomical basis for the present regenerative study, which has the goal of describing the morphology and morphogenesis of the regenerated forelimb muscles in axolotls.

Among the very few morphological investigations of limb musculature regeneration done in these amphibians, most were done by Carlson and colleagues (see, e.g., Grim and Carlson, 1974a,b; Carlson, 2003, 2007; and references therein). Grim and Carlson (1974b) is the most detailed publication, and mainly served as a basis for subsequent analyses of and discussions about the regeneration of forelimb muscles in axolotls. Other authors did provide extensive morphological studies of limb regeneration in salamanders, but these were mainly focused on other structures, such as nerves (e.g., Weiss and Walker, 1934; Piatt, 1957; Stephens and Holder, 1987). Interestingly, those studies indicated that, contrary to the common belief that salamanders provide an illustrative example of perfect or almost perfect limb regeneration (see above), the regeneration of nerves was far from perfect. For instance, Piatt (1957) showed that when the forelimb is amputated through the arm or the forearm, the nerve pattern which develops in the regenerated limb is clearly not normal. That is, the general pathways of the nerves may seem to be essentially normal but the identity of the nerves is lost because of repeated branching and anastomosing of the major nerve trunks. In many cases nerves which normally are found in a subcutaneous position run intramuscularly for long distances; others which normally run between muscles are in many instances also intramuscular in their course. Anomalous/supernumerary nerves are also present in a number of the regenerates. Although Piatt's study was mainly focused on nerves, she stated that the regeneration of muscles seemed to be more normal than that of nerves (see also Stephens and Holder, 1987, for a similar view). The main exception was the coracoradialis muscle, which in salamanders is normally tendinous throughout the entire arm, but in regenerates often is fleshy at the level of the arm.

The idea that the limb muscles of salamanders, including axolotls, almost always, or always, regenerate normally after amputation ("epimorphic mode of regeneration" sensu e.g., Carlson, 2003) is usually accepted by other authors (e.g., Wigmore and Holder, 1985). In fact, this idea plays a crucial role in the theoretical justification for why salamanders in general and axolotls in particular are the most used model organisms in limb regenerative studies. However, this idea mainly comes from aside notes and/or from mainly superficial observations of a few authors, because the only detailed morphological study that was performed to specifically test this idea (with a focus on muscles) was that of Grim and Carlson (1974b). In that study Grim and Carlson suggested that the regenerated muscles of amputated forelimbs were essentially normal, that is, indistinct from the original muscles before amputation. For instance, they stressed that both in the regeneration and the ontogeny of the forelimb muscles of this species there was a proximo-distal and a radio-ulnar gradient (see below). However, their study also had some limitations (see Diogo and Tanaka, 2012) that were addressed in the present study. For example, Grim and Carlson's study only analyzed the regeneration of the axolotl forearm and hand muscles. However, as will be seen below, many of the current cell, molecular and/or genetic studies on axolotl regeneration also include the arm muscles. Moreover, even when the amputation is at the level of the arm, some muscles that are connected to the most medial portion of the pectoral girdle (e.g., coracoradialis) are also regenerated and anatomically affected after injury. Additionally, papers such as that of Grim and Carlson's (1974b) focus on more mature axolotls, so one aim of the present study was to include observations about the timing and mode of forelimb muscle regeneration in less mature axolotls. Another purpose for undertaking the current study is that, as explained by Diogo and Tanaka (2012), there are also some differences between our observations of the normal (i.e., non-regenerated) forearm and hand musculature of *A. mexicanum* and the descriptions of Grim and Carlson (1974a). The present study provides, for the first time, a morphological investigation of the regeneration of the axolotl forelimb muscles that includes dissections and analyses of transgenic animals that express GFP in muscle fibers. In particular, for the context of the present work, by including GFP-transgenic axolotls and visualizing these animals with and without simultaneous transmission laser light, one can have a more complete and clearer understanding of the exact limit of the fleshy (shown as fluorescent green in GFPs) and tendinous (examined in dissections and also by using the transmission light) parts of the muscles and their specific connections with the skeletal elements (Diogo and Tanaka, 2012).

The purposes of this study were: (1) to test whether regeneration of the amputated forelimb in axolotls leads to a completely normal configuration of the muscles; and (2) to set the basis for future evolutionary and particularly regenerative, developmental and morphogenetic studies on the forelimb of axolotls and other tetrapods. This is accomplished by providing anatomical and morphogenetic descriptions of the regeneration of the arm, forearm, hand and some pectoral (e.g., coracoradialis)

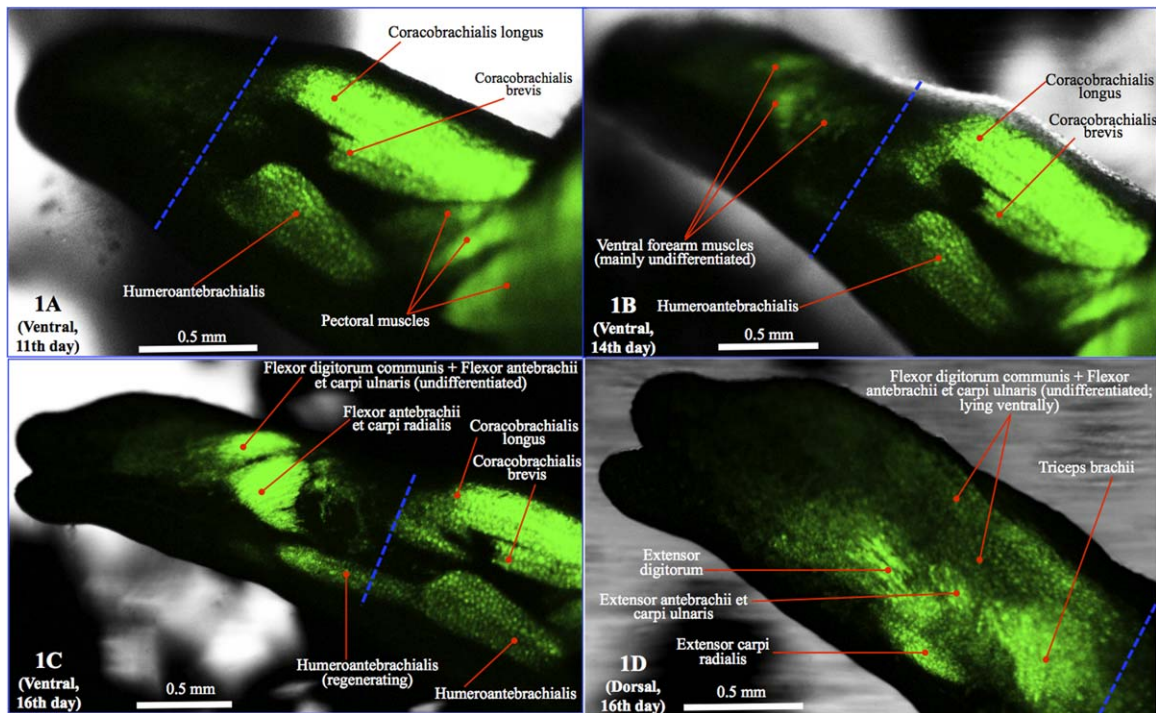


Fig. 1. Right limb of GFP-transgenic axolotl CRTD AM10; in this figure and in Figs. 2 and 3, ulnar is to the top, radial to the bottom (head of the animal is also to the bottom), distal to the left, and proximal to the right (horizontal flop done with Photoshop on the pictures showing the dorsal view, in order to have the same orientation that those

showing dorsal view have, to facilitate comparisons), and the blue dashed line indicates the approximate place of amputation. (A) Ventral view at 11 days of regeneration (dr). (B) Same view at 14 dr. (C) Same view at 16 dr. (D) Dorsal view at 16 dr.

muscles, in particular using GFP-transgenic animals. Four specific questions were addressed in this study: (a) does the regeneration of the forelimb really lead to a configuration that is 100% similar to that of the original forelimb musculature?; (b) how is muscle regeneration done topologically and morphogenetically, that is, which muscles develop first and how are they related to each other 3-dimensionally?; (c) does this detailed analysis confirm the morphogenetic observations done by authors such as Grim and Carlson (1974b) about the occurrence of both a radio-ulnar and a proximo-distal gradient, and does it also provide evidence for a possible dorso-ventral or ventro-dorsal gradient?; and (d) how does muscle morphogenesis in regeneration compare with what is known about the ontogeny of the forelimb muscles in axolotls and other salamanders?

MATERIALS AND METHODS

A total of 46 *Ambystoma mexicanum* forelimbs were examined for this study. Two of these were adult wildtype specimens (HU AM1, right forelimb examined; HU AM2, both forelimbs examined) that were from the Anatomy Department of Howard University. The others specimens analyzed and/or dissected were obtained from the CRTD (Center for Regenerative Therapies Dresden). These include an adult wildtype (CRTD AM0, both forelimbs examined) and 20 transgenic animals that express GFP in muscle fibers (under the Cardiac-Alpha-Actin promoter): CRTD AM1, 2 and 6, 3-month post-fertilization, no amputation; CRTD AM3, 4, 7 and 8, 3-month post-fertilization, amputations in both forelimbs due to natural bites of

other specimens; CRTD AM5, 3-month post-fertilization, left forelimb amputated for study; CRTD AM9 and 10, 3-month post-fertilization, left and right forelimbs amputated for study; CRTD AM11 to 20, adults, left forelimb amputated for study. The study was approved by the CRTD animal ethics committee. No animal was purposely sacrificed for this anatomical study. The animals were in general examined alive, and those that were dissected were sacrificed for reasons related to research/work of other individuals (e.g., colony keeping, experiments: see Diogo and Tanaka, 2012, for more details). All amputations were done at the level of the arm (see Figs. 1A, 4A,B, and 5A) and all experiments conformed to the relevant regulatory standards. In total, we therefore examined 23 regenerated and 23 non-amputated forelimbs, which were used as controls (these latter forelimbs were described in detail by Diogo and Tanaka, 2012). The 23 regenerated forelimbs may be grouped into three major groups, each studied for different purposes. The five regenerated forelimbs of CRTD AM5, 9 and 10 (animals born on 26 February 2012, amputations at the level of the arm done on 25 May 2012) were analyzed everyday from the 7th day until the 34th day after the regeneration started (dr). This was done in order to describe in detail the anatomy and morphogenesis of each forelimb muscle during the regeneration process. Figures 1–3 illustrate this process in the left forelimb of CRTD AM10, which can be used as an illustrative example of the general pattern observed in the five forelimbs (see below). The 10 regenerated forelimbs of CRTD AM11–20 (animals born on 8 November 2011, 9 November 2011, and 10 November 2011, amputations at

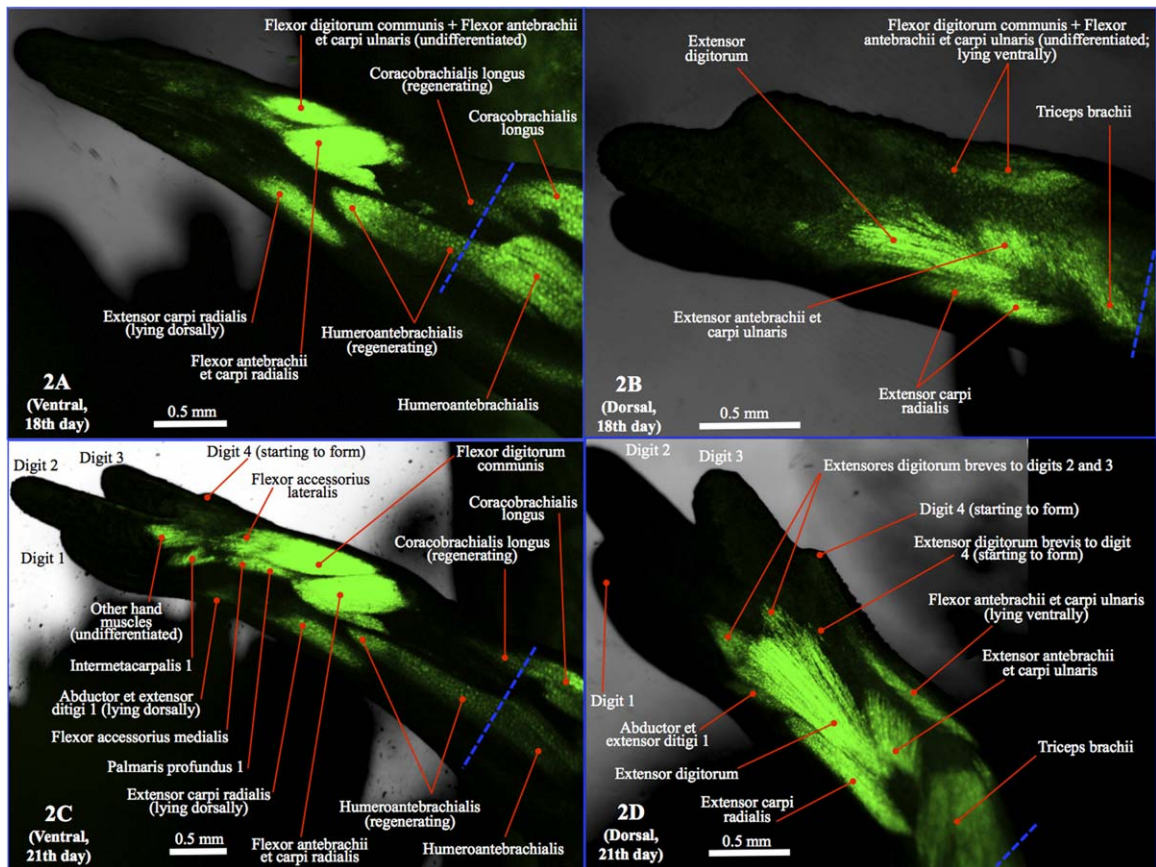


Fig. 2. Right limb of GFP-transgenic axolotl CRTD AM10 (for orientation, see Fig. 1). (A) Ventral view at 18 dr. (B) Dorsal view at 18 dr. (C) Ventral view at 21 dr. (D) Dorsal view at 21 dr.

the level of the arm done on 7 February 2012) were studied at later stages of regeneration (128dr). At this stage these forelimbs were already fully formed and their size was similar to that of the contralateral forelimb (see, e.g., Fig. 4B). The eight regenerated forelimbs of CRTD AM3, 4, 7, and 8 (animals born on 26 February 2012) had amputations provoked by natural bites of other specimens at different times during the first 105 days of life (analysis done on 28 June 2012). These forelimbs were studied to check if the regeneration process seen in these animals where the amputations were done by natural bites of other specimens is in general similar to that seen in the other animals where the amputations were done artificially. Because the aggressions from other specimens occurred naturally when the animals were living in a same container, it was not possible to determine the exact day after amputation. Each animal was then moved to a new container so the forelimbs could regenerate without further aggressions and each animal was analyzed in detail when all or almost all the muscles were already formed in the regenerated forelimbs (see, e.g., Fig. 5B).

The imaging of the GFP-transgenic animals was performed with a Leica TCS LSI confocal microscope at the light microscopy facility of the CRTD-BIOTEC. Images were acquired using a 1x zoom objective. The GFP fluorescence was excited with the 488-nm laser line and fluorescence were collected between 500 and 520 nm with the standard PMT. Simultaneously transmitted laser

light was detected with a T-PMT to create a transmitted light image. Overlay of both channels was created using LAS-AF software (V 2.6) (see Figs. 1–5, all of them showing both channels). The nomenclature of the muscles mainly follows Diogo and Tanaka (2012). When we refer to the anterior, posterior, dorsal and ventral regions of the body, we therefore do so in the sense the terms are used for pronograde tetrapods (e.g., the forelimb is anterior to the hindlimb, and in each limb the extensor muscles are dorsal to the flexor muscles).

RESULTS

The results are shown in detail in Figs. 1–5 and Table 1. Therefore this section will provide a brief description of these results, which are divided into the three major subsections. It should be noted that, with exception to those specific cases of anomalous muscle regeneration described below and discussed in the Discussion, there were no significant differences concerning the specific insertion and origin of the other muscles analyzed, among the other regenerated limbs studied.

Regeneration of Forelimbs of CRTD AM5, 9 and 10, From 7 Days of Regeneration (dr) to 34dr

The regeneration of the right forelimb of specimen CRTD AM10, which in general illustrates the normal

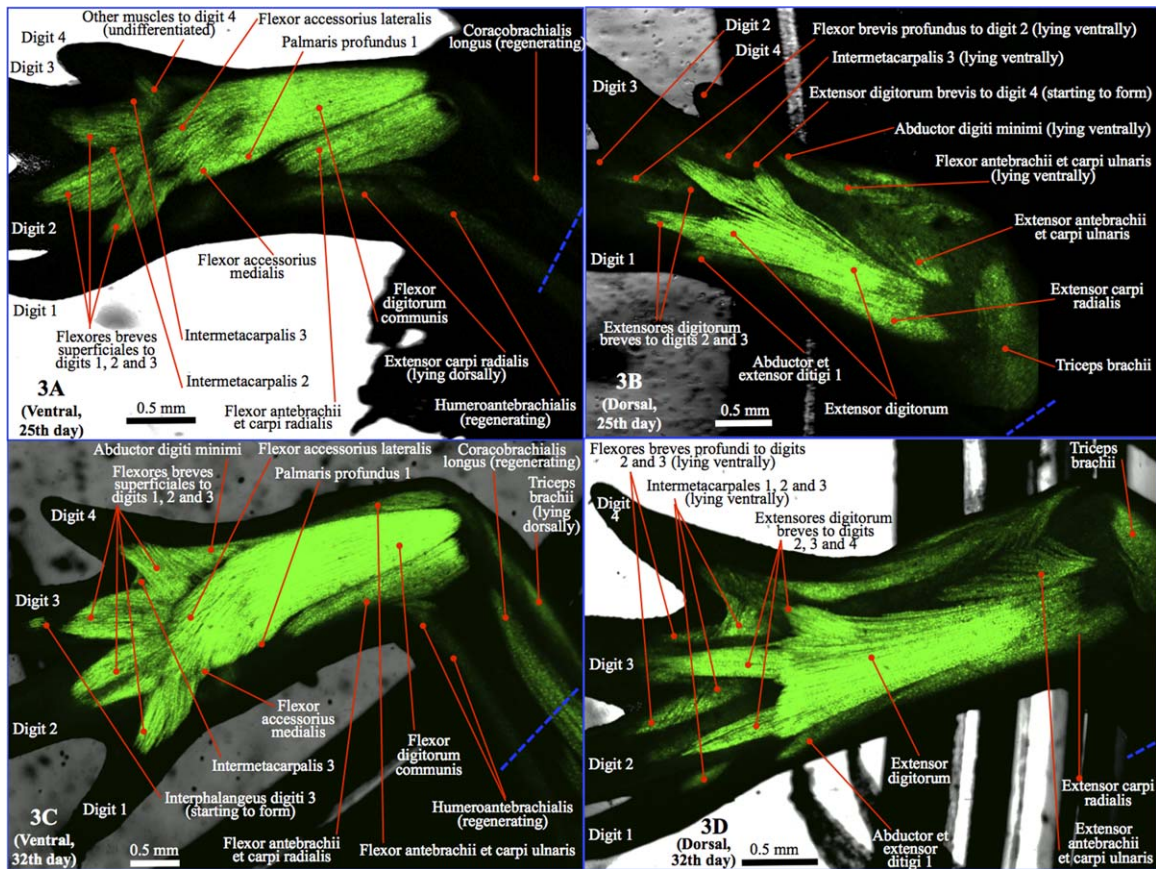


Fig. 3. Right limb of GFP-transgenic axolotl CRTD AM10 (for orientation, see Fig. 1); it should be noted that in **A** and **C** the ventral arm muscles appear darker than in the photographs of Figs. 1 and 2 not because they are less developed or they are degenerating (well on the contrary; they are clearly regenerating), but simply because of the orientation of the limb when the photographs were taken. (A) Ventral view at 25 dr. (B) Dorsal view at 25 dr. (C) Ventral view at 32 dr. (D) Dorsal view at 32 dr.

pattern of regeneration observed in the other specimens, will be used as the basis for the comparisons with and descriptions of the other specimens provided in Table 1.

The coracobrachialis, humeroantibrachialis and triceps brachii were cut in the amputations of both limbs of this specimen, and during the first 11dr the region of these muscles that is just proximal to the amputation site degenerated (see, e.g., Fig. 1A), as is usually the case in axolotl limb regeneration (e.g., Carlson 2003, 2007). On the right forelimb regeneration started to be clearly noted at 14dr when the forearm muscles started to form but were still not differentiated from each other (Fig. 1B). The most relevant anatomical changes observed to this stage are summarized in Table 1. It is important to stress that even at the stages shown in this table and described below in which all the muscles are formed, the total size of the regenerated forelimb was much smaller than that of the controls, that is, at this stage the regenerated limbs were essentially a miniature limb (see, e.g., Fig. 5A).

At 16 dr there was a newly formed distal part of the humeroantibrachialis, which was somewhat discontinuous with the main body of the muscle. At this stage, the flexor antibrachii et carpi radialis are differentiated from a mass that corresponds to the flexor antibrachii

et carpi ulnaris + flexor digitorum communis. The extensor carpi radialis, extensor digitorum and extensor antibrachii et carpi ulnaris were also differentiated (it is not certain if the supinator [= "extensor antibrachii radialis"] is already differentiated as a distinct muscle because this muscle is deeper, that is, more ventral, than these latter three muscles) (Fig. 1C,D). At 21 dr (Fig. 2C,D) the flexor digitorum communis and flexor antibrachii et carpi ulnaris were differentiated and these two muscles and the flexor antibrachii et carpi radialis were now much longer and developed than at 16dr. At this state, the beginning of differentiation of the more distal and deep ventral forearm muscles (e.g., flexor accessorius lateralis, flexor accessorius medialis and palmaris profundus 1), of the intermetacarpalis 1, and of a muscle mass that will give rise to the other hand muscles, can be seen. The abductor et extensor digiti 1 and the extensores breves digitorum to digits 2, 3, and 4 were differentiated, the muscle going to digit 4 being much smaller than the muscles going to the other digits. At 23 dr the short flexors to digits 1, 2, and 3, as well as the intermetacarpales 2 and what seems to be an intermetacarpalis 3, were differentiated (the short flexors include the flexores breves superficiales, the contrahentes, which is sometimes designated as "flexores

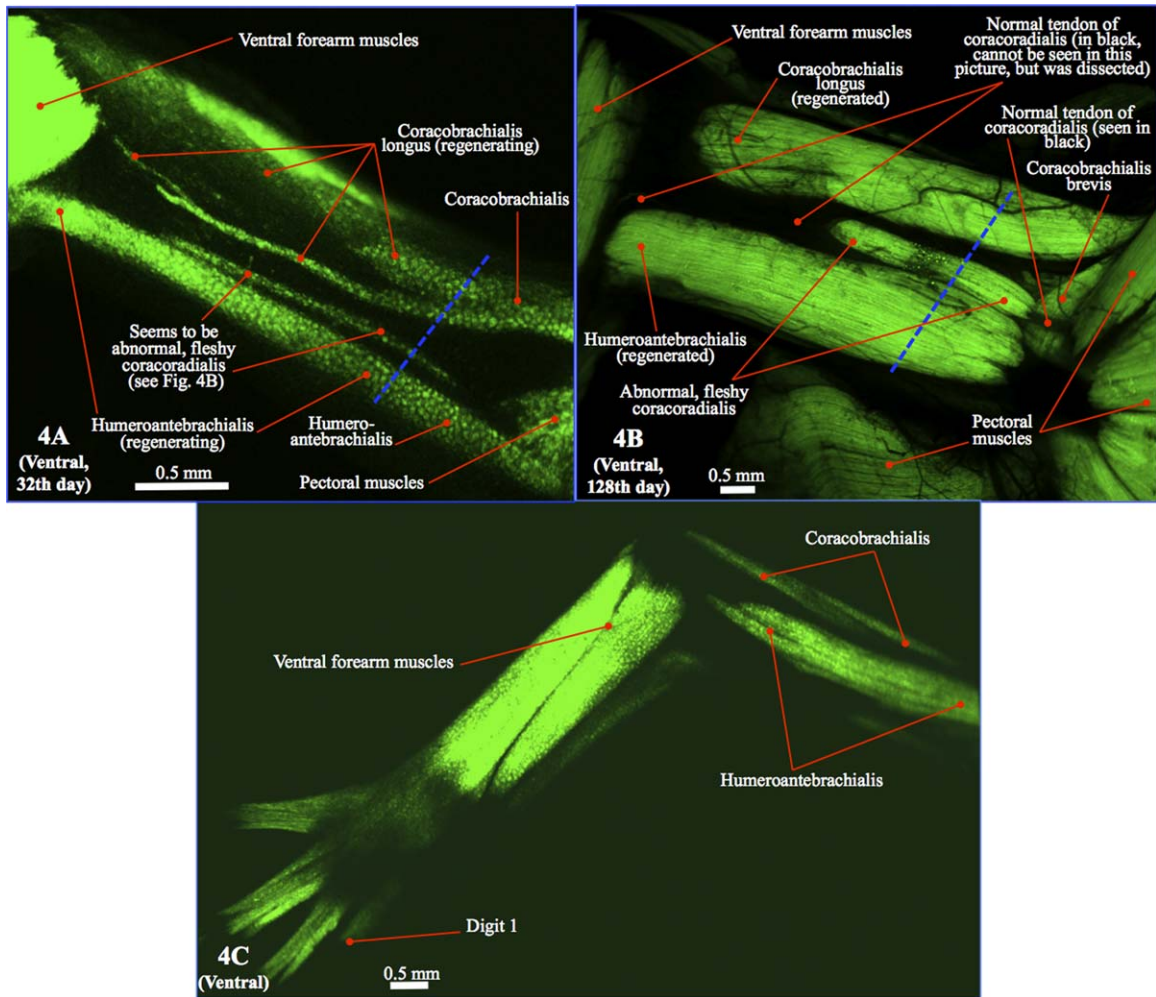


Fig. 4. GFP-transgenic axolotls. (A) CRTD AM5, ventral view of ventral arm muscles of left limb (ulnar is to the top, radial to the bottom—so, head of the animal is also to the bottom—, distal to the left, and proximal to the right) at 32dr showing radio-ulnar gradient (regeneration of humeroantibrachialis more advanced than that of the coracobrachialis) and also what seems to be a fleshy muscle coracoradialis. (B) CRTD AM14, ventral view of ventral arm muscles of left limb (ulnar is to the top, radial to the bottom—so, head of the animal is also to the bottom—, distal to the left, and proximal to the right) at 128dr showing a fleshy muscle coracoradialis. (C) CRTD AM35, control, non-

regenerated specimen, same view as in A, illustrating the fact that the coracoradialis does usually not have fleshy fibers in nonregenerated axolotl forelimbs, that is, that the two only fleshy muscles that are usually seen in the nonregenerated forelimbs, in a ventral view of the arm, are the humeroantibrachialis and the coracobrachialis, with a relatively wide space between these two muscles (where normally lie the abnormal, fleshy coracoradialis fibers that are usually present in regenerated forelimbs, as seen in A and B; for more details on the normal configuration of axolotl forelimb muscles, see Diogo and Tanaka, 2012).

breves intermediales,” and the flexores breves profundi. At 25 dr the abductor digiti minimi was observed for the first time (Fig. 3B). Lastly, at 32 dr the interphalangeus digiti 3 was present and all the muscles were in their expected anatomical location and had their normal adult attachments (Fig. 3C,D).

Regeneration of Left Forelimbs of CRTD AM11-20, at 128dr

This subsection refers only to the major anomalies seen in these specimens at 128dr. In CRTD AM11 the configuration of the regenerated forelimb muscles was normal (i.e., similar to that of nonamputated limbs) with three exceptions. First, the coracoradialis had fleshy fibers at the level of the arm (Fig. 4A). Second, there

was a separate muscle running from the lateral epicondyle of the humerus to the olecranon process of the ulna, which was thus similar to the anconeus of other tetrapods and was clearly not normal. This latter muscle was blended with both the extensor antibrachii et carpi ulnaris and the triceps brachii, and thus might well derive from the fibers of the extensor antibrachii et carpi ulnaris (as seemingly does the anconeus in other tetrapods: e.g., Diogo and Abdala, 2010). Third, the coracobrachialis longus did not extend as distal as it usually does (i.e., to the elbow joint), extending instead to about 3/4 of the total length of the humerus. In CRTD AM12, 14, and 19 the only muscle anomaly was that the coracoradialis had fleshy fibers at the level of the arm (Fig. 4B). In CRTD AM20 the single muscle anomaly was that the fleshy part of the coracobrachialis longus only

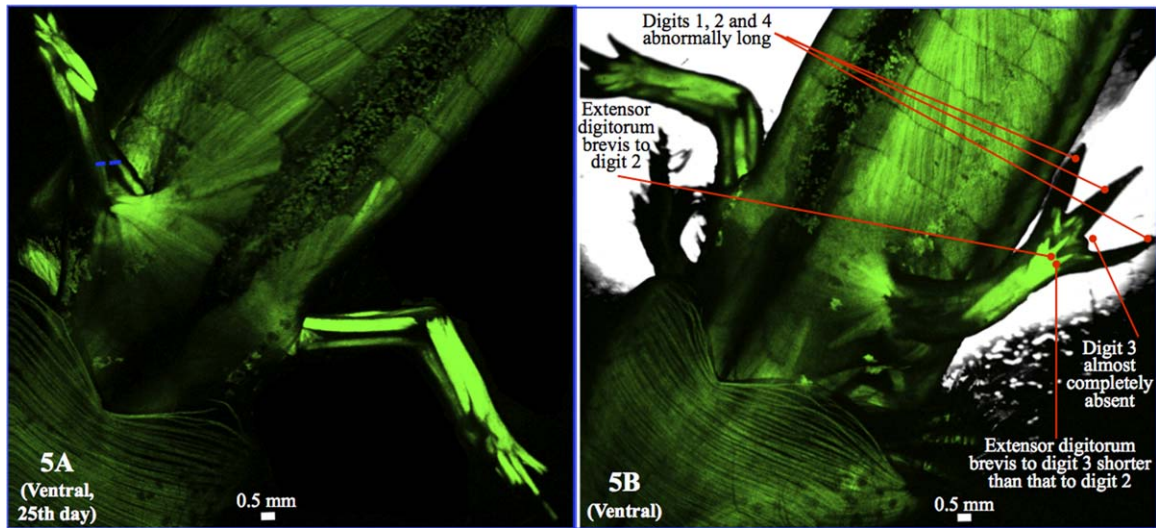


Fig. 5. GFP-transgenic axolotls. (A) CRTD AM5, same view as in Fig. 4A, but at 25dr, to show how, although almost all the muscles have already appeared in the regenerated left limb, this limb is still much smaller than the nonamputated right limb, that is, it somewhat resembles a miniature limb. (B) CRTD AM3, same view as in A, showing how regeneration of the right limb due to natural (uncontrolled;

therefore we do not know since how many days the regeneration has been occurring) bites performed by other specimens lead to the formation of a highly abnormal limb and limb muscles (e.g., digits 1, 2, and 4 are abnormally long, and digit 3 and at least some of its muscles are poorly developed).

extended to about 2/3 of the humerus. That is, the distal 1/3 of the humerus had no fleshy fibers of this muscle although the muscle did send a distal tendon that reached the elbow joint, but the fleshy part was clearly shorter than normally. No muscle anomalies were found in CRTD AM13, 15, 16, 17, and 18.

Regeneration of Forelimbs of CRTD AM3, 4, 7, and 8

On the regenerated left forelimb of CRTD AM3 all the muscles were in place and had a normal configuration. However, there were notable anomalies on the right forelimb. First, digit 3 was abnormally reduced in size, while digits 1, 2, and 4 were abnormally long. Second, apart from the missing interphalangeus digiti 3, all the other muscles associated with this digit seemed to be actually formed, although obviously they did not extend as distal as they normally do in this digit because of its reduced size (see, e.g., Fig. 5B). In CRTD AM4 and AM7 the only clear muscle anomaly was the presence of a fleshy coracoradialis at the arm level in their right forelimbs. No muscle anomalies were observed in CRTD AM8.

DISCUSSION

Do the Regenerated Forelimb Muscles Have a Normal Configuration?

Within the 10 regenerated forelimbs of the axolotls examined at 128 days (CRTD AM11–20) there were seven muscle anomalies distributed in five forelimbs, so there were anomalies in 50% of the ten forelimbs. Four of these five forelimbs (CRTD AM12, 14, 19, and 20) only had one anomaly affecting a specific muscle (fleshy coracoradialis at the level of the arm in CRTD AM12, 14, and 19 and short fleshy coracobrachialis longus in CRTD 20). The other forelimb (CRTD AM11) had three

anomalies affecting three different muscles (fleshy coracoradialis at the level of the arm, presence of anconeus, and short coracobrachialis longus). If we consider the total number of muscles analyzed ($N = 36$, i.e., the coracoradialis plus the 35 arm, forearm, and hand muscles reported by Diogo and Tanaka, 2012) one then concludes that in average each of the ten examined forelimbs only displayed anomalies in 1.9% of the total number of muscles. Regarding the five regenerated limbs that were examined from the moment of amputation until 34 dr (i.e., of CRTD AM5, 9, and 10), there were two muscle anomalies in total. These were the presence of fleshy coracoradialis at the level of the arm on the left forelimbs of CRTD AM5 and of CRTD AM9. Therefore, there were anomalies in 40% of these five forelimbs, and in average each of these five forelimbs had anomalies in 1.1% of the total number of muscles. Concerning the eight regenerated forelimbs that were amputated due to natural bites from other specimens (i.e., forelimbs of CRTD AM3, 4, 7, and 8), there were eleven muscle anomalies. These were the presence of coracoradialis on the right forelimbs of CRTD AM4 and of CRTD AM7 and absence/shortness of the intermetacarpalis 2, the intermetacarpalis 3, the interphalangeus digiti 3 and of the tendon of the extensor digitorum, the tendon of the flexor digitorum communis, the extensor digitorum brevis, the flexor brevis superficialis, the contrahens and the flexor brevis profundus to digit 3 of the right forelimb of CRTD AM3, associated to the very reduced size of digit 3. Therefore, there were anomalies in 38% of these eight forelimbs, and on average each of these eight forelimbs thus displayed anomalies in 3.9% of the total number of muscles. Within the 23 regenerated forelimbs that were examined in the present work as a whole, there were thus muscle anomalies in 10 (43%) forelimbs. This is a surprising result of the present study, because such a high percentage contradicts the idea that the

TABLE 1. Summary of most relevant anatomical changes during the regeneration of the forelimbs of CRTD AM5, 9 and 10, from 7 to 34 days of regeneration (dr, i.e. after amputation; for more details see text and Figures 1-5)

CRTD AM 10, right forelimb	CRTD AM 10, left forelimb	CRTD AM 9, right forelimb	CRTD AM 9, left forelimb	CRTD AM 5, left forelimb
<p>14dr: forearm muscles start to form but are still not differentiated from each other</p>	<p>27dr: regeneration of the muscles just starts to be noted at this stage, when the coracobrachialis (seemingly brevis and longus), the humeroantebrachialis and the triceps starting to regenerate</p>	<p>16dr: configuration similar to that reported on the right for the left forelimb of this specimen, at 13dr; in addition, one can also see the differentiation of the extensor carpi radialis, flexor antebrachii et carpi radialis and of an undifferentiated mass including the flexor digitorum communis + flexor antebrachii et carpi ulnaris</p>	<p>13dr: regeneration starts to be clearly noted, i.e. the coracobrachialis and particularly the humeroantebrachialis have now extended distally to the amputation site due to the formation of fibers that extend proximally and towards the ulnar side to reach the distal margin of the main body of these muscles, thus forming a discontinuity with this main body</p>	<p>18dr: 1) the humero-antebrachialis is apparently fully regenerated, the newly formed part of the muscle being thinner and its axis being more radial than the main body of the muscle, and the coracobrachialis longus has a newly formed part that is much less developed than the newly formed part of the humero-antebrachialis; 2) the newly formed triceps already extends distally to attach onto the ulna; 3) the flexor antebrachii et carpi radialis is already differentiated from the muscle mass including the flexor digitorum communis + flexor antebrachii et carpi ulnaris, and dorsally the supinator [= 'extensor antebrachii radialis'], extensor digitorum, extensor carpi radialis and extensor antebrachii et carpi ulnaris are also differentiated</p>
<p>16dr: 1) there is a newly formed distal part of the humeroantebrachialis, which is somewhat discontinuous with the main body of the muscle; 2) one can now differentiate the flexor antebrachii et carpi radialis from a mass that corresponds to the flexor antebrachii et carpi ulnaris + flexor digitorum communis; 3) the extensor carpi radialis, extensor digitorum and extensor antebrachii et carpi ulnaris are differentiated</p>	<p>29dr: appearance of the extensor carpi radialis, the extensor digitorum, the extensor antebrachii et carpi ulnaris, the flexor antebrachii et carpi radialis, and the undifferentiated muscle mass of the flexor digitorum communis + flexor antebrachii et carpi ulnaris</p>	<p>18dr: one can begin to see the differentiation of the contrahentium caput longum, flexor accessorius lateralis, flexor accessorius medialis and palmaris profundus 1 and of an undifferentiated mass of hand muscles</p>	<p>21dr: one can see a differentiated extensor carpi radialis and a differentiated flexor antebrachii et carpi radialis lying radially to the seemingly undifferentiated masses of the extensor digitorum + extensor antebrachii et carpi ulnaris and of the flexor digitorum communis + flexor antebrachii et carpi ulnaris, respectively; the contrahentium caput longum, flexor accessorius medialis, flexor accessorius lateralis and palmaris profundus 1 are starting to differentiate</p>	<p>20dr: 1) the newly formed humeroantebrachialis is more complete and well defined, being continuous to and just a little bit thinner than the main body of this muscle; 2) the newly formed coracobrachialis longus is still fragmented and lies more radial than the main body of this muscle; 3) the flexors and extensors of the forearm are longer; 4) one can now see the contrahentium caput longum, flexor accessorius lateralis, flexor accessorius lateralis and palmaris profundus 1; 5) one can seemingly also</p>

TABLE 1. (continued).

CRTD AM 10, right forelimb	CRTD AM 10, left forelimb	CRTD AM 9, right forelimb	CRTD AM 9, left forelimb	CRTD AM 5, left forelimb
<p>already differentiated as a distinct muscle or not because this muscle is deeper - i.e. more ventral - than these latter three muscles) (Fig. 1C,D)</p> <p>21dr (Fig. 2C,D): 1) the flexor digitorum communis and flexor antebrachii et carpi ulnaris are differentiated and these two muscles and the flexor antebrachii et carpi radialis are now much longer and developed than at 16dr; 2) one can begin to see the differentiation of the more distal and deep ventral forearm muscles (e.g., flexor accessorius lateralis, flexor accessorius medialis and palmaris profundus 1), of the intermetacarpalis 1, and of a muscle mass that will give rise to the other hand muscles; 3) the abductor et extensor breves digitorum to digits 2 and 3 and 4 are differentiated, the muscle going to digit 4 being much smaller than the muscles going to the other digits</p> <p>23dr: the short flexors to digits 1, 2 and 3, as well as the intermetacarpales 2 and what seems to be an intermetacarpalis 3, are differentiated</p>	<p>32dr: 1) the humeroantebrachialis is now fully formed (i.e. it has its normal attachments) while the coracobrachialis is regenerating fast but is still not clearly defined and not continuous from its normal origin from the humerus to its normal insertion onto the elbow region; 2) the flexor antebrachii et carpi ulnaris, flexor digitorum communis, flexor accessorius lateralis, flexor accessorius medialis and palmaris profundus 1, extensores digitorum breves to digits 2 and 3 and abductor et extensor digiti 1 are differentiated; 3) one can see the undifferentiated muscle masses that later will form the intermetacarpales 1 and 2 and the short flexors of digits 1, 2 and 3</p> <p>34dr: the coracobrachialis is still not fully formed, while the short flexors for digits 1, 2 and 3 and also the intermetacarpales 1 and 2 are now well differentiated; the abductor digiti minimi, the intermetacarpalis 3, the short flexors to digit 4, the</p>	<p>23dr: 1) distally the coracobrachialis longus now seems continuous to the elbow joint, but its distal portion is still very thin and with fibers clearly still joining it; 2) the abductor et extensor digiti 1 is differentiated; 3) the intermetacarpalis 1 is longer and radially to it one can already see the differentiated short flexors going to digit 1 as well; 4) one can also now see the intermetacarpales 2 and 3 and also the short flexors of digits 2 and 3, with their characteristic radial, central and ulnar portions to each of these digits, but there are seemingly no well defined short flexors going to digit 4 yet; 5) one can now clearly see the extensor digitorum brevis to digit 3, while that to digit 4 is just starting to form</p> <p>25dr: the short flexors to digit 4 and the abductor digiti minimi are differentiated, while on the left forelimb there is now a separation between an undifferentiated muscle mass that will later give rise to the short flexors of digit 1 and an</p>	<p>23dr: the flexor antebrachii et carpi ulnaris, the abductor intermetacarpalis 1 are now differentiated, the latter muscle lying near an undifferentiated mass that will give rise to the short flexors of the hand</p> <p>27dr: the following muscles are differentiated: intermetacarpalis 2, short flexors of digits 2 and 3, and extensores digitorum breves to digits 2 and 3; interestingly, the dorsal forearm muscles extensor digitorum, extensor carpi radialis and</p>	<p>see what seems to be intermetacarpalis 1 just next to the undifferentiated mass that will give rise to the other hand muscles, as well as a differentiated flexor antebrachii et carpi ulnaris and a differentiated abductor et extensor digiti 1 lying next to the undifferentiated muscle mass of the extensores digitorum breves</p> <p>23dr: the humeroantebrachialis and coracobrachialis have essentially a normal (but miniaturized: see, e.g., Fig. 5A and text) configuration and one can already see at least some flexores breves superficiales and contraheutes to digits 1, 2 and 3 as well as an intermetacarpalis 2 and what seems to be an intermetacarpalis 3 starting to form; there are also extensores breves digitorum to digits 2 and 3 but not to digit 4</p> <p>25dr: 1) the coracobrachialis longus is still not a continuous structure extending to the elbow region, being clearly less developed than the humeroantebrachialis; 2) importantly, there are well-defined muscle fibers that follow the path of the</p>

TABLE 1. (continued).

CRTD AM 10, right forelimb	CRTD AM 10, left forelimb	CRTD AM 9, right forelimb	CRTD AM 9, left forelimb	CRTD AM 5, left forelimb
extensor digitorum brevis of digit 4, and the interphalangeus digiti 3 are still not differentiated at this stage	undifferentiated muscle mass that will later give rise to the short flexors of the other digits	particularly the extensor antebrachii et carpi ulnaris are still very poorly developed, particularly compared with the ventral forearm muscles flexor digitorum communis, flexor antebrachii et carpi radialis and flexor antebrachii et carpi ulnaris	coracoradialis tendon; 3) the short flexors for digit 4, the intermetacarpalis 3 and the abductor digiti minimi are differentiated; 3) the extensor digitorum brevis to digit 4 is now starting to form; 4) the extensor antebrachii et carpi ulnaris extends far less distally than the extensor digitorum and the extensor carpi radialis	In the subsequent days, and until 34dr, the dorsal forearm muscles extensor digitorum, extensor carpi radialis and particularly the extensor antebrachii et carpi ulnaris are still very poorly developed, particularly compared with the ventral forearm muscles flexor digitorum communis, flexor antebrachii et carpi radialis and flexor antebrachii et carpi ulnaris; the coracobrachialis longus is also much less developed and disorganized than the humeroantebrachialis, which lies just next to the fleshy coracoradialis (Fig. 4A)
25dr: the abductor digiti minimi is observed for the first time (Fig. 3B)	—	32dr: One can see the interphalangeus digiti 3, so all the forelimb muscles have been formed	29dr: the abductor digiti minimi and the extensor digitorum brevis to digit 4 are first seen	
32dr: the interphalangeus digiti 3 is present; all the muscles are thus in place and all the arm muscle including the coracobrachialis longus already have their normal attachments (Fig. 3C,D)	—	—	34dr: the interphalangeus digiti 3 is still not differentiated	

axolotl forelimbs that are regenerated after amputation almost always display a muscle configuration that is similar to that of the original limbs (see Introduction). However, if one analyses these numbers from a different angle, the total number of anomalies observed in the 23 regenerated forelimbs examined was 20, so in average each forelimb had anomalies in only 2.5% of the total ($N = 36$) number of muscles examined. It should be noted that none of the muscle defects seen in the regenerated limbs analyzed for the present work was seen in the non-regenerated (i.e., original) limbs analyzed by Diogo and Tanaka (2012). For instance, a fleshy coracoradialis was not found in any of these nonregenerated limbs (see, e.g., Fig. 5C).

The average of anomalies per total number of muscles was greater in the eight forelimbs that suffered from natural bites (3.9%) than in the ten regenerated forelimbs of CRTD AM11–20 (1.9%) and in the five regenerated forelimbs of CRTD 5, 9, and 10 (1.1%). One explanation for this could be that the recurrent aggressions provoked by different animals and affecting different regions of the forelimb would result in a greater number of regenerative anomalies compared with a experimental setting where a specimen is subject to a single amputation in a specific region of the limb and then can regenerate the limb without suffering further aggressions. If this would be so, then the percentage of limbs displaying muscle anomalies after regeneration in wild animals should be greater than what the results of experimental studies might suggest. However, in order to test this hypothesis one would clearly have to study a far greater number of specimens that have been subject of aggression by other specimens in laboratories and, if possible, in the wild. What is clear from the present study is that a great part of the muscle anomalies observed in the specimens that were and that were not subject to natural bites concerned the presence of a fleshy coracoradialis at the level of the arm: in total, this happened in eight of the 23 (35%) regenerated forelimbs examined. This phenomenon has been greatly overlooked in the field of regenerative biology. One of the reasons for this is that the very few morphological analyses that have been done on the regeneration of the forelimb muscles (e.g., Grim and Carlson 1974b) did not include observations of the arm and pectoral muscles. This is therefore also one of the reasons why these studies have given the idea that the regeneration of the forelimb muscles in axolotls is more accurate than what the present study suggests.

Piatt (1957), in a study mainly focus on the regeneration of nerves in axolotl limbs, stated that a fleshy coracoradialis was commonly found at the level of the arm of the regenerated forelimbs, as was the case in the present study. According to Piatt the occurrence of such muscle anomalies, and particularly of the numerous morphological anomalies seen in the regenerated nerves of the salamander limbs (see Introduction) does not necessarily mean that the regenerated limbs display functional problems. Piatt's observations, and the observations done by us, actually seem to indicate that the movements of the limbs are essentially normal. That is, in this sense they do not contradict the idea that the regenerated limbs of model organisms such as axolotls are "fully functional." What these observations contradict is the idea that having a "fully functional" limb

equates to having a morphologically completely normal limb. For instance, the presence of some fleshy fibers associated with the tendon of the coracoradialis (e.g., Fig. 4B; cf., with Fig. 4C) does not necessarily change the main function of this muscle, which is to flex the forearm, because the proximal and distal attachments of the muscle remain exactly the same (Diogo and Tanaka, 2012; see below).

Morphogenesis

The regeneration of urodele limbs is a classic case study for the investigation of the morphogenesis of both hard and soft tissues (see, e.g., review in chapter 7 of Carlson's 2007 book). In general, one can say that the tempo and mode of the morphological regenerative events observed in the present study (see, e.g., Figs. 1–3) are similar to those reported by Grim and Carlson (1974b). As noted by these and other authors (see, e.g., reviews by Carlson 2003, 2007) in the course of the regeneration of the arm, forearm and hand muscles and also of the associated skeletal structures there is a proximo-distal and a radioulnar gradient. These morphogenetic gradients are also observed during the formation of the musculoskeletal elements during the ontogeny of the limbs of urodeles. That is, in this respect there is a parallel between regeneration and ontogeny in these animals (e.g., Grim and Carlson, 1974b). The radio-ulnar gradient observed in these amphibians is strikingly different to the ulno-radial gradient seen during the ontogenesis of the limb skeletal structures in other tetrapod groups and during the ontogenesis of limb muscles seen in at least some of these groups (e.g., chickens and frogs: see, e.g., Carlson, 2007; Diogo and Ziermann, in press).

According to Grim and Carlson (1974b) during the ontogeny of the axolotl forearm and hand the first muscles to differentiate are the extensor digitorum and flexor antebrachii et carpi radialis (both at stage 43). Then at stages 45 and 46 all the forearm muscles are differentiated. Only from stage 46 the abductor et extensor digiti 1 and the hand muscles start to differentiate, first the abductor digiti minimi and the intermetacarpales, and later muscles such as the flexores breves superficiales, the flexores breves profundi, the contrahentes, and the interphalangeus digiti 3. According to these authors, during regeneration the first muscle to differentiate is the supinator [=extensor antebrachii radialis]. Then there is a differentiation of the flexor antebrachii et carpi radialis, flexor antebrachii et carpi ulnaris and flexor digitorum communis, the other forearm muscles differentiating later, followed by the abductor et extensor 1 and then by the hand muscles. Therefore, according to these authors not only the morphogenetic gradients, but also the specific order of differentiation of the different muscles, are mainly similar during regeneration and ontogeny. The fact that in both processes at least some forearm muscles differentiate before the hand muscles is a clear example of the proximo-distal gradient. The fact that the long flexors and extensors that lie on the ulnar side (i.e., flexor antebrachii et carpi ulnaris and extensor antebrachii et carpi ulnaris) always differentiate after the differentiation of at least some more radial muscles (e.g., flexor antebrachii et carpi ulnaris and supinator) is an example of the radio-ulnar gradient. This gradient can be also be easily

seen by the order in which the digits are formed (from the two most radial to the two most ulnar digits: see, e.g., Figs. 2C,D).

Within the ventral forearm muscles of the regenerated forelimbs studied in the present study the first muscle to differentiate was usually the flexor antebrachii et carpi radialis, followed by the flexor digitorum communis and the flexor antebrachii et carpi ulnaris, and then by the deeper forearm flexors (i.e., the flexor accessorius lateralis, flexor accessorius medialis, palmaris profundus 1 and contrahentium caput longum) (Figs. 1–5). Regarding the dorsal ventral muscles, in the regenerated forelimbs the first muscle to differentiate was usually the extensor carpi radialis, followed by the extensor digitorum and the extensor antebrachii et carpi ulnaris. The abductor et extensor digiti 1 only differentiated later, usually at about the same time when the extensores digitorum breves to digits 2 and 3 differentiated. The extensor digitorum brevis to digit 4 was the last to differentiate (N.B., it is difficult to know if the supinator usually differentiated together with the extensor carpi radialis, or only later, because it lies deep—that is ventral—to the long forearm flexors: Figs. 1–5). Concerning the hand, during regeneration the first muscle to differentiate was usually intermetacarpalis 1, followed by the intermetacarpalis 2 and the short flexors of digits 1, 2, and 3, then by the muscles associated with digit 4 (e.g., abductor digiti minimi, intermetacarpalis 3 and short flexors to digit 4), and then by the interphalangeus digit 3. This latter muscle is the most distal muscle of the forelimb and the last to form (Figs. 1–5). Therefore, these observations clearly support the existence of both the radio-ulnar and the proximo-distal morphogenetic gradients described by other authors. Our observations of the regeneration of the arm muscles, which were not examined by Grim and Carlson (1974b), also support these gradients. This is because the regeneration of the triceps brachii, humeroantebrachialis, and coracobrachialis longus mainly proceeded in a proximo-distal direction, and the humeroantebrachialis (which is more radial) almost always regenerated faster, and reached its distal attachment quicker, than the coracobrachialis longus (which is more ulnar) (see, e.g., Fig. 1C). In this regard, it is worthy to mention that when the regenerated fibers of these two latter muscles were being formed and join the main body of each muscle, they usually were first in a position that is clearly more radial than the main body of the muscle (compare, e.g., the position of the newly formed fibers of the coracobrachialis longus shown in Fig. 4A with the position of the main body of this muscle in this figure and also with the position of the fully formed muscle in Fig. 4B, which is similar to the condition found in the limb shown in Fig. 4A at later stages of regeneration, i.e., at 34dr). This is one of the reasons that leads to the characteristic discontinuity between the main body of the original muscles and the newly formed fibers of these muscles, reported by previous authors (e.g., Tank et al., 1976).

Importantly, apart from the radioulnar and proximo-distal morphogenetic gradients, the observations of the present study seem to indicate that there was also a marked ventro-dorsal gradient during the regeneration of at least the forearm muscles. This is because, as explained in the Results, during the regeneration of these muscles the extensor carpi radialis, extensor digi-

torum and particularly the extensor antebrachii et carpi ulnaris were usually poorly developed when compared to the long forearm flexors (i.e., flexor antebrachii et carpi radialis, flexor antebrachii et carpi ulnaris, and flexor digitorum communis). This is particularly outstanding because the forearm extensors originate topologically from the lateral (radial) epicondyle of the humerus, while the long flexors originate from the medial (ulnar) epicondyle. Therefore, following the radio-ulnar gradient, the proximal portion (which is the first to form) of the extensors should be more developed than that of the flexors, and not the opposite. In particular, the extensor antebrachii et carpi ulnaris remained remarkably poorly developed during long periods of regeneration (being clearly shorter than the flexor antebrachii et carpi ulnaris; Figs. 2B,C and 3B), including at late stages when all the forelimb muscles were already differentiated (Fig. 3D). To our knowledge, such a ventro-dorsal gradient was not reported in previous studies of limb muscle regeneration or ontogeny in any tetrapod taxon. In fact, an opposite, dorso-ventral gradient has been reported in Kardon's (1998) study of the ontogeny of the hindlimb muscles of chickens. We plan to investigate whether such a ventro-dorsal gradient is, or is not, also seen during the ontogeny of the axolotl forelimb muscles. If this is so, then this would be a further difference between the ontogeny of muscles in these amphibians and in other tetrapods such as chickens (in addition to the radioulnar ontogenetic gradient, which is clearly distinct from the ulno-radial ontogenetic gradient seen in other tetrapods: see above).

At least the proximo-distal and radioulnar gradients are so marked in the regeneration and seemingly in the ontogeny of salamander limbs that they constituting what seems to be a clear parallelism between ontogeny and regeneration in urodeles. Therefore, it would also be interesting to undertake a comparative analysis including the fossil record in order to investigate if there is also a parallelism between ontogeny and the phylogenetic history of these amphibians. However, it is important to stress that there are some crucial differences between the ontogeny and the regeneration of the axolotl limbs. For instance, as reported by other authors (e.g., Tank et al., 1976; Carlson 2003, 2007) and corroborated in the present study during regeneration there is a clear dissociation between morphogenesis and growth. When all the muscles were already differentiated in the regenerated limb, the limb was still a "miniature" version of the original limb (Fig. 5A,B). The original limb then undergoes a prolonged phase of growth that may take several months, in which some internal changes (e.g., ossification of the cartilaginous skeleton) occur (e.g., Tank et al., 1976). Interestingly, the large degree of disparity in cross-sectional area between the blastema and the stump that is seen in older animals is not seen in larval axolotls nor in newts (Tank et al., 1976). According to Tank et al. (1976) the marked disparity seen in larger animals may be a reflection of an upper limit of absolute size that a blastema can attain and yet carry on normal morphogenetic processes. Another difference between the ontogeny and regeneration of the axolotl limbs concerns the well-known process of intercalation seen in the regenerative process. As explained in the recent review by Nacu and Tanaka (2011), the results of regenerative studies indicate that the major molecular

players for the patterning of the limb during development are usually reestablished during limb regeneration. But Gardiner et al. (1995) proposed that the initial expression of *hoxa* genes occurs in a different order in regeneration when compared with development. Whole-mount *in situ* hybridization of axolotl *hoxa9* and *hoxa13* showed simultaneous, early expression within 24 hr after amputation (Gardiner et al., 1995). In later stages of regeneration, the *hoxa9* and *hoxa13* domains segregated into PxDs nested domains within the blastema, reminiscent of development. These data were interpreted to confirm that, unlike development, some distal structures appear first than more proximal structures in regeneration, with subsequent intercalation of intermediate values. What can be said about the results of the present study is that in general the muscles of each region (arm, forearm and hand) form in a proximo-distal gradient as stated above. But some of the structures of a more distal region started to form before the structures of the more proximal region were fully formed (e.g., the long forearm flexors and extensors started to form well before the arm muscles were fully formed [e.g., Figs. 1B,C, 2A,C, and 4A]. Also, some hand muscle masses started to form before the forearm muscles were fully formed [e.g., Figs. 1A,C]).

The frequent presence of an anomalous, fleshy coracodialis at the level of the arm in the regenerated forelimbs (see above and e.g. Figs. 4A,B; cf. with Fig. 4C) might actually be due to another notable difference between the ontogenetic and regenerative processes. It is seemingly not due to transdifferentiation of ligament/tendon progenitors into myogenic cells as such transdifferentiation across cell lineages were shown to not take place during axolotl regeneration (Kragl et al., 2009). Thus what might be happening is that myogenic progenitors end up interacting with the coracodialis ligament/tendon during regeneration. This could happen in two ways: (1) during early stages of regeneration the myogenic progenitors end up in the prospective region of the coracodialis; or (2) during later stage of regeneration, the mature or differentiating muscle fibers from the coracobrachialis or the humeroantibrachialis are attracted to or attract the regenerating coracodialis tendon. In support of the first hypothesis, there is evidence that during development, tendon progenitors, marked by the expression of the *scleraxis* (*scx*) gene, and myogenic progenitors, marked by *pax3* expression, are located in overlapping regions of the limb bud (Schweitzer et al., 2001). Also, early patterning of tendon and myogenic progenitors is achieved by similar molecules, such as *Tbx5* and *BMPs* (Hasson et al., 2010, Schweitzer et al., 2001). Thus it is possible that during early stages of regeneration myogenic progenitors arising from the coracobrachialis or humeroantibrachialis read out the tendon patterning cues of the coracodialis as myogenic patterning cues and thus end up associating with the coracodialis. In support of the second hypothesis, it was shown that in *Drosophila* muscle development, cells at muscle attachment sites will secrete *Slit* which acts through *Robo* receptors to cause muscle cell migration to the muscle attachment sites (Kramer et al., 2001). A molecular mechanism by which tendon cells attract muscle fibers could exist in vertebrates, too. Indeed, it seems that at later stage of development interaction between differentiating muscle fibers and differentiating tendons

is crucial for their survival (Kardon, 1998). It was also shown that muscles express *Fgf4* at their extremities, in the regions where the tendons will attach (Edom-Vovard et al., 2002). In turn *Fgf4* has been shown to promote *Egr1* and *Egr2* expression in tendon progenitors and drive their further differentiation (Lejard et al., 2010). The second hypothesis would therefore explain why in some of the regenerated forelimbs examined by us and by authors such as Piatt (1957) those muscle fibers associated with the coracodialis tendon seem to be also closely associated with the humeroantibrachialis (e.g., Fig. 4A).

Evolutionary Implications

The detailed study of the muscle anomalies found in the regenerated axolotl limbs examined has implications for the understanding of the musculature of tetrapods and of morphological macroevolution in general. This is because if at least part of the amniote biceps brachii corresponds to the amphibian coracodialis (see Diogo and Tanaka, 2012), this would mean that at some point in evolution the tendinous part of the coracodialis of amphibians that lies in the forearm and arm regions had to be/become associated with fleshy fibers (the biceps brachii of amniotes usually has fleshy fibers at these regions). As Alberch (1989) has shown, there is often a parallel between the anatomical anomalies/variations occurring in biological organisms (naturally, or after experimentation) and the normal phenotype found in their closest relatives. That is, evolution is highly constrained and similar phenotypes are therefore often created (see also Diogo and Wood, 2012). In this line of thought, the occurrence of an abnormal regeneration of fleshy fibers associated with the tendinous portion of the coracodialis that lies at the level of the arm in urodeles makes it more feasible to envisage that this portion of the coracodialis corresponds to at least part of the fleshy biceps brachii of amniotes. Alberch's ideas are also corroborated by the analysis of other muscle anomalies displayed by the regenerated axolotl limbs examined by us, which are effectively found in at least some non-urodele living taxa. For instance, most non-urodele taxa, and even some urodeles, have an anconeus, while a coracobrachialis longus not extending to the elbow joint and/or antibrachium is actually the most common configuration found in tetrapods, as can be deduced by the name of this structure (Diogo and Tanaka, 2012).

CONCLUSIONS

One of the most relevant and surprising results of the present study was the high percentage (43%) of regenerated forelimbs showing muscular anomalies. Such a high percentage contradicts the idea that the axolotl forelimbs that are regenerated after amputation almost always display a muscle configuration that is similar to that of the original limbs. Recent studies have also reinvestigated the gross anatomy of the regenerated muscles of parts of other animals that are said to function in a normal way after regeneration, e.g., the tail of lizards, and have also concluded that when one looks to the fine details the configuration of these muscles is clearly not normal (e.g., Ritzman et al., 2012).

Interestingly, the average of anomalies per total number of muscles was greater in the examined forelimbs that suffered from natural bites (3.9%) than in the 10 regenerated forelimbs of CRTD AM11-20 (1.9%) and in the five regenerated forelimbs of CRTD 5, 9, and 10 (1.1%). This could be due to the recurrent aggressions provoked by different animals and affecting different regions of the forelimb, compared with an experimental setting where a specimen is subject to a single amputation in a specific region of the limb.

Importantly, apart from the radioulnar and proximo-distal morphogenetic gradients described in previous regenerative studies of axolotls, the present study indicates that there is also a marked ventro-dorsal gradient during the regeneration of at least the forearm muscles.

This study stresses that the detailed study of the muscle anomalies found in the regenerated axolotl limbs has implications for the understanding of the musculature of tetrapods and of morphological macroevolution in general, including the origin and evolution of the amniote forelimb muscles.

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