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Development of Fore- and Hindlimb Muscles in GFP-Transgenic Axolotls: Morphogenesis, the Tetrapod Bauplan, and New Insights on the Forelimb-Hindlimb Enigma



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ABSTRACT

The axolotl is becoming one of the most used model organisms in developmental and regenerative studies but no publication has described in detail the development of its forelimb and hindlimb muscles. We describe and illustrate the ontogeny of these muscles in transgenic axolotls that express GFP in muscle fibers and discuss our results and data previously obtained by us and by other authors about limb regeneration in axolotls and ontogeny in frogs and other tetrapods. Our observations and comparisons: (1) demonstrate radio-ulnar and ventro-dorsal morphogenetic gradients in the order of axolotl forelimb muscle formation and differentiation, while in axolotl hindlimb ontogeny there are only proximo-distal and tibio-fibular gradients; some of the axolotl gradients are therefore different from the ulno-radial/fibulo-tibial fore- and hindlimb and the dorso-ventral hindlimb ontogenetic morphogenetic gradients seen in frogs and amniotes such as chickens; (2) provide a potential explanation for the usual presence, in both limbs of taxa from all major tetrapod groups, of more radial/tibial muscles than ulnar/fibular muscles; (3) support the "in-out" developmental mechanism of appendicular muscle formation; (4) offer new insights about the ancestral Bauplan of tetrapod limbs, including the striking similarity of the zeugopodial (forearm/leg) and autopodial (hand/foot) muscles of the two limbs and the ventro-dorsal symmetry of the zeugopodial muscles of a same limb; and (5) provide further evidence to corroborate the hypothesis that these similarities are due to derived homoplastic events that occurred during the fins-limbs transition and not due to forelimb-hindlimb serial homology. *J. Exp. Zool. (Mol. Dev. Evol.)* 9999B: XX-XX, 2013. © 2013 Wiley Periodicals, Inc.

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The neotenuous axolotl *Ambystoma mexicanum* (Amphibia: Urodela) is becoming one of the most used model organisms in evolutionary, developmental, and regenerative studies, being a particularly powerful regenerative model (e.g., Carlson, 2007; Kragl et al., 2009; Nacu and Tanaka, 2011; Stocum and Cameron, 2011). However, there is not a single paper or monograph that describes the development of the fore- and hindlimb muscles of this species. The very few studies providing some details about the development of axolotl limb muscles refer only to a specific group of muscles, such as Grim and Carlson ('74a,b) ontogenetic

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work of the forearm and hand muscles or Boisvert et al.'s (2013) study on the ontogeny of the pelvic muscles.

The present paper provides a detailed morphological investigation of the ontogeny of the axolotl pectoral, arm, forearm, hand, pelvic, thigh, leg, and foot muscles, which is based on analyses of transgenic animals that express GFP in muscle fibers (Figs. 1–12; see Materials and Methods section). Diogo and Tanaka (2012) and Diogo et al. (Diogo et al., in press, submitted for publication) recently explained the major advantages of including GFP-transgenic animals in a study such as the present one. For instance, 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.

The main goals of the present publication are therefore: (1) to set the basis for future regenerative, developmental, and morphogenetic studies on the limbs of axolotls and other tetrapods; (2) to analyze the topological and morphogenetic sequence of axolotl limb muscle ontogeny, that is, which muscles develop first and how are they related to each other three-dimensionally (e.g., is there a tibio-fibular, a proximo-distal, and/or a ventro-dorsal gradient?); (3) to discuss, based on comparisons with the data provided in the literature and obtained in our recent studies of axolotl limb regeneration (Diogo et al., in press, submitted for publication) and frog limb development (Diogo and Ziermann, in press), broad developmental and evolutionary topics such as the evolution and morphogenesis of tetrapod limbs and the supposed serial homology between the fore- and hindlimbs.

MATERIALS AND METHODS

A total of 104 hindlimbs and 104 forelimbs of *Ambystoma mexicanum* were examined for this study. Except two adult wildtype specimens (HU AM1, right hindlimb and forelimb examined; HU AM2, right hindlimb and forelimb examined) dissected at the Anatomy Department of Howard University, all the *A. mexicanum* specimens analyzed were from the CRTD (Center for Regenerative Therapies Dresden). These included 51 transgenic animals that express GFP in muscle fibers (under the Cardiac-Alpha-Actin promoter, described by Khattak et al. (2013): CRTD AM11-20, adults, four limbs of each animal examined during 1 day; CRTD AM10, 3 months post-fertilization, four limbs examined during 1 day; CRTD AM110-124, 1.5-months post-fertilization, four limbs of each animal examined by live imaging every day during 1.5 months; CRTD AM125, 10 cm total length when the analyses of the four limbs of this animal were done; CRTD AM126-139, 0.5 months post-fertilization, four limbs of each animal examined by live imaging every day during 1.5 months). No animal was purposely sacrificed for this anatomical

study: 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 experiments conformed to the relevant regulatory standards. 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 1× zoom objective, the GFP fluorescence being excited with the 488 nm laser line and fluorescence being 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). Staging followed Bordzilovskaya et al. ('89) and Nye et al. (2003).

The nomenclature of the muscles follows that of Diogo and Abdala (2010), Diogo and Tanaka (2012), and Diogo (in press), which takes into account the evolution and homologies of the forelimb and hindlimb muscles of all the major tetrapod groups. In cases in which a synonym has been commonly used in the amphibian literature by other authors, that synonym will be given in the description of the respective muscle, in the Results section. When we refer to the anterior, posterior, dorsal and ventral regions of the body, we therefore used the terminology 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). Tables 1–4 summarize the “topological correspondences” between the forelimb and hindlimb, and specifically the leg versus forearm and foot versus hand, muscles of axolotls, following Diogo et al. (2013) and Diogo (in press) (no apparent “topological correspondences” were found in the pelvic and thigh versus pectoral and arm muscles: see Discussion section). The methodology used to build these tables was described in detail in Diogo et al. (2013), and basically uses the criteria that have been used historically to search for the “correspondence” between body parts of an organism, that is, “positional or topographical similarity” sensu Owen (1866) or “morphological congruence” sensu Shubin and Alberch ('86). That is, each muscle (distal attachments, proximal attachments, number of divisions, relations to other hard and soft-tissue structures, orientation of fibers, and number of crossed joints) was analyzed in order to investigate which hindlimb muscles shown in Tables 1–4 have a clear, direct (one to one) “topological equivalent” in the forelimb of the same taxa (for more details, see Diogo et al., 2013; Diogo, in press).

RESULTS

The results of our analyses are shown in detail in Figures 1–12 and Tables 1–4; therefore in this section we will provide a brief textual description of these results, which is divided into eight major subsections: pectoral/arm muscles, ventral forearm muscles, dorsal forearm muscles, hand muscles, pelvic/thigh muscles, ventral leg muscles, dorsal leg muscles, and foot muscles. It should

be noted that we did not find any significant differences concerning the order in which the different muscles develop and differentiate or regarding the specific insertion and origin of the muscles, among the forelimbs and hindlimbs analyzed. For each subsection, we provide first a short description of the attachments of the hindlimb and forelimb muscles of adults, followed by a description of the ontogeny of the muscles. The forelimb muscles of adult axolotls were described and shown in detail by Diogo and Tanaka (2012); therefore the descriptions of the adult forelimb muscles provided here are simplified from those given in that paper. Regarding the hindlimb muscles of adult axolotls, this is the first paper to describe in detail the muscles of the pelvis/thigh, leg, and foot.

Pectoral and Arm Muscles

The pectoral and arm muscles seen in the pictures obtained from imaging the developing axolotls we analyzed are the procoracohumeralis, pectoralis, coracobrachialis, supracoracoideus, humeroantibrachialis, latissimus dorsi, deltoideus scapularis, and triceps brachii. All these muscles are appendicular muscles sensu Diogo and Abdala (2010). That is, none of the two axial pectoral muscles sensu Diogo and Abdala (i.e., serratus anterior and levator scapulae; Table 1) could be clearly seen in the forelimb developmental stages examined, that is, from stage 46 to stage 54. This is likely because the axial pectoral muscles are often deep muscles superficially covered by the appendicular pectoral muscles (Fig. 1; see also e.g., Diogo and Abdala, 2010; Valasek et al., 2011) and not due to the absence of all the axial pectoral muscles in these stages of development (e.g., these muscles appear early in the ontogeny of the amphibian urodele *Necturus*; see, e.g., Chen, '35; see Discussion section). This idea is also supported by the fact that the muscle subcoracoscapularis, which is an appendicular but very deep pectoral muscle, could not be seen in the pictures of the developing axolotls obtained by us.

In adults, the procoracohumeralis, supracoracoideus, coracoradialis, and pectoralis cover the ventral surface of the pectoral region, the procoracohumeralis and pectoralis being respectively the most anterior and posterior structures within these muscles (Fig. 1). The pectoralis (Fig. 1) originates from the coracoid near the midline and from the rectus abdominis and sternum and inserts onto the proximal portion of the humerus. The supracoracoideus (or "coracohumeralis"; Fig. 1) runs from the superficial ventral surface of the coracoid to the proximal portion of humerus. The procoracohumeralis (or "procoracohumeralis longus"; Fig. 1) runs from the procoracoid cartilage to the proximal portion of the humerus. The coracoradialis (or "sterno-radialis") has a well developed fleshy belly that originates from the ventral surface of the coracoid deep (dorsal) to the superficial (ventral) layers of the pectoralis and supracoracoideus and that gives rise to a long and thin tendon that inserts onto the proximal portion of the radius. The humeroantibrachialis and coracobrachialis lie respectively on the radial and ulnar sides of the ventral surface of the arm (Figs. 1

Table 1. Scheme illustrating the "topological correspondences" between the pelvic/thigh and pectoral/arm muscles of axolotls (modified from Diogo et al., 2013; Diogo, in press; see text).

Pectoral/arm and pelvic/thigh muscles

Iliofemoralis

Extensor ilirotibialis

Tenuissimus

Puboischiofemoralis internus

Adductor femoris

Pubotibialis

Gracilis

Femorofibularis

Ischioflexorius

Caudofemoralis

Puboischiofemoralis externus

Ischiotrochantericus

[Serratus anterior]

[Levator scapulae]

[Pectoralis]

[Supracoracoideus]

[Deltoideus scapularis]

[Procoracohumeralis]

[Subcoracoscapularis]

[Latissimus dorsi]

[Triceps brachii]

[Humeroantibrachialis]

(Continued)

Table 1. (Continued)

Pectoral/arm and pelvic/thigh muscles

[Coracoradialis]

[Coracobrachialis]

As can be seen in this table, none of the pelvic/thigh muscles/muscle seems to "correspond topologically" directly to forelimb muscles/muscle groups (the forelimb muscle/muscle groups are shown between square brackets).

and 2). The humeroantibrachialis (or "brachialis"; Figs. 1 and 2) connects the proximal portions of the humerus and radius. The coracobrachialis is differentiated into two bundles that originate from the posteroventral and posterolateral margin of the coracoid:

the coracobrachialis longus inserts onto the distal humerus and the elbow joint, while the coracobrachialis brevis inserts onto the medial side of approximately the proximal 1/3 of the humerus. The deltoideus scapularis and latissimus dorsi lie on the dorsal side of the pectoral region, while the triceps brachii lies mainly on the surface of the arm (Figs. 1 and 2). The deltoideus scapularis (or "dorsalis scapulae"; Fig. 1) extends from the lateral and dorsal surfaces of the suprascapular cartilage to the ventral margin of the proximal portion of the humerus. The latissimus dorsi (Fig. 1) is divided into a broader, posterior bundle and a smaller, anterior bundle, which run from the dorsal fascia and insert onto the proximal portion of the humerus. The triceps brachii (sometimes erroneously designated as "anconeus"; Figs. 1 and 2) is divided into four bundles, all being blended distally to insert onto the olecranon process of the ulna: the triceps coracoideus originates

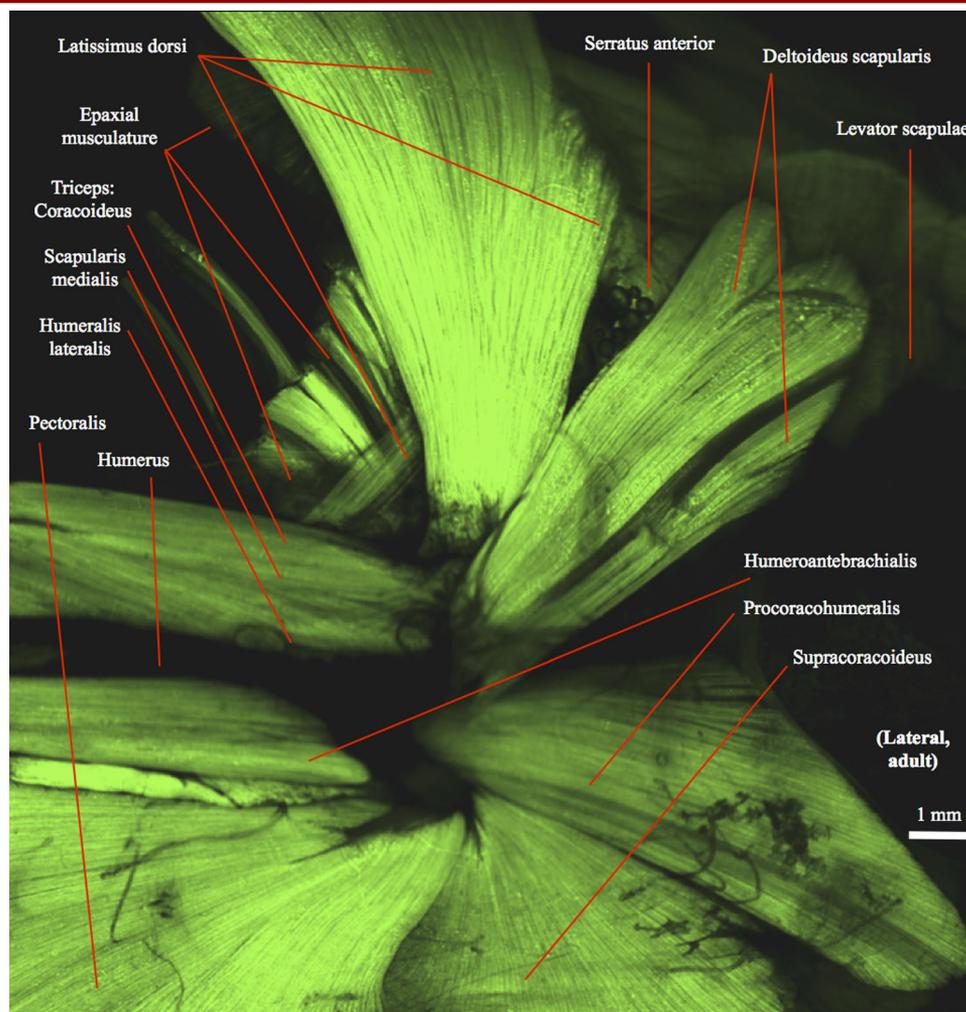
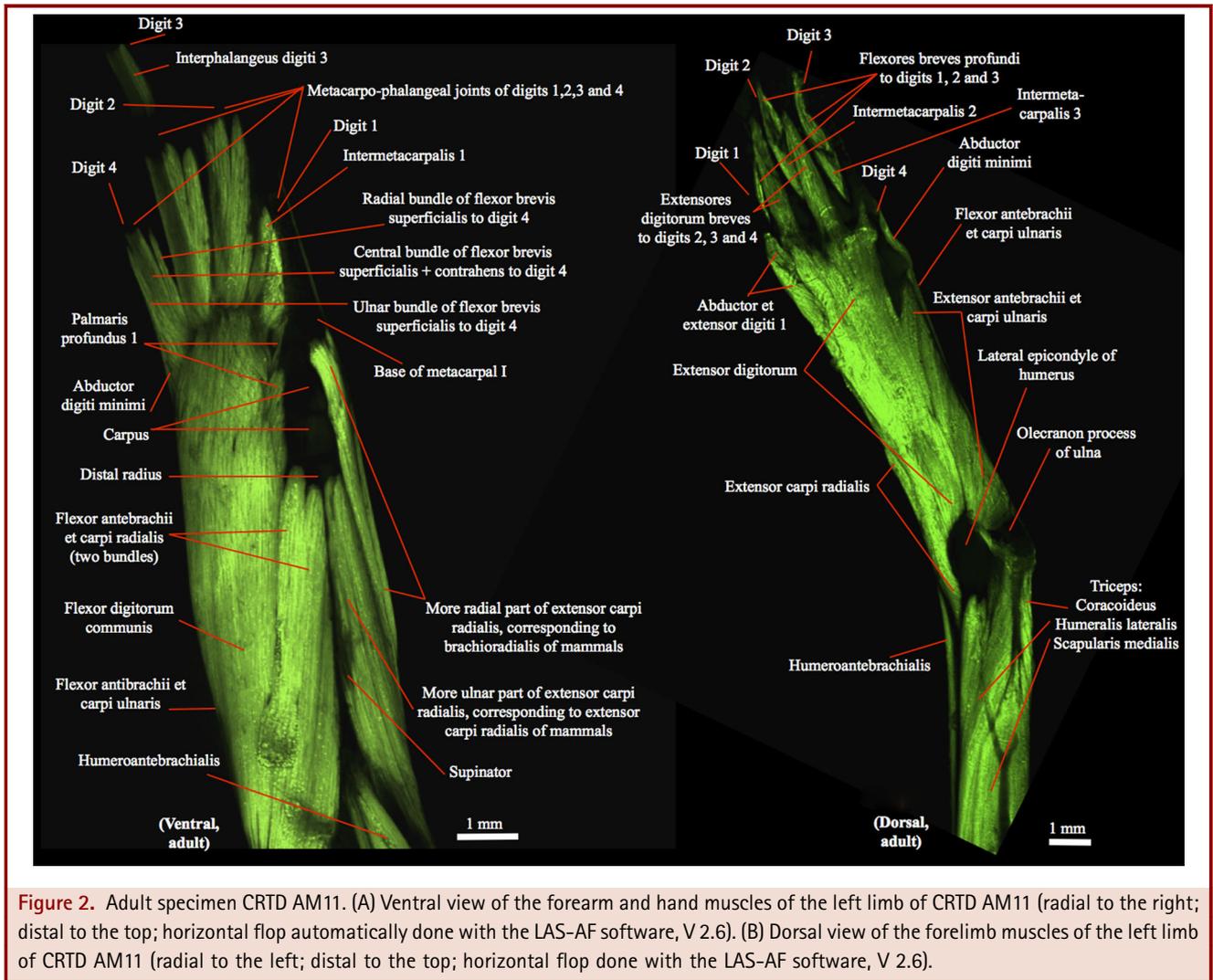


Figure 1. Adult specimen CRTD AM11. Lateral view of the left pectoral and arm muscles (anterior is to the right, dorsal to the top); the protractor pectoralis (not a pectoral nor a forelimb muscle) was removed (horizontal flop done with the LAS-AF software, V 2.6).



from a prominent posterior process of the coracoid and is medial to the triceps scapularis medialis, which originates from the scapula and from connective tissue surrounding the shoulder joint capsule; the triceps humeralis lateralis originates from the lateral surface of the proximal portion of the humerus, while the triceps humeralis medialis originates from about 1/3 of the proximo-distal length of the humerus.

Regarding the development of the pectoral and arm structures, the first appearance of muscles can be seen at stage early 46, in which the anlage of the pectoralis (and possibly the coracobrachialis) and the anlage of the supracoracoideus plus the coracoradialis and probably also of the procoracohumeralis are seen in a ventral view, while the anlage of the deltoideus scapularis plus the latissimus dorsi are seen in a dorsal view. Importantly, all these muscle anlages lie near the distal region of the arm. At stage mid 46 the pectoralis, coracobrachialis,

supracoracoideus + coracoradialis, procoracohumeralis, deltoideus scapularis, and latissimus dorsi are differentiated and more elongated proximo-distally (Fig. 3A). All these muscles become considerably broader at later stages (e.g., Figs. 3B,C and 4A), and, with exception of the coracobrachialis, they all extend proximally towards the midline of the body to cover a significant part of the dorsal and ventral sides of the thoracic region, as they do in adults (Fig. 1). The coracobrachialis brevis and coracobrachialis longus become differentiated at stage mid 47, that is, at the stage in which the humeroantebrachialis (Fig. 3C) and triceps brachii become clearly visible. At later stages these four arm structures extend distally (e.g., Fig. 4A,B), and the various bundles of the triceps brachii become clearly differentiated from stage late 47 (e.g., Fig. 4B); at stage 50 the overall configuration and attachments of these four muscles are similar to those seen in adults (Fig. 5A,B).

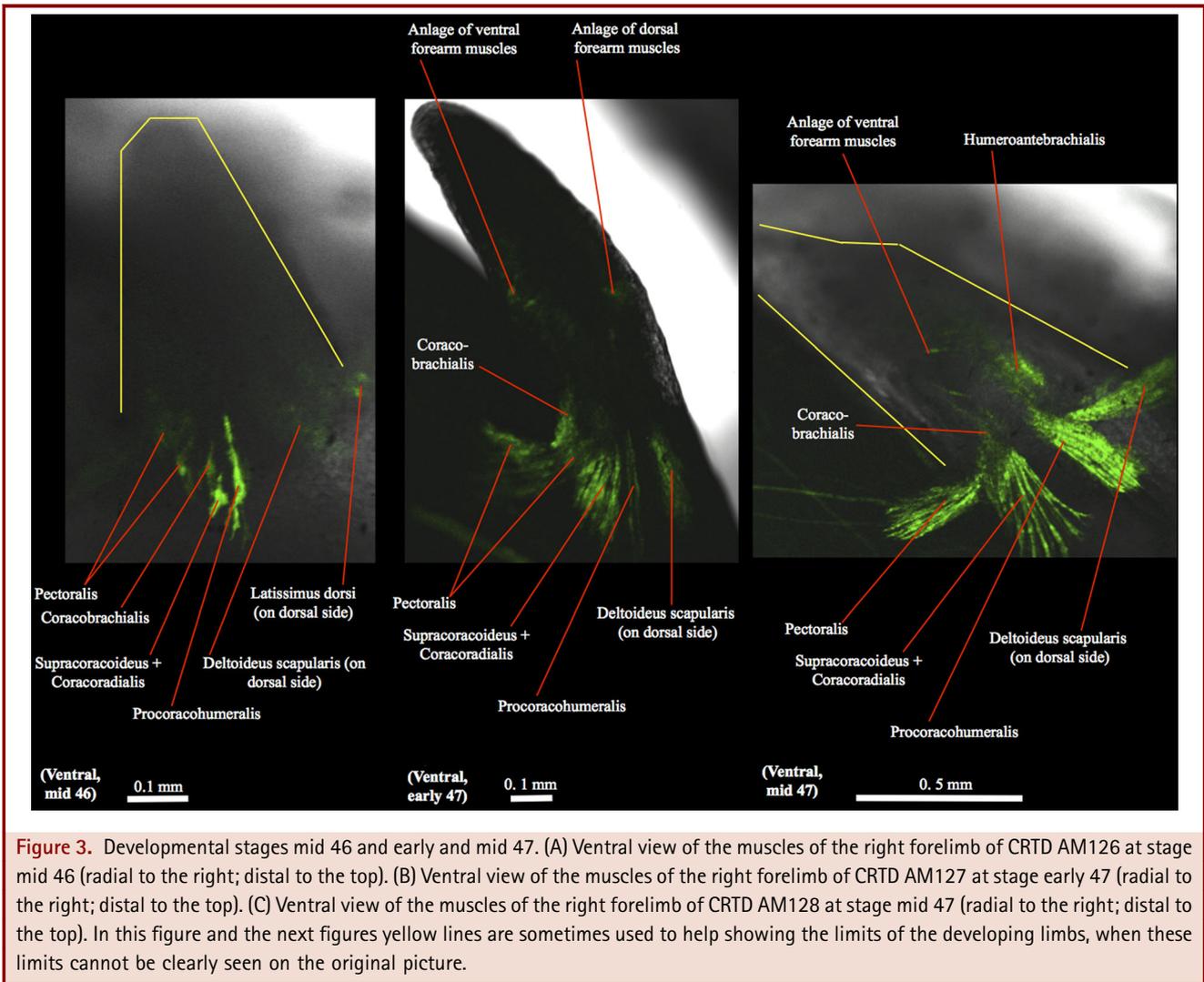


Figure 3. Developmental stages mid 46 and early and mid 47. (A) Ventral view of the muscles of the right forelimb of CRTD AM126 at stage mid 46 (radial to the right; distal to the top). (B) Ventral view of the muscles of the right forelimb of CRTD AM127 at stage early 47 (radial to the right; distal to the top). (C) Ventral view of the muscles of the right forelimb of CRTD AM128 at stage mid 47 (radial to the right; distal to the top). In this figure and the next figures yellow lines are sometimes used to help showing the limits of the developing limbs, when these limits cannot be clearly seen on the original picture.

Ventral/Flexor Forearm Muscles

In adults the palmaris profundus 1 (or “pronator profundus”; Fig. 2A) is deep to the contrahentium caput longum and runs from the ulna, intermedium and ulnare to the carpus and base of metacarpal I. The pronator quadratus (or “interosseous antebrachii”) lies deep to the palmaris profundus 1 and thus to the other ventral forearm muscles, running from the ulna to the radius. The contrahentium caput longum (or “ulnocarpalis”) lies deep (dorsal) to the flexor antebrachii et carpi ulnaris and to the flexor digitorum communis and originates from the proximal portion of the ulna and inserts onto the carpus through a tendon, being continuous with the contrahentes digitorum. The flexor accessorius lateralis (or “palmaris profundus 3”) extends from the distal portion of the ulna and the ulnare to the dorsal surface of the palmar fascia, passing superficially (ventrally) to the contrahentium caput longum. The flexor accessorius medialis (or

“palmaris profundus 2”) connects the distal portion of the ulna and the intermedium to the dorsal surface of the palmar fascia, originating deep (dorsal) to the contrahentium caput longum. The flexor digitorum communis (or “palmaris superficialis”; Fig. 2A) is the most superficial (ventral) muscle of the ventral forearm, originating from the medial epicondyle of the humerus, and sending a thin tendon to the distal phalanx of each of the four digits. The flexor antebrachii et carpi ulnaris (Fig. 2A) lies on the ulnar side of, and is partially covered ventrally by, the flexor digitorum communis, running from the medial epicondyle of the humerus to the ulna and ulnare. The flexor antebrachii et carpi radialis (Fig. 2A) is divided into a longer, more superficial (ventral), and proximo-distally oriented bundle that runs from the medial epicondyle of the humerus to the radius and radiale, and a shorter, deeper (dorsal), and more oblique bundle that runs from the medial epicondyle of the humerus to about half of the length of the radius,

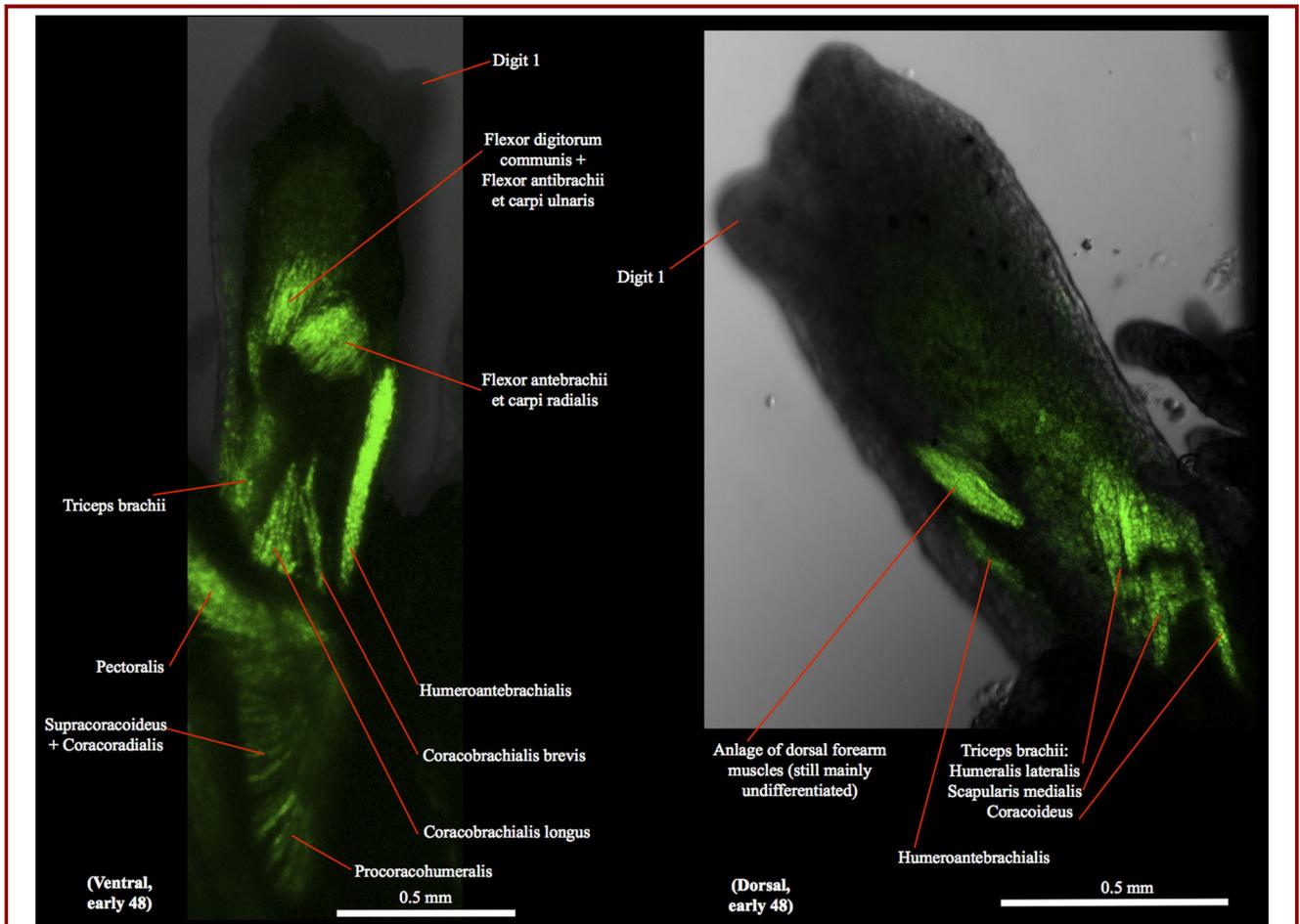


Figure 4. Developmental stage early 48, CRTD AM 129. Ventral (A: radial to the right; distal to the top) and dorsal (B: radial to the left; distal to the top) views of the muscles of the right forelimb.

but that instead of inserting onto the radius directly, is mainly associated distally with the longer bundle (Table 2).

Concerning development, the first appearance of an anlage—still undifferentiated—of ventral forearm muscles is at stage early 47 (Fig. 3B). At stage late 47 the flexor antibrachii et carpi radialis is a separate muscle, but at this stage and even at stage early 48 the flexor antibrachii et carpi ulnaris is apparently still undifferentiated from the anlage of the flexor digitorum communis or is just a small muscle covered ventrally by the latter muscle (Fig. 4A). At stage early 48 the flexor antibrachii et carpi radialis starts to be differentiated into the two heads that are found in adults (Fig. 4A; see also Figs. 5A, 6A, 7A, and 2A). The flexor antibrachii et carpi ulnaris, as well as the deeper muscles palmaris profundus 1, flexor accessorius lateralis, flexor accessorius medialis, and contra-hentium caput longum, are clearly visible at stage 50 (Fig. 5A; N.B., in the images of the developing forelimb, the deeper pronator quadratus could not be seen). At stage late 52 the overall

configuration and attachments of these muscles as well as of the other ventral forearm muscles are basically similar to those found in adults (Fig. 6A).

Dorsal/Extensor Forearm Muscles

In adults the extensor carpi radialis (Fig. 2B) is divided into two bundles: the more radial one runs from the distal 1/2 of the radial side of the humerus to the radial side of the distal radius, carpus, and base of metacarpal I; the more ulnar one runs from the lateral epicondyle of the humerus to the radial side of the distal radius and radiale. The supinator (or “extensor antibrachii radialis”) originates from the lateral epicondyle of the humerus and is distally separated from the extensor carpi radialis by the whole ventral side of the radius, because it inserts onto the medial surface of the distal 1/3 of the radius. The extensor antibrachii et carpi ulnaris (Fig. 2B) extends from the lateral epicondyle of the humerus to the ulna and ulnare. The extensor digitorum (or

Table 2. Scheme illustrating the “topological correspondences” between the ventral (flexor) leg and forearm muscles of axolotls (modified from Diogo et al., 2013; Diogo, in press; see text).

Long flexors

Flexor digitorum communis
[Flexor digitorum communis]
Flexor accessorius medialis
[Flexor accessorius medialis]
Flexor accessorius lateralis
[Flexor accessorius lateralis]
Contrahehium caput longum
[Contrahehium caput longum]
Interosseus cruris
[Pronator quadratus]
Tibialis posterior
[Palmaris profundus 1]

[Flexor antebrachii et carpi ulnaris]

[Flexor antebrachii et carpi radialis]

As can be seen in this table, all six ventral leg muscles/muscle groups seem to “correspond topologically” directly to forelimb muscles/muscle groups (the forelimb muscle/muscle groups are shown between square brackets). However, the ventral forearm muscles flexor antebrachii et carpi ulnaris and flexor antebrachii et carpi radialis have no clear “equivalents” in the hindlimb.

“humerodorsalis”/“humerometacarpalis”; Fig. 2B) is the most superficial (dorsal) of the dorsal forearm muscles, originating from the lateral epicondyle of the humerus and sending thin tendons to the metacarpals I, II, III, and IV. There are three extensores digitorum breves (Fig. 2B), which lie deep (ventral) to the main body of the extensor digitorum, originate from carpal bones, and send thin tendons to the distal phalanx of digits 2, 3, and 4. The abductor et extensor digiti 1 (or “supinator manus”; Fig. 2B) originates from carpal bones and from the distal portions of the radius and ulna, and has two bundles: the most radial one inserts onto the radial side of the base of metacarpal I and corresponds to the abductor part of this muscle; the most ulnar one sends a thin tendon to the distal phalanx of digit 1 and corresponds to the extensor breves of digit 1 (Table 3).

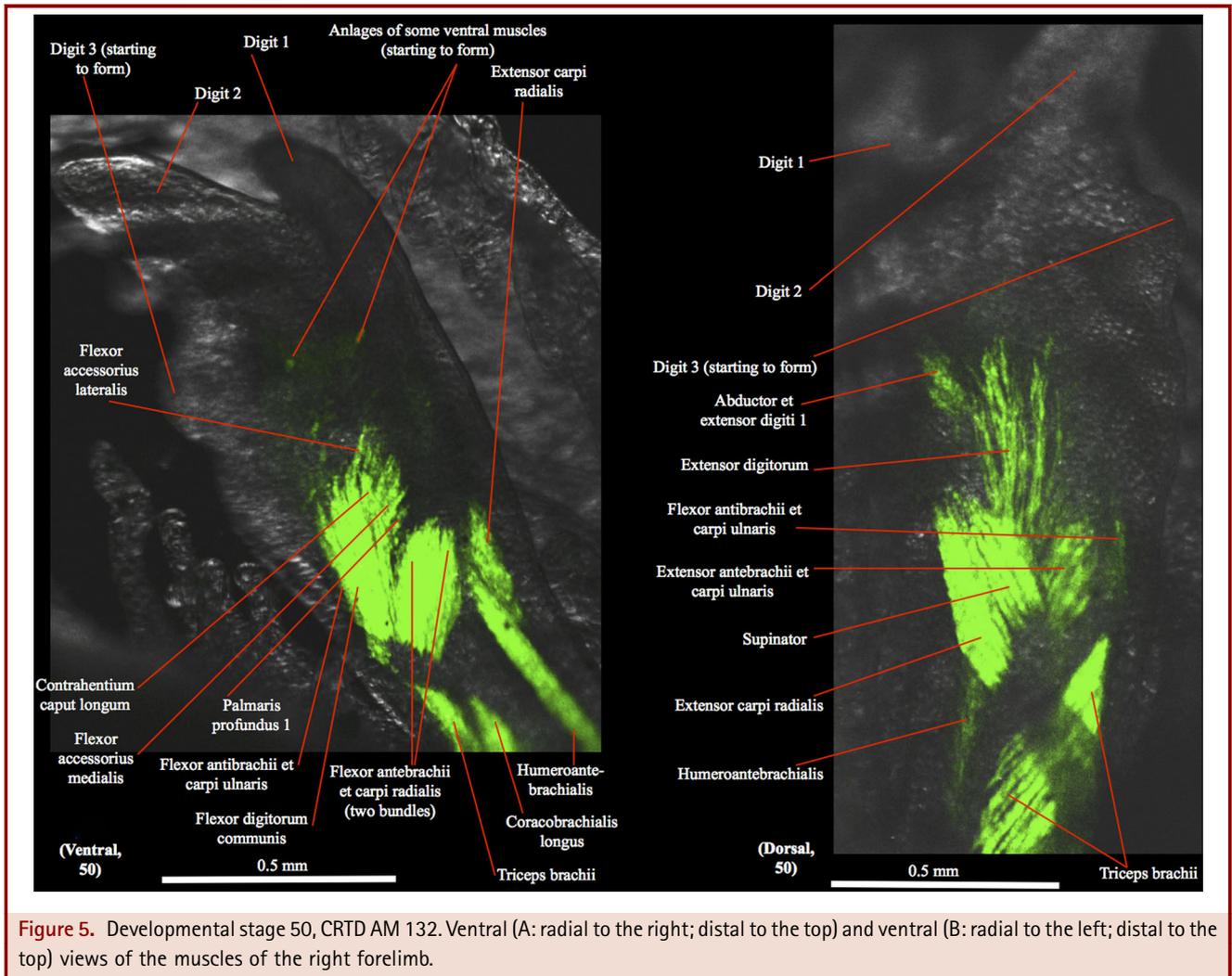
Concerning development, the first appearance of an anlage—still undifferentiated—of dorsal forearm muscles is at stage early 47 (Fig. 3B). During stages mid and late 47 and early 48 this anlage becomes broader but remains mainly undifferentiated (Fig. 4B). The abductor et extensor digit 1, extensor digitorum, supinator, extensor carpi radialis, and the extensor antebrachii et carpi ulnaris are present as separate muscles at stage 50 (Fig. 5B). At mid stage 52 the short extensor of digit 2 is present and the short extensor of digit 3 is starting to form, and one can

see the differentiation of the “brachioradialis” and “extensor carpi radialis” bundles of the extensor carpi radialis; the short extensor of digit 4 does not start to form until stage late 53 (Figs. 6B and 7B). At later stages the overall configuration and attachments of the dorsal forearm muscles are similar to those found in adults.

Hand Muscles

In adults the flexores breves superficiales (Fig. 2A) are the most ventral intrinsic muscles of the hand, running from the aponeurosis and tendons of the flexor digitorum communis and the palmar fascia to the metacarpophalangeal joint of each digit; all digits receive an ulnar, a radial, and a central bundle from these muscles, the latter bundle being blended with the contrahehium going to the same digit. The contrahehiumes digitorum (or “flexores breves medii”; Fig. 2A) constitute the second layer of intrinsic hand muscles and run from the carpus to the metacarpophalangeal joints of digits 1–4. The flexores breves profundi form the third layer, running from the carpals at the base of their respective digits to the distal portion of the metacarpals of digits 1–4, being mainly attached to the ulnar and radial sides of each metacarpal. The flexores digitorum minimi are two short, thin muscles that extend from the central part of the ventral surface of metacarpals I and IV to the metacarpophalangeal joints of digits 1 and 4, respectively, together with the flexores breves superficiales and the contrahehiumes digitorum. The interphalangeus digiti 3 (Fig. 2A) runs from the ventral margin of the base of the proximal phalanx of digit 3 to the proximal interphalangeal joint of this digit. The abductor digiti minimi (or “extensor lateralis digiti IV”; Fig. 2A) extends from the ulnare to the ulnar side of the base of metacarpal IV, being blended proximally to the tendon of the flexor carpi ulnaris and distally to the ulnar bundle of the flexor brevis profundus of digit 4. There are three intermetacarpales (Fig. 2A), which run distally and radially from the radial sides of metacarpals II–IV to the ulnar sides of metacarpals I–IV, respectively (Table 4).

During development, the first appearance of anlage—still undifferentiated—of hand muscles is seen at stage 50 (Fig. 5A). From stage mid 52 on, the intermetacarpalis 1 and the contrahehiumes and flexores breves superficiales of digits 1 and 2 are clearly visible, the intermetacarpalis 2 and the contrahehiumes and flexores breves superficiales of digit 3 being visible at stage late 52 (Fig. 6A). At stage early 53 the intermetacarpalis 3 and the anlage of the other muscles of digit 4 are starting to form. At stage late 53 the contrahehiumes and flexores breves superficiales of digit 4 are differentiated and elongated, as is the abductor digiti minimi (Fig. 7A), and the interphalangeus of digit 3 is just starting to form. The flexores breves profundi are clearly visible at stage early 54 and the interphalangeus digit 3 becomes more elongated proximodistally and from stage 55 on the configuration of the hand muscles is essentially similar to that seen in adults (N.B., the deep flexores digitorum minimi could not be seen in the images of the developing forelimb obtained by us).



Pelvic and Thigh Muscles

The ventral pelvic/thigh muscles that were visible in the images of the developing hindlimbs obtained by us are the pubotibialis, femorofibularis, gracilis, ischioflexorius, and caudofemoralis. Within these muscles, the gracilis (or “puboischiotibialis”) is the most ventral thigh muscle in adults (Fig. 8A). This muscle has proximal and distal heads (only the latter head can be seen in Fig. 8A) and runs from the ventral midline of the puboischiac plate to the proximal two-thirds of the anteromedial face of the tibia. On the fibular side of this muscle lies the ischioflexorius (Fig. 8A), which runs from the posterolateral corner of the puboischiac plate to the plantar aponeurosis and is divided into proximal and distal portions separated by a tendinous plate at the level of one-third to one-half the distance between the origin and insertion of the muscle. On the tibial side of the gracilis lies the pubotibialis (Fig. 8A), which connects the anterolateral border of the

puboischiac plate to the proximal tibia. The femorofibularis lies on the deep ventral side of the thigh and runs from the posteroventral border of the femur, at a point approximately half-way to the knee, to the posterolateral border of the fibula between the insertion of the extensor cruris tibialis and the fibular portion of origin of the flexor digitorum communis. The caudofemoralis runs from the caudal vertebrae to the femur (Fig. 8B). The dorsal pelvic/thigh muscles that were visible in the images of the developing hindlimbs obtained by us are the extensor iliotibialis, tenuissimus, and puboischiofemoralis internus. Within these muscles, the extensor iliotibialis (or “iliotibialis”) is the most dorsal thigh muscle in adults (Fig. 8B). This muscle is divided into anterior (“iliotibialis”) and posterior (“ilioextensorius”) heads and runs from the ilium to a wide tendon inserting onto the crista tibialis and blending with the distal portions of the extensor cruris tibialis and the extensor digitorum longus. On the

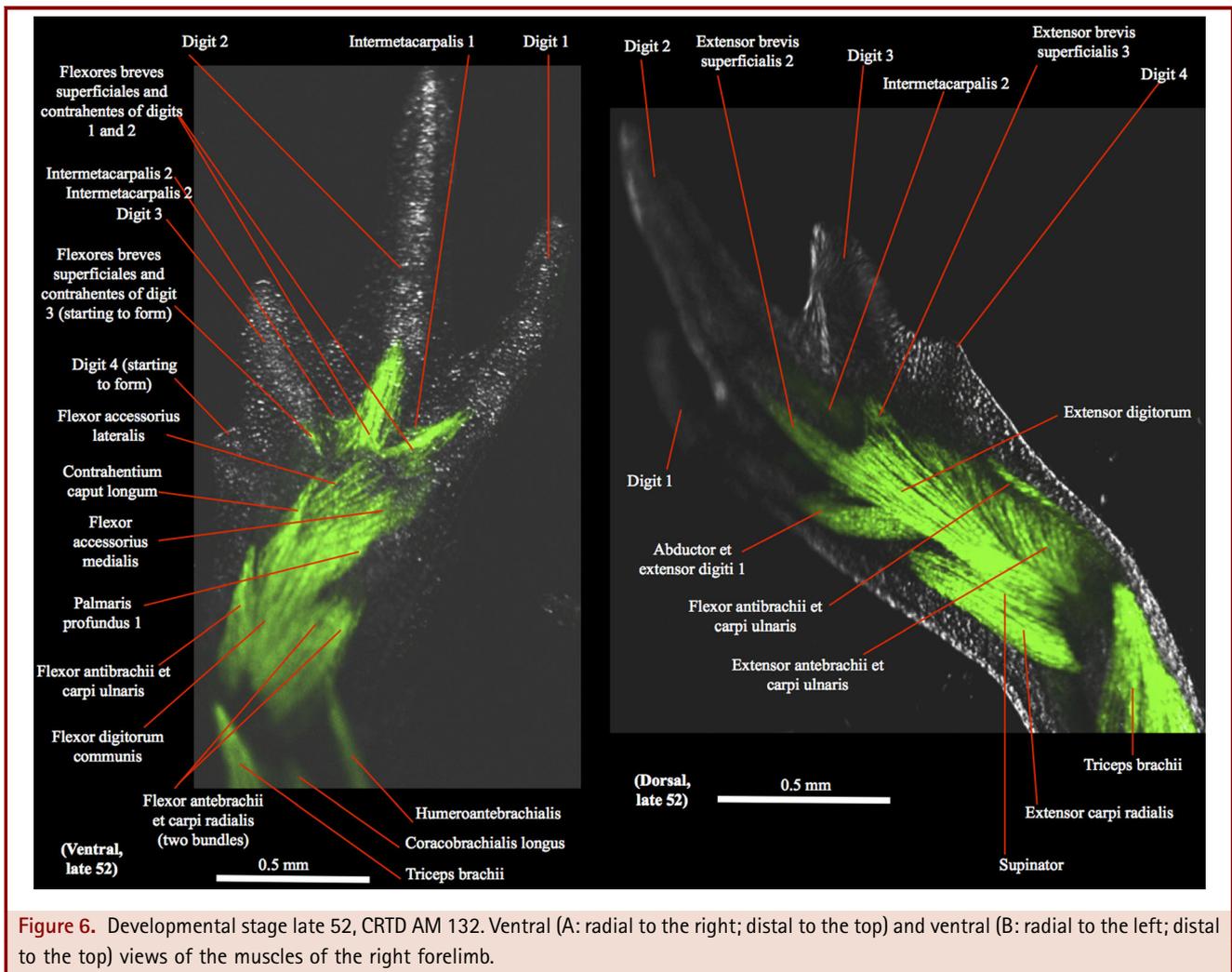


Figure 6. Developmental stage late 52, CRTD AM 132. Ventral (A: radial to the right; distal to the top) and ventral (B: radial to the left; distal to the top) views of the muscles of the right forelimb.

fibular side of the extensor iliobtibialis lies the tenuissimus (often also designated as “iliofibularis”) (Fig. 8B), which runs from the tendon of the extensor iliobtibialis to the posterior border of the fibula. On the tibial side of the extensor iliobtibialis lies the puboischiofemorialis internus (Fig. 8B), which connects the anterior portion of the dorsal midline of the pubo-ischiac plate, ypsiloid cartilage, and pubis to the femur (Table 1).

During development of the hindlimb, the first appearance of pelvic and thigh muscles is seen at stage early 54, in which the caudofemoralis and the dorsal muscles tenuissimus, extensor iliobtibialis (seemingly with only one head), and puboischiofemorialis internus are present but still very small. At stage mid 54 the two heads of the iliobtibialis seem to be differentiated, and at stage early 55 all these three dorsal muscles become more elongated (Fig. 9A,C). Regarding the ventral pelvic/thigh muscles, at stage mid 54 at least one anlage of these muscles was present, but it is

difficult to discern if some muscles are already differentiated or not. At stage late 55 the ventral muscles ischioflexorius, gracilis and pubotibialis are clearly differentiated (Fig. 9B). At stage late 56 the configuration and attachments of all the analyzed pelvic/thigh muscles, including the femorofibularis, are basically similar to those found in adults (Fig. 11A,B).

Ventral/Flexor Leg Muscles

The ventral (flexor) leg muscles found in adults are the flexor digitorum communis, flexor accessorius medialis, flexor accessorius lateralis, contrahentium caput longum, interosseus cruris, and tibialis posterior. The most ventral of these muscles is the flexor digitorum communis (Fig. 8A), which originates from the fibular condyle of the femur and sends a broad tendon to the distal phalanges of digits 1–5. The deeper ventral leg muscles can be seen in Figure 8A because this broad tendon appears as transparent in

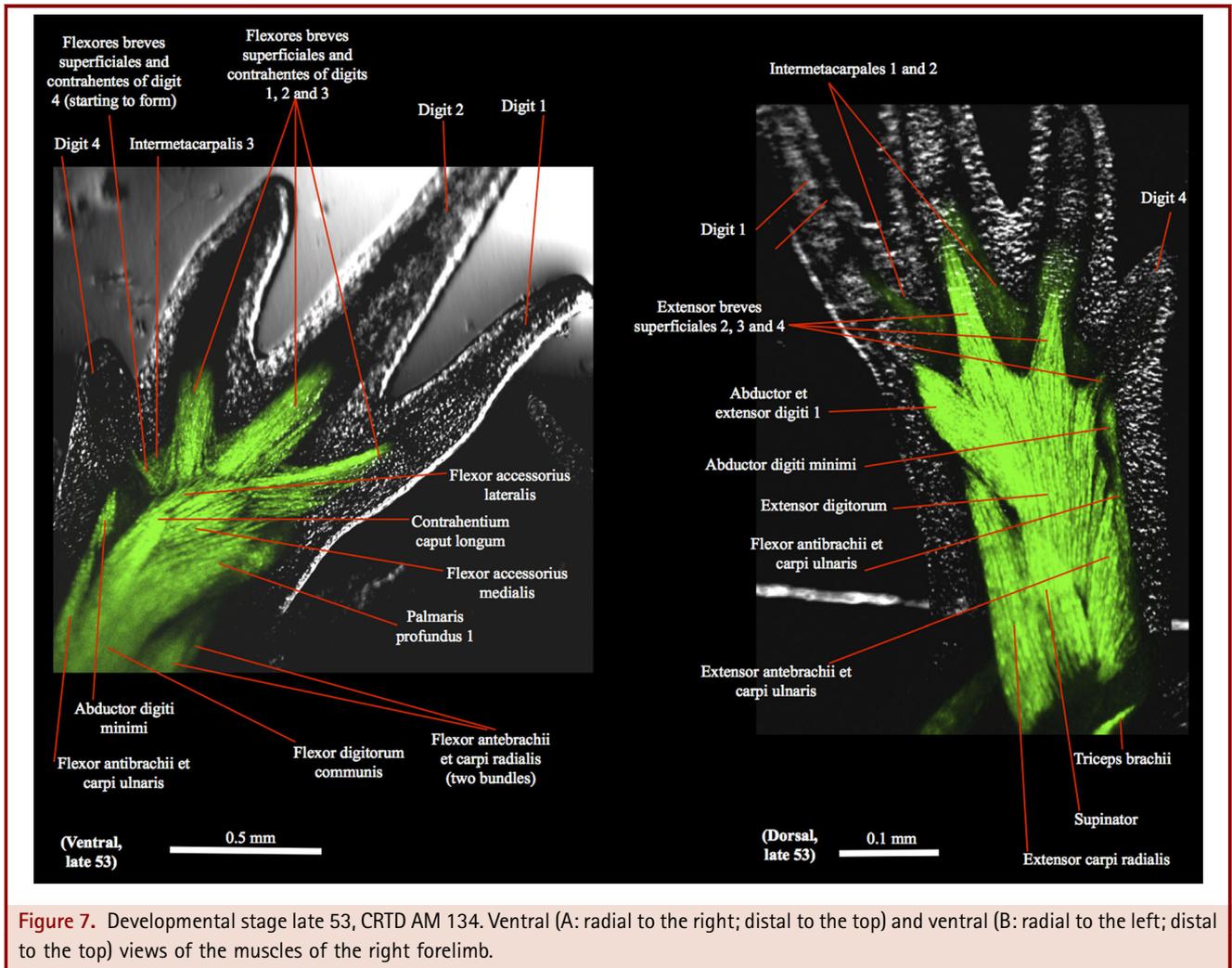


Figure 7. Developmental stage late 53, CRTD AM 134. Ventral (A: radial to the right; distal to the top) and dorsal (B: radial to the left; distal to the top) views of the muscles of the right forelimb.

the figure. The tibialis posterior (or “pronator profundus”) is the most tibial of these deeper muscles (Fig. 8A), running from the medial part of the fibula to the distal portion of the tibia, the tibiale and the base of metatarsal I. On the fibular side of the tibialis posterior lies the flexor accessorius medialis (Fig. 8A), which runs from the distal region of the fibula, the fibulare, and intermedium to the plantar fascia, then the contrahentium caput longum (Fig. 8A), which lies deep to the plantar fascia and connects the distal portion of the fibula to the distal tarsal bones and the contrahentes, and then the flexor accessorius lateralis (Fig. 8A), which runs from the fibulare to the plantar fascia. The interosseus cruris is the deeper ventral leg muscle, running from the proximal part of the fibula to the distal portion of the tibia (Table 2).

Regarding development, the first appearance of ventral leg muscles is seen at stage late 55, in which the flexor digitorum communis is present (Fig. 9B). At stage early 56 one can see, deep

(dorsally) to the flexor digitorum communis, a well developed muscle anlage that does not seem differentiated yet into the flexor accessorius lateralis, flexor accessorius medialis, contrahentium caput longum, interosseus cruris, and tibialis posterior muscles. These deep muscles become differentiated at stage mid 56 (Fig. 10A), and at stage late 56 the overall configuration and attachments of the ventral leg muscles are essentially similar to those found in adults (Fig. 11A).

Dorsal/Extensor Leg Muscles

The dorsal (extensor) leg muscles found in adults are the extensor digitorum longus, extensor tarsi tibialis, extensor cruris tibialis, extensor cruris et tarsi fibularis, extensores digitorum breves, and abductor et extensor digiti 1. The most dorsal of these muscles is the extensor digitorum longus (or “extensor digitorum communis”) (Fig. 8B), which runs from the femoral condyles to the

Table 3. Scheme illustrating the “topological correspondences” between the dorsal (extensor) leg and forearm muscles of axolotls (modified from Diogo et al., 2013; Diogo, in press; see text).

Long extensors

Extensor digitorum longus
[Extensor digitorum]
Extensor tarsi tibialis
[Extensor carpi radialis]
Extensor cruris tibialis
[Supinator]
Extensor cruris et tarsi fibularis
[Extensor antebrachii et carpi ulnaris]

Short extensors

Extensores digitorum breves
[Extensores digitorum breves]
Abductor et extensor digiti 1
[Abductor et extensor digiti 1]

As can be seen in this table, all six dorsal leg muscles/muscle groups seem to “correspond topologically” directly to forelimb muscles/muscle groups (the forelimb muscle/muscle groups are shown between square brackets).

proximal end of metatarsals I–V. On the tibial side of this muscle lies the extensor cruris tibialis (Fig. 8B), running from the tibial epicondyle of the femur to the anteroventral and anterodorsal margins of the tibia, and then the extensor tarsi tibialis (Fig. 8B), running from the tibial epicondyle condyle of the femur to the

Table 4. Scheme illustrating the “topological correspondences” between the foot and hand muscles of axolotls (modified from Diogo et al., 2013; Diogo, in press; see text).

Intrinsic foot muscles

Flexores breves superficiales
[Flexores breves superficiales]
Abductor digiti minimi
[Abductor digiti minimi]
Contraheentes pedis
[Contraheentes digitorum]
Flexores digitorum minimi
[Flexores digitorum minimi]
Interphalangei
[Interphalangei]
Flexores breves profundi
[Flexores breves profundi]
Intermetatarsales
[Intermetacarpales]

As can be seen in this table, all seven foot muscles/muscle groups seem to “correspond topologically” directly to forelimb muscles/muscle groups (the forelimb muscle/muscle groups are shown between square brackets).

tibiale bone. On the fibular side of the extensor digitorum longus lies the extensor cruris et tarsi fibularis (Fig. 8B), running from the femoral condyles to the posterodorsal face of the fibula (the posterior part of the muscle) and the fibulare (the anterior part of the muscle). Distal to these four muscles lie the four extensores digitorum breves (Fig. 8B), which connect the distal tarsal bones to the dorsal surface of the proximal end of the distal phalanx of digits 2–5 through a long tendon. The extensor digitorum brevis of digit 1 is fused with an abductor muscle to form the abductor et extensor digiti 1 muscle (Fig. 8B), which runs from the distal tarsal bones to the metatarsal I and distal phalanx of digit 1 (Table 3).

With respect to development, the first appearance of dorsal leg muscles is at stage early 55, in which the extensor digitorum longus, extensor cruris et tarsi fibularis and an undifferentiated extensor cruris tibialis + extensor tarsi tibialis are present (Fig. 9A). At stage late 55 the extensor cruris tibialis and extensor tarsi tibialis start to differentiate (Fig. 9C). At stage early 56 the abductor et extensor digiti 1 is present and the short extensors of digits 2 and 3 start to be clearly visible, the short extensor of digit 4 starting to be clearly visible at stage mid 56 (Fig. 10B). At stage late 56 the short extensor of digit 5 starts to be visible (Fig. 11B), and at stage early 57 the overall configuration and attachments of the dorsal leg muscles are essentially similar to those seen in adults (Fig. 12B).

Foot Muscles

The intrinsic foot muscles found in adults are the flexores breves superficiales, flexores breves profundi, abductor digiti minimi, contraheentes pedis, flexores digitorum minimi, interphalangei, and intermetatarsales. The most ventral of these muscles are the flexores breves superficiales (Fig. 8A), which run from the dorsal side of the plantar fascia to metatarsals I–V and digits 2–4 (the first muscle only goes to metacarpal I, not to digit 1), and the abductor digiti minimi (Fig. 8A), which runs from the distal end of the fibula to the fibulare, basale V and the base of metatarsal V. The five contraheentes pedis are deep to, and lie between, the flexores breves superficiales (Fig. 8A), connecting the tendon of the contraheentium caput longum and tarsal bones to the proximal phalanx of digits 1–5. Deep to these muscles lie the flexores breves profundi, running from the carpal/metacarpal region to each side of digits 1–5, and then the flexores digitorum minimi, which are deep and small muscles running from the metatarsals to the ventral side of the base of the proximal phalanx of digits 2–5. The four intermetatarsales connect the metatarsals of digits 1–5 (Fig. 8A,B). The interphalangei are the most distal foot muscles (Fig. 8A). Digit 3 has one interphalangeus connecting the metatarsophalangeal and first interphalangeal joints of this digit. Digit 4 has two muscles, one similar to the interphalangeus digiti 3, connecting the metatarsophalangeal and first interphalangeal joints of digit 4, and the other connecting the first and second interphalangeal joints of this digit 4 (Table 4).

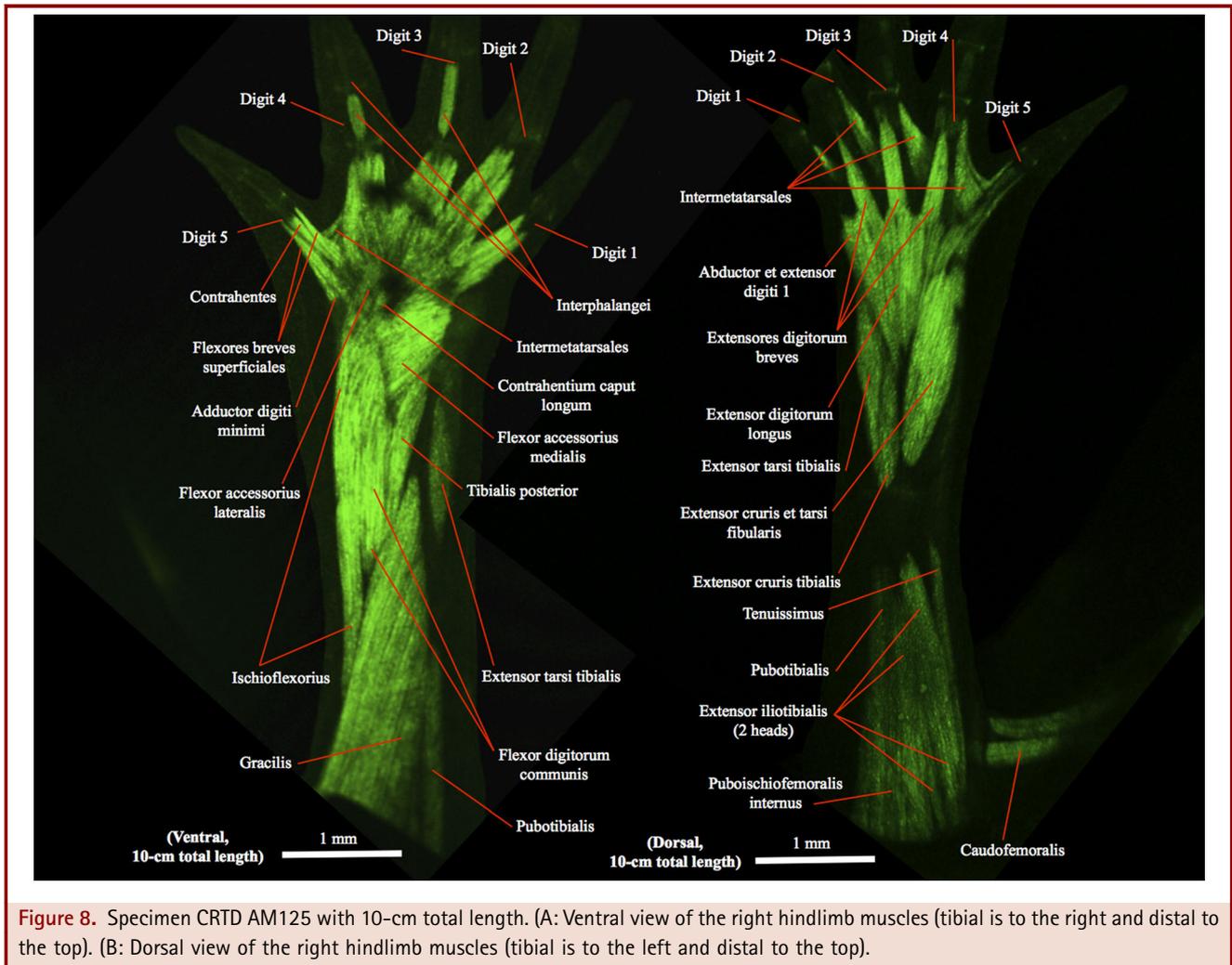


Figure 8. Specimen CRTD AM125 with 10-cm total length. (A: Ventral view of the right hindlimb muscles (tibial is to the right and distal to the top). (B: Dorsal view of the right hindlimb muscles (tibial is to the left and distal to the top).

Concerning development, the first appearance of intrinsic foot muscles is at stage early 56, in which an undifferentiated muscle anlage can be seen in the proximal and central region of the foot. At stage mid 56 the contrahentes and flexores breves superficiales of digits 1 and 2 are differentiated, as is the intermetatarsalis 1 lying between these two digits (Fig. 10A). At stage late 56 the flexores breves superficiales, flexores breves profundi and contrahentes of at digits 1–4 are formed, as are the intermetatarsales 1–4 and the abductor digiti minimi, and the flexores breves superficiales and the contrahens of digit 5 are starting to form (Fig. 11A). At stage early 57 the interphalangeus of digit 3 is clearly visible, and the proximal interphalangeus of digit 4 is starting to form (Fig. 12A). At stage late 57 the distal interphalangeus of digit 4 starts to be visible, and at later stages the overall configuration and attachments of all foot muscles are thus similar to those found in adults.

DISCUSSION

In the text provided below we will explicitly follow a similar order, and discuss some of the same issues, that were discussed in our recent paper on muscle limb development in frogs (Diogo and Ziermann, in press). This will allow us to directly compare the results obtained in urodeles and anurans and to point out if the data obtained in the present study supports, or not, the major evolutionary and developmental hypotheses supported by the data obtained from the study of frogs. In addition, we discuss various issues that were not discussed in that paper.

Morphogenesis and Myological Patterns

In general, our observations of the development of the axolotl fore- and hindlimb muscles are similar to the scarce detailed information provided in the literature about the ontogeny of these muscles in this and other urodele amphibian taxa. For instance, as

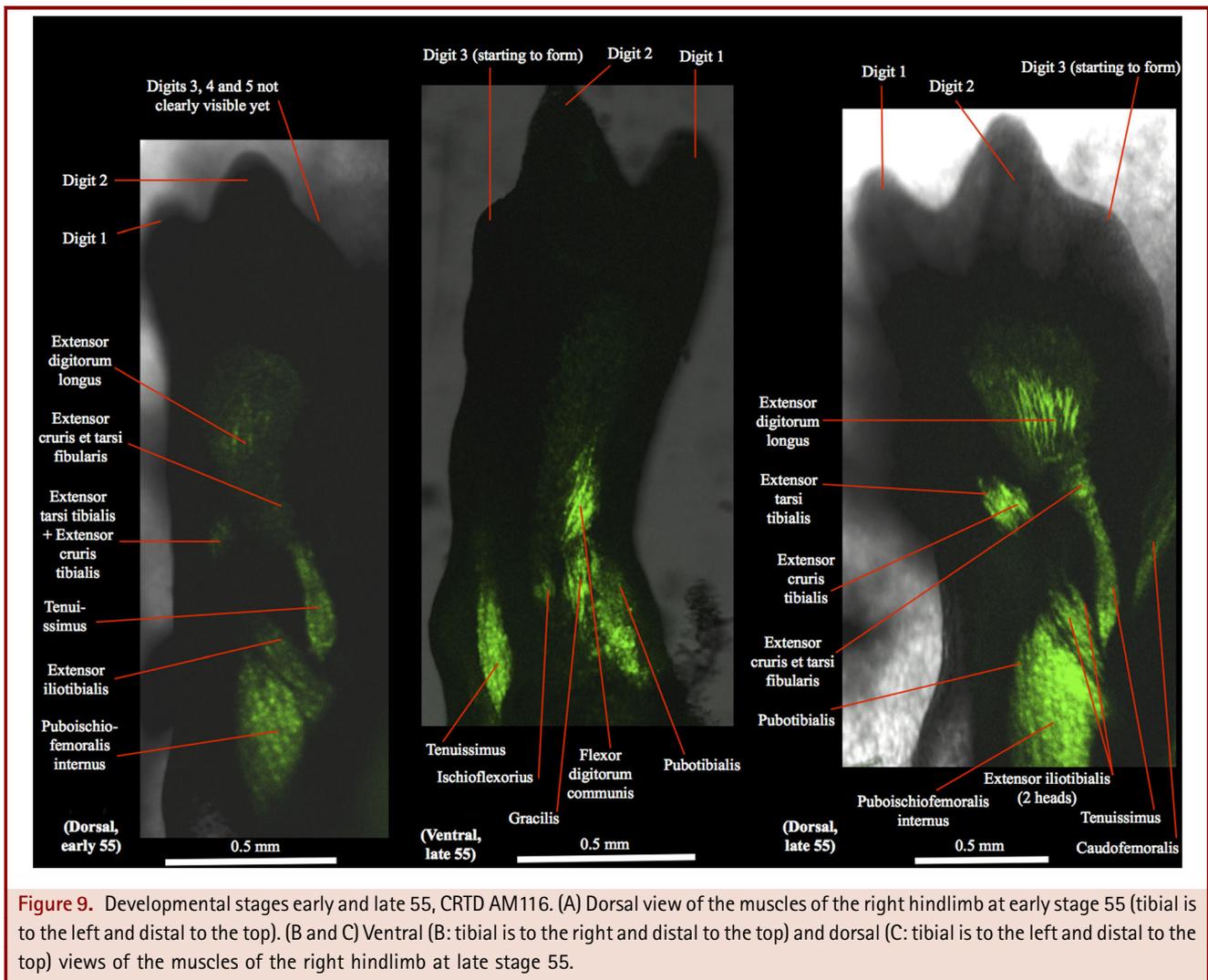


Figure 9. Developmental stages early and late 55, CRTD AM116. (A) Dorsal view of the muscles of the right hindlimb at early stage 55 (tibial is to the left and distal to the top). (B and C) Ventral (B: tibial is to the right and distal to the top) and dorsal (C: tibial is to the left and distal to the top) views of the muscles of the right hindlimb at late stage 55.

observed in our study, Grim and Carlson ('74a, b) reported that the long flexors and extensors of the forearm form before muscles such as the abductor et extensor digiti 1 (Tables 2 and 3), which in turn forms before most intrinsic hand muscles (Figs. 3–7). As reported by those authors and also by Diogo et al. (2013), in axolotl regeneration a similar pattern of formation of the forearm and hand muscles is also observed. Our observations also support Chen's ('35) statements concerning his ontogenetic study of the aquatic salamander *Necturus* (Chen, '35), according to which the latissimus dorsi, deltoideus scapularis and triceps brachii develop from dorsal embryonic anlagen while the pectoralis, supra-coracoideus, coracoradialis, procoracohumeralis, coracobrachialis, and humeroantibrachialis develop from ventral embryonic anlagen (Figs. 3–5). Both Chen's and our ontogenetic studies of salamanders thus contradict the idea, defended by authors such as

Howell ('35) and mainly based on comparisons of adult morphology, that the procoracohumeralis is a dorsal pectoral muscle. Interestingly, in the present study the coracobrachialis seems to develop from the anlage of, or from an anlage that lies very near the anlage of, the pectoralis (Fig. 3; see Results). One difference between our observations in axolotls and Chen's ('35) observations in *Necturus* is that in our study the coracobrachialis and humeroantibrachialis appear at different stages (respectively at stages mid 46 and mid 47), while in Chen's study the two muscles appear at the same stage (17.5 mm). As Chen's ('35) study of *Necturus*, our study did show that the long forearm extensors form before the short extensors and that the superficial forearm flexors form before the deep forearm flexors (i.e., flexor accessorius medialis and lateralis, contrahentium caput longum, palmaris profundus 1 and pronator quadratus: Tables 2 and 3).

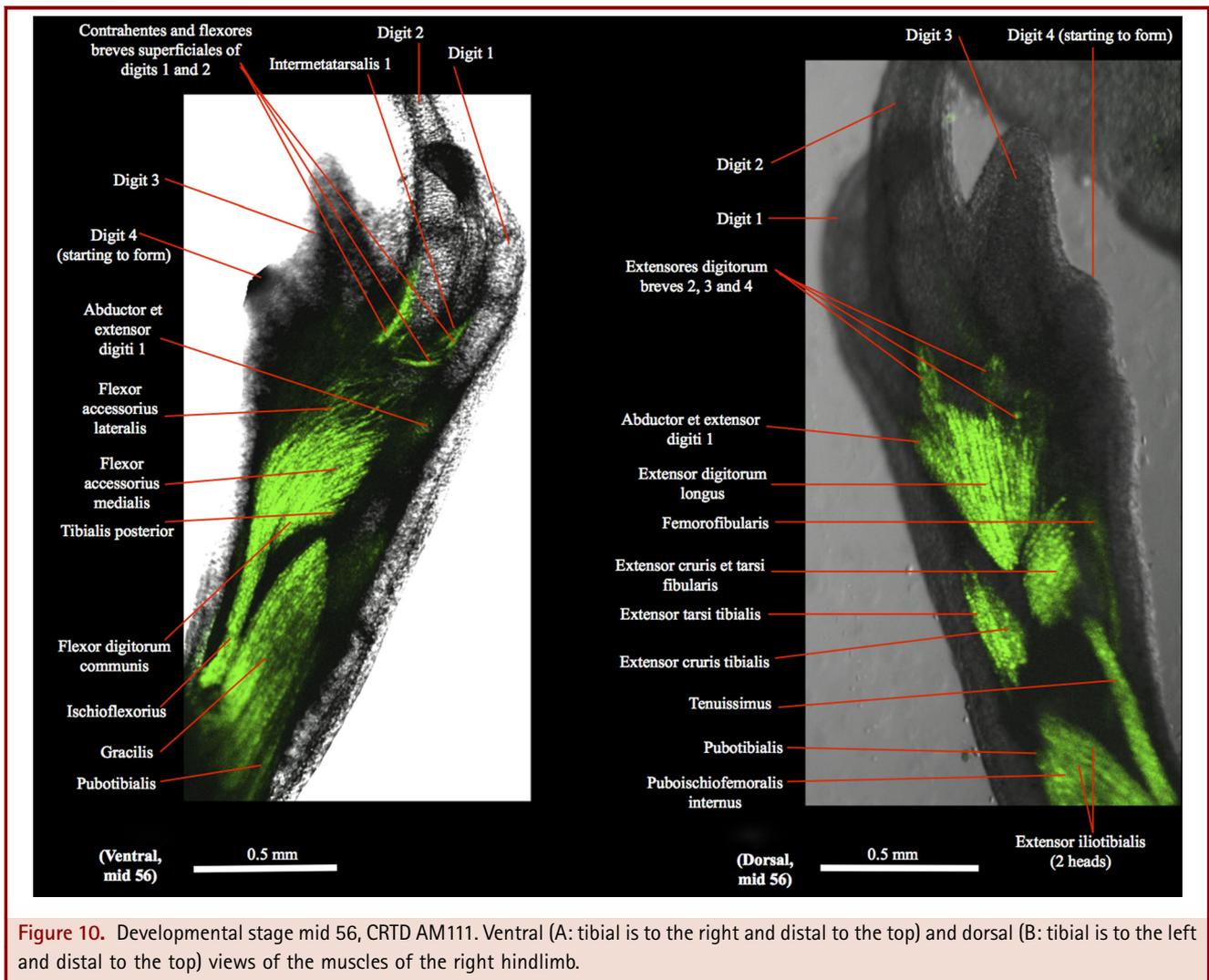


Figure 10. Developmental stage mid 56, CRTD AM111. Ventral (A: tibial is to the right and distal to the top) and dorsal (B: tibial is to the left and distal to the top) views of the muscles of the right hindlimb.

In general, our observations are also in agreement with Boisvert et al.'s (2013) descriptions of the development of the axolotl pelvic/thigh muscles, but some of the captions of the figures provided by those authors seem to be wrong. For instance, their Figure 4BL is an external (lateral) view of the hindlimb and therefore can only mainly show dorsal muscles (ventral hindlimb muscles have usually to be seen from a medial/ventral view). However, in their caption they wrote ischioflexorius, which is one of the most ventral pelvic/thigh muscles. In fact, our ontogenetic observations support the idea that the extensor iliobtibialis, puboischiofemoralis internus, and tenuissimus develop from dorsal, and that the gracilis, pubotibialis, and ischioflexorius develop from ventral, pelvic/thigh anlagen, as shown in Diogo's (in press) (Table 1). Still concerning muscles associated with the girdles, our observations support Valasek et al.'s (2011) "in-out"

mechanistic hypothesis, which was based on a review of ontogenetic data from various tetrapod taxa. According to those authors the superficial girdle muscles ("appendicular pectoral muscles" sensu Diogo and Abdala, 2010) develop by an "in-out" mechanism whereby migration of myogenic cells from the somites into the limb bud is followed by their extension from the proximal limb bud out onto the thorax; the deep girdle muscles ("axial pectoral muscles" sensu Diogo and Abdala, 2010) are induced by the forelimb field that promotes myotomal extension directly from the somites. As explained in the Results section, the fact that the axial pectoral muscles are often deep girdle muscles probably explains why the levator scapulae and serratus anterior could not be seen in the pictures obtained by us of the developing muscles of axolotls from stages 46 to 54. As our ontogenetic study of frog limbs (Diogo and Ziermann, in press), the present study of axolotls

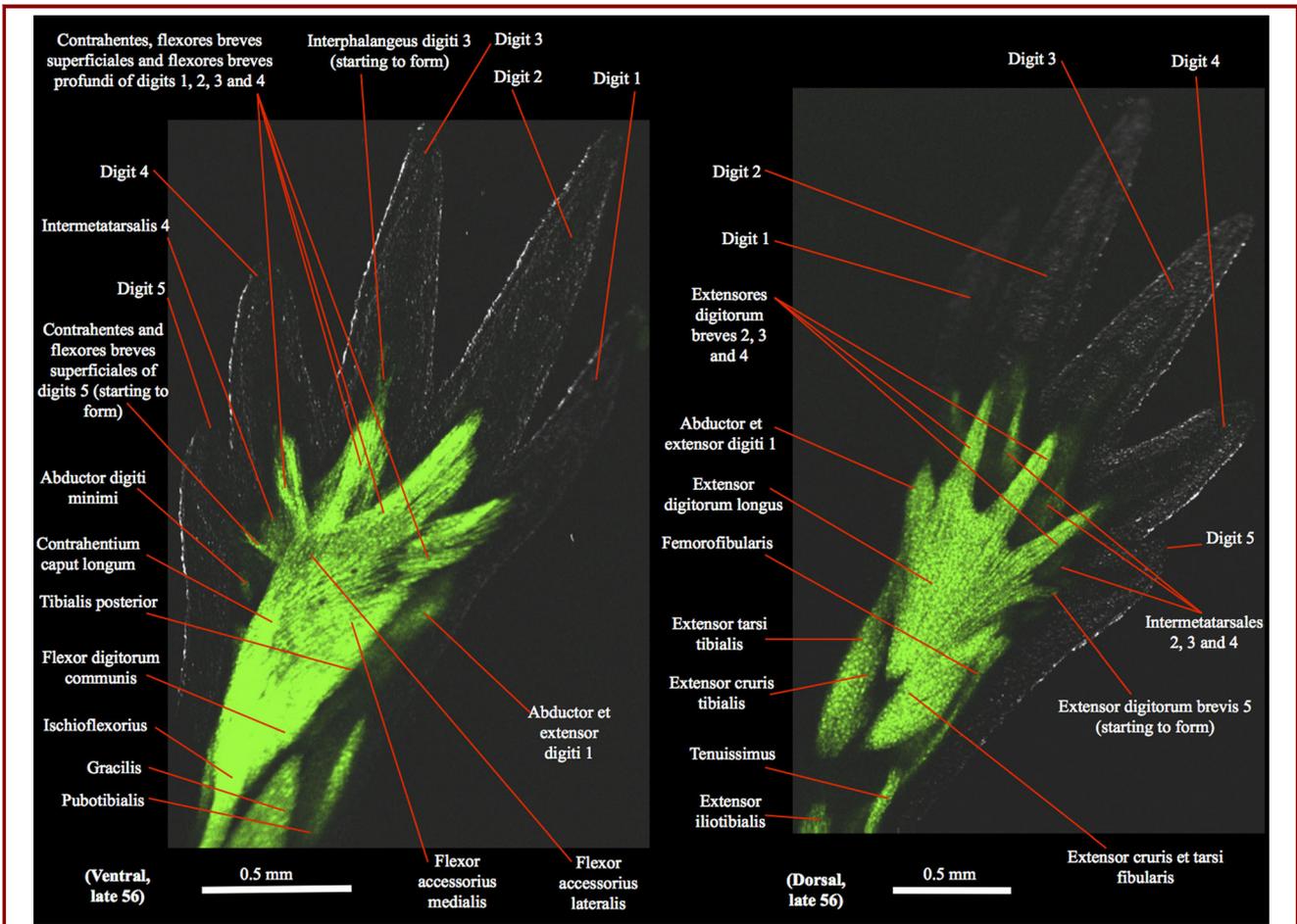


Figure 11. Developmental stage late 56, CRTD AM112. Ventral (A: tibial is to the right and distal to the top) and dorsal (B: tibial is to the left and distal to the top) views of the muscles of the right hindlimb.

also supports Valasek et al.'s (2011) "in-out" mechanism because at earlier ontogenetic stages the appendicular pectoral muscles start to develop far from the midline, at the level of the proximal region of the arm; only later in development they extend medially to cover a substantial part of the ventral and dorsal surfaces of the thoracic region (compare, e.g., Fig. 1 with Fig. 3).

The regeneration of urodele forelimbs is a classic case study for the investigation of the morphogenesis of both hard and soft tissues (see, e.g., Carlson's 2007). In our previous study of axolotl forelimb regeneration (Diogo et al., in press) the tempo and mode of the morphological events observed during regeneration were similar to those reported by other authors, that is, the formation and differentiation of the muscles followed a proximo-distal and a radio-ulnar gradient (e.g., Grim and Carlson, '74a, b). However, apart from these two morphogenetic gradients described in the literature, the results of Diogo et al.'s (Diogo et al., in press) study

indicated that there is also a marked ventro-dorsal gradient during the regeneration of at least some axolotl forelimb muscles. This contrasts with the results of Diogo et al.'s (submitted for publication) study of axolotl hindlimb regeneration, in which we found proximo-distal and tibio-fibular morphogenetic gradients but not a ventro-dorsal gradient.

The results of the present ontogenetic study of axolotl limb muscles reveal a pattern similar to that found in regeneration, that is, proximo-distal and radio-ulnar/tibio-fibular morphogenetic gradients found in both limbs, but ventro-dorsal gradient only found in the forelimb. The proximo-distal gradient is clearly seen in Figures 3–7 and 9–12: in the forelimb the pectoral/arm muscles form first, followed by the forearm muscles and then by the hand muscles; in the hindlimb the pelvic/thigh muscles are the first to form, followed by the leg and then the foot muscles. Regarding the radio-ulnar/tibio-fibular gradients, they can be seen for example

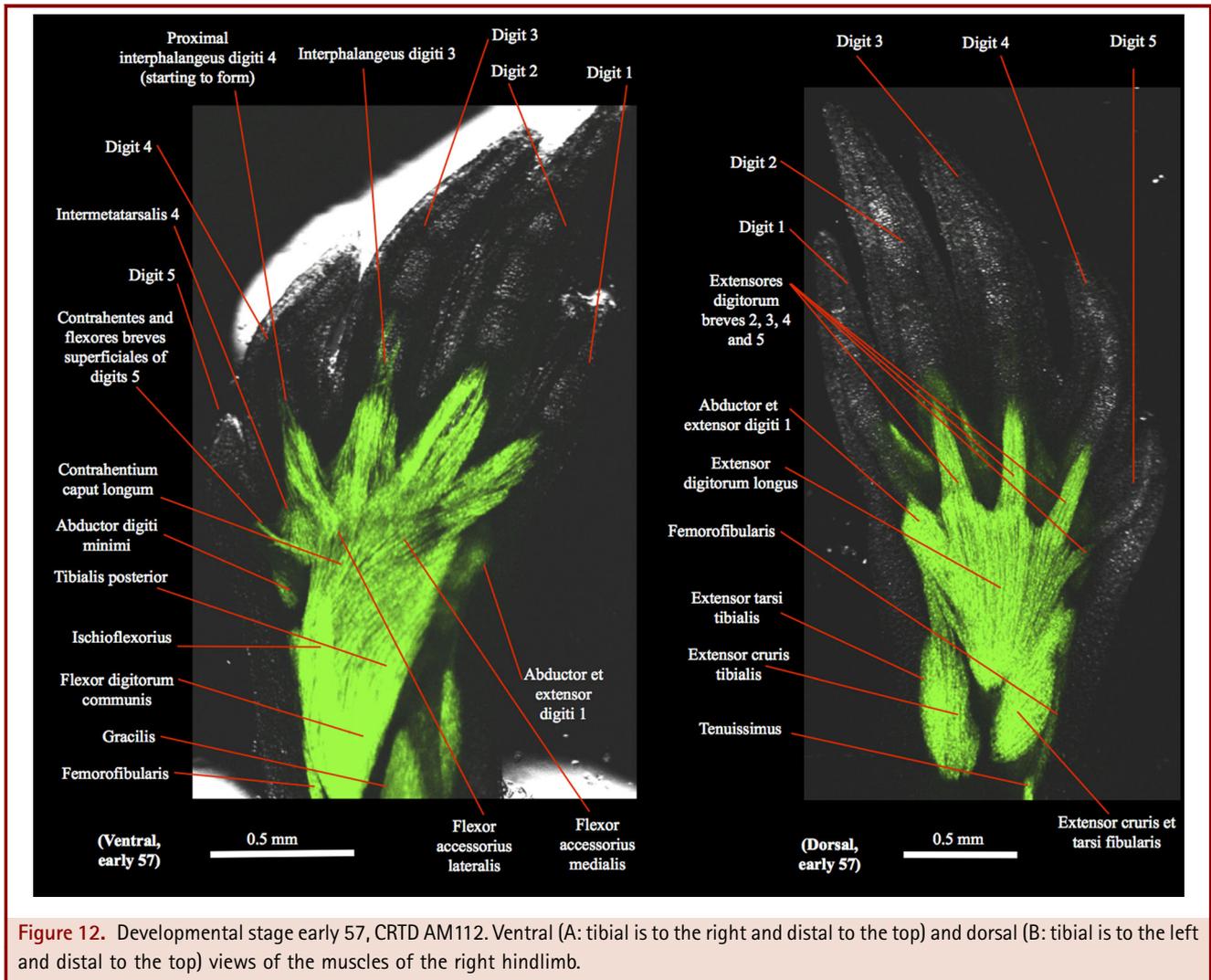


Figure 12. Developmental stage early 57, CRTD AM112. Ventral (A: tibial is to the right and distal to the top) and dorsal (B: tibial is to the left and distal to the top) views of the muscles of the right hindlimb.

in the zeugopodium (forearm/leg), for example, at stage early 48 the flexor antebrachii et carpi radialis is differentiated and well developed but the flexor antebrachii et carpi ulnaris is apparently still undifferentiated from the anlage of the flexor digitorum communis or is just a very small muscle covered ventrally by the latter muscle (Fig. 4A). But these gradients are particularly evident in the region of the autopodia (hand/foot), for example, the first intrinsic hand/foot muscles and the short extensors to form and differentiate are those of the most radial/tibial digits, the last ones to form and differentiate being those of the most ulnar/fibular digit (digit 4 in the hand, digiti 5 in the foot) (Figs. 5–7 and 10–12). Regarding the ventro-dorsal gradient seen in forelimb ontogeny, this is clearly illustrated at stage early 48, in which the anlage of the dorsal forearm muscles is still relatively thin and not differentiated, while the ventral forearm muscles flexor digitorum

communis and flexor antebrachii et carpi radialis are already differentiated and well developed (Fig. 4A,B).

The occurrence of a ventro-dorsal gradient in the regeneration and ontogeny of the forelimb, but not of the hindlimb, muscles could be seen as potential genuine difference between the forelimbs and hindlimbs. That is, this could potentially be added to the list of differences between the tetrapod fore- and hindlimbs provided by Diogo et al. (2013), whom proposed that these limbs are not serial homologues but instead the result of homoplasy (convergence/parallelism: see The Fore-Hindlimb Enigma and the Ancestral Bauplan of Tetrapods section). However, it should be noted that a dorso-ventral gradient was reported in Kardon's ('98) study of the ontogeny of the hindlimb muscles of chickens. Moreover, our recent ontogenetic study of the frog *Eleutherodactylus coqui* (Diogo and Ziermann, in press) has shown that

both limbs display a proximo-distal muscle morphogenetic gradient and that the hindlimb displays a dorso-ventral gradient, as reported in chickens by Kardon ('98). Also, instead of a radio-ulnar/tibio-fibular gradient as seen in the regeneration and ontogeny of the musculature of both limbs of axolotls, there is mainly a ulno-radial/fibulo-tibial gradient in the ontogeny of the musculature of the fore- and hindlimbs of this frog. The ulno-radial muscle morphogenetic gradient observed in frogs is thus more similar to the ulno-radial gradient seen during the ontogenesis of the limb skeletal structures in other non-urodele tetrapod groups and also during the ontogenesis of limb muscles seen in at least some of these groups (e.g., chickens: see, e.g., Carlson, 2007).

The existence of different morphogenetic gradients of muscle formation and differentiation in the same limbs of different taxa and in different limbs of the same taxon shows a remarkable plasticity regarding the morphogenesis of tetrapod limbs. This plasticity reads difficult to discern and discuss plesiomorphic states and general morphogenetic patterns in tetrapods. For instance, an idea that occurred to us while doing the present study is that the existence of a general radio-ulnar/tibio-fibular gradient could help explaining why in general there are for instance more radial/tibial than ulnar/fibular muscles in tetrapod taxa from all major groups, that is, amphibians, reptiles, and mammals. That is, due to the radio-ulnar gradient the radial musculature would become more differentiated than the ulnar musculature before reaching adulthood. For instance, in the axolotl hindlimb there are two tibial dorsal long extensors (extensor cruris tibialis and extensor tarsi tibialis) and only one fibular long extensor (extensor cruris et tarsi tibialis) (Fig. 8). A similar pattern is seen in the adult axolotl forearm, in which there are two radial dorsal long extensors (supinator and extensor carpi radialis, which is in turn divided into two bundles) and only a ulnar long extensor (extensor antebrachii et carpi ulnaris). A similar pattern is also seen in the adult human forearm, which has four radial long extensors (supinator, brachioradialis, extensor carpi radialis longus, and extensor carpi radialis brevis) versus two ulnar long extensors (anconeus and extensor carpi ulnaris) (Diogo and Wook, 2012). A similar pattern is seen in the adult forelimb autopodia of humans and birds, such as chickens, for instance, in which the most radial digit (thumb in humans) has more muscles associated with it than the most ulnar digit does (Diogo and Abdala, 2010; Diogo and Wook, 2012).

However, the problem for this theory is that, as explained above, in amphibians such as frogs and amniotes such as chickens, there is instead a ulno-radial muscle and skeletal morphogenetic gradient. But it could be argued that the occurrence of radio-ulnar/tibio-fibular, as well as of proximo-distal, gradients in both limbs can effectively represent the plesiomorphic condition for tetrapods and that this condition could be explained by a dependence of muscle patterning upon the patterning of connective tissue. This is because both in axolotl development

and regeneration, as well as in the ontogeny of other tetrapods, there are many markers and patterning genes that have been extensively implicated in patterning of limb connective tissue and that are upregulated in proximo-distal (e.g., *Hox*, *FGFs*, *RA*) and radio-ulnar and/or tibio-fibular fashion (e.g., *Shh*) (e.g., Gilbert, 2006; Carlson, 2007). It is therefore possible that either these molecules have direct impact on muscle patterning or they can influence muscle patterning via patterning of connective tissue, and that this could explain, at least in part, the usual presence of more radial/tibial than ulnar/fibular muscles in tetrapods. In fact, it is worthy to mention that some early amphibian fossils (that are likely phylogenetically more basal than the last common ancestor of extant urodeles and anurans) seem to have a mainly preaxial (radio-ulnar) type of development of the digits; this evidence also raises questions about whether the postaxial (ulno-radial) type of digit development seen in extant anurans and amniotes represents, or not, the plesiomorphic condition for tetrapods (Fröbisch and Shubin, 2011).

The Fore-Hindlimb Enigma and the Ancestral Bauplan of Tetrapods

Based on an extensive anatomical comparison of the adult muscles of the pectoral and pelvic appendages of numerous vertebrate taxa and on a review of other lines of evidence available (e.g., from paleontology, functional morphology, evo-devo, and genetics), Diogo et al. (2013) and Diogo (in press) argued that, contrary to the commonly accepted dogma, there is no serial homology between these appendages. That is, according to those authors the enigmatic similarity between many forelimb and hindlimb structures ("fore-hindlimb enigma"), including muscles, was acquired during the "fins-limbs transition." This is because during that transition there was a *derived* cooption of some similar genes for the development of the more distal parts of both the forelimb and the hindlimb ("genetic piracy" sensu Roth, '94; see also the recent review of Pavlicev and Wagner, 2012).

We recently discussed this subject, based on our ontogenetic study of frog limbs (Diogo and Ziermann, in press), in which we stated that there is effectively a marked similarity, both in the larvae and the froglets/adults of *E. coqui*, between many forearm/hand and leg/foot muscles. The similarity is much more striking in axolotls, in which all the leg/foot and forearm/hand muscles have a clear "topological equivalent" in the other limb, with exception to the flexor antebrachii et carpi radialis and flexor antebrachii et carpi ulnaris (Tables 2–4; see also Diogo, in press). The limb musculature of urodeles such as axolotls is much more similar to the limb musculature that was plesiomorphically present in tetrapods, than is the musculature of frogs (Diogo and Abdala, 2010). Therefore, the striking similarity between the zeugopodial and autopodial musculature of the fore- and hindlimb of axolotls indicates that this similarity was very likely acquired in the transitions from fins to limbs, supporting Diogo et al. (2013) and Diogo (in press) hypothesis. Importantly, Diogo's (in press) hypothesis is also supported by

the fact that concerning the musculature of the girdles, even in anatomically plesiomorphic tetrapods such as salamanders there is in fact no clear similarity or correspondence between any pelvic/thigh and any pectoral/arm muscle (Table 1; compare, for example, Figs. 1 and 2 with Fig. 8). According to Diogo et al. (2013), this is due to a phylogenetic constraint, that is, the musculature of the girdles is phylogenetically very ancient (contrary to the zeugopodial and autopodial musculature, which was only acquired in the node leading to tetrapods), and the pelvic and pectoral muscles were very different from the beginning. The muscles of the pectoral girdle are effectively extremely different from the muscles of the pelvic girdle in not only tetrapods but also in non-tetrapod gnathostomes, reinforcing the idea that the pelvic and pectoral appendages are not serial homologues (Diogo et al., 2013).

The ontogenetic observations of the present work are very important in this context, because one possible explanation, according to the serial homology hypothesis, would be that the adult configuration found in these taxa is highly modified from the configuration seen at earlier stages of development, which could in theory provide more evidence of the similarities between the muscles of the two girdles. However, as seen in the present study, even in anatomically plesiomorphic tetrapods such as salamanders, from early ontogenetic stages the muscles of the two girdles are also markedly different from each other. Such a marked difference was also found in our recent study of frog limb development (Diogo and Ziermann, in press). As explained in that study, faced with these facts, a defender of the serial homology hypothesis could still argue that the evidence may be interpreted by a change of the developmental mechanisms during evolutionary history, that is, that serial homologous muscles now develop in totally different ways. There is, for instance, a huge anatomical divergence in the adult forelimb of tetrapods, from the wings of reptiles such as birds to the pectoral fins of mammals such as dolphins, and there is no doubt that the forelimbs of different tetrapod taxa are homologous to each other.

However, as explained by Diogo and Ziermann (in press), if one studies in detail the development of the muscles of the forelimbs of reptiles and mammals, there are some developmental differences (e.g., heterochronic changes where some muscles develop before others do, in different taxa), but there is still a clearly recognizable pattern/bauplan of the forelimb in all tetrapods. So, likewise, if there is a true serial homology between the pectoral and pelvic girdle hard and soft tissues in fish and tetrapods, one should expect to still find at least some type of recognizable common features and bauplan in the ontogeny of the structures of these girdles. However, so far no researcher was able to find any clear resemblances between not only the adult configuration but also the ontogeny of any single pectoral and pelvic muscle in any tetrapod taxon. As explained above, we could not also find any resemblance in the present study, in which we directly compared

the development of the fore- and hindlimbs of the very same, anatomically plesiomorphic, tetrapod species (*Ambystoma mexicanum*).

These new insights on the serial homology versus homoplasy of the structures of the tetrapod limbs and the new data obtained in the present work about the development of the limb muscles of the myologically plesiomorphic axolotls allow us to have a more comprehensive picture about the ancestral Bauplan of tetrapods. That is, it is now clear that the pelvic/thigh muscles of the first tetrapods were very different topologically to the pectoral/arm muscles, as the pectoral and pelvic girdle muscles had/have been in the vast majority of fish. Although the humerus and femur clearly correspond topologically to each other, these structures have very likely appeared in fish much earlier than the origin of tetrapods, and moreover the thigh/arm muscles originate topologically from the pelvic/pectoral girdles, which were anatomically very different in the fish that gave rise to tetrapods (e.g., Coates and Cohn, '98; Diogo et al., 2013). This explains why not only the pelvic/pectoral, but also the thigh/arm muscles are topologically very different (Table 1; see above). In contrast, the forearm/hand and leg/foot muscles are very similar due to a *derived* cooption, during the fin-limb transition, of some similar genes for the development of the zeugopodia and autopodia, which are mainly *de novo* structures, as explained above.

But how similar were the zeugopodial/autopodial muscles of the two limbs in the first tetrapods? As explained above, and shown in Tables 2–4, in myologically plesiomorphic tetrapods such as salamanders all the leg/foot and forearm/hand muscles have a clear “topological equivalent” in the other limb, with exception to the flexor antibrachii et carpi radialis and flexor antibrachii et carpi ulnaris. However, there is some evidence indicating that in the first tetrapods the musculature of the leg-foot might have been even more strikingly similar to the musculature of the forearm-hand than is the case in these anatomically plesiomorphic extant tetrapods. For instance, the comparative studies of McMurrich ('05) and Bardeen ('06) suggested that in extant salamanders the distal (leg) portion of the ischioflexorius corresponds to an ancestral muscle flexor cruris et tarsi tibialis, while the femoral head of the flexor digitorum communis corresponds to an ancestral muscle flexor cruris et tarsi fibularis. These and most other authors consider that the femorofibularis is a thigh muscle, and that is why we show this latter muscle in Table 1. However, Howell ('36) described a “femorocruralis” muscle in birds that is very similar to the femorofibularis of salamanders and that is innervated by the tibial nerve (and not by the obturator nerve as suggested by Gadow, 1882). As stressed by Howell, this avian muscle seems to be the result of a proximal migration of part of the gastrocnemius complex, thus providing some support to the idea that the femorofibularis of amphibians might correspond to at least part of the gastrocnemius complex of reptiles and is effectively a leg, and not a thigh, muscle. But the avian

“femorocruralis” could alternatively correspond to part of, or the whole, distal (leg) portion of the ischioflexorius that has migrated proximally.

Our ontogenetic study of the axolotl limb muscles sheds light on this issue because the way in which the femorofibularis develops and its topology, including its fibular position in the ventral side of the leg and the fact that its distal portion lies near and just proximal to the abductor digiti minimi, do indicate that it may well correspond topologically to the flexor antebrachii et carpi ulnaris of the forearm (e.g., Figs. 10B, 11B, 12B, compare with Figs. 5B, 6B, 7B). That is, our study supports the idea that the distal (leg) portion of the ischioflexorius corresponds to an ancestral muscle flexor cruris et tarsi tibialis and points out that the femorofibularis might correspond to an ancestral muscle flexor cruris et tarsi fibularis. However, we have to stress here that the more consensual view is that the salamander femorofibularis is a thigh muscle, particularly because it is said to be innervated by the sciatic nerve (e.g., Francis, '34) and because there is no such muscle or any apparent derivative of it in the leg of anurans (Diogo, in press), thus supporting the idea that this is actually a peculiar, derived thigh muscle of urodeles and not an important part of the tetrapod leg Bauplan that gave rise to amniote leg muscles. But it should also be noted that some previous authors, such as Humphry (1872a, b), have also proposed that the femorofibularis is a leg muscle that might have given rise to at least part of the short head of the biceps femoris of mammals.

Be that as it may, what seems to be clear is that either if a flexor cruris et tarsi fibularis is represented by the femorofibularis and/or by part of the flexor digitorum communis (as suggested by McMurrich, '05; Bardeen, '06), the ancestral Bauplan of the zeugopodial and autopodial musculature of both limbs is even more strikingly similar than it is usually assumed. That is, it is very likely that, due to the *derived* (homoplastic) cooption of similar genes for the development of the fore- and hindlimb zeugopodia and autopodia in the fin-limb transition, in the first tetrapods all the forearm/hand muscles had a topological “equivalent” in the hindlimb. This striking similarity is still seen, to a lesser degree, in myologically plesiomorphic tetrapods such as salamanders, because they have flexor antebrachii et carpi radialis/ulnaris anlagen also seem to have flexor cruris et tarsi tibialis/fibularis anlagen. But this similarity is not as evident when one analyses salamander adult morphology because in the forearm the muscle/muscles derived from each anlage are present as distinct muscles, while in the leg the flexor cruris et tarsi tibialis is fused with the thigh portion of the muscle ischioflexorius, and the flexor cruris et tarsi fibularis either migrated slightly proximally (forming the femorofibularis) and/or became fused with the flexor digitorum communis.

Importantly, these comparisons indicate that in the first tetrapods the dorsal forearm musculature was not only strikingly similar to the dorsal leg musculature but also to the ventral forearm and to the ventral leg musculature (dorso-ventral

symmetry). This is because the forelimb also has extensor antebrachii et carpi radialis/ulnaris anlagen and the hindlimb also has extensor cruris et tarsi tibialis/fibularis anlagen. That is, the first tetrapods probably had a very similar configuration in the eight sides of the four zeugopodia (i.e., dorsal and ventral sides of the four limbs), with a recognizable central muscle (extensor digitorum (longus)/flexor digitorum communis) surrounded by radial/tibial and ulnar/fibular muscles (derived by the flexor/extensor antebrachii/cruris et carpi/tarsi radialis/tibialis/ulnaris/fibularis anlagen). However, one probably cannot refer to 16 symmetrical parts within the four adult zeugopodia of these first tetrapods (i.e., also to a radio-ulnar and tibio-fibular symmetry). This is because, as explained above, the flexor/extensor antebrachii/cruris et carpi/tarsi radialis/tibialis anlagen usually gives rise to more adult muscles and bundles than do the flexor/extensor antebrachii/cruris et carpi/tarsi ulnaris/fibularis anlagen. Interestingly, our ontogenetic study of axolotls revealed that the dorso-ventral symmetry of the zeugopodia might concern not only the central, the radial/tibial and the ulnar/fibular anlagen, but also the anlagen of the other, shorter muscles of the zeugopodia. This is due to the fact that during the development of the axolotl muscles, for example, in stage 50 (Fig. 5), the morphogenesis of, for instance, the forearm abductor et extensor digiti 1 is very similar to that of the palmaris profundus 1; both muscles form distally and deep to the central extensor/flexor (extensor digitorum/flexor digitorum communis) and run distally and radially to reach the region of the thumb (digit 1). In this sense, the short and deep dorsal forearm muscles extensor digitorum breves 2–4 (which are part of the group that includes the abductor et extensor digiti 1) could correspond topologically to the short and deep ventral forearm muscles flexor accessorius medialis, contrahentium caput longum, and flexor accessorius lateralis (which are part of the group that includes the palmaris profundus 1). However, further studies, including (ideally) mechanistic developmental studies, are clearly needed to test this hypothesis.

In summary, it can thus be said that the more data is obtained about the comparative anatomy and developmental biology of tetrapod limbs, the better we understand the origin and ancestral Bauplan of the tetrapod limbs, and the further the fore-hindlimb serial homology hypothesis is contradicted. However, much remains to be done, and in this respect it is striking that some essential aspects of tetrapod limb morphogenesis (e.g., the occurrence of a ventro-dorsal morphogenetic gradient within axolotl forelimb muscle formation and differentiation) are only now being discovered and reported. We plan to continue studying the ontogeny of tetrapod limbs; specifically, after studying frog and axolotl development we plan to analyse the ontogeny of limb muscles in reptiles and mammals and to undertake mechanistic developmental studies in all tetrapod groups. However, a major goal of this paper is to stimulate other researchers to investigate limb comparative anatomy and developmental biology. Only a

multidisciplinary, comprehensive, collaborative approach can help solve the crucial and fascinating questions that remain to be answered about the origin and evolution of tetrapod limbs. In this sense, it is worthy mentioning that together with other colleagues we are now studying in detail the appendicular muscles of dipnoans and coelacanth (including developmental studies of dipnoans) and of key fossil sarcopterygian fish and early tetrapods (including discussions about polydactyly; Diogo et al., in preparation).

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