

# Development of Fore- and Hindlimb Muscles in Frogs: Morphogenesis, Homeotic Transformations, Digit Reduction, and the Forelimb–Hindlimb Enigma



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## ABSTRACT

Here we provide the first detailed description, based on immunohistochemistry and dissections, of the limb muscle development in the direct developing frog *Eleutherodactylus coqui*. We compare *E. coqui* with other tetrapods and discuss our results in a broad evolutionary and developmental context to address some major questions concerning the origin, evolution, and ontogeny of the tetrapod limbs. Our observations and comparisons: (1) support the “in–out” developmental mechanism of the appendicular pectoral muscles; (2) show that the protractor pectoralis and its amniote derivatives trapezius and sternocleidomastoideus clearly develop, anatomically, from the branchial muscles; (3) corroborate that the similarity between the forearm/hand and the leg/foot muscles in tetrapods is due to derived homoplastic events that occurred during the fins–limbs transition and not due to serial homology; (4) lend some support for the hypothesis that the morphological transformation of the anuran tibiale and fibulare represents a distal shift in the zeugo–autopodial border; (5) provide evidence that the identity of the tetrapod hand and foot muscles is mainly related to the topological position of the digits to which they attach; and (6) for the first time, show that apart from a proximo–distal morphogenetic gradient there is also an ulno–radial/fibulo–tibial gradient within the development of the fore- and hindlimb muscles and a dorsoventral gradient within the ontogeny of the hindlimb (but not forelimb) muscles of the frog *E. coqui*; the two latter gradients are seen in the ontogeny of amniotes such as chickens but are markedly different to those seen in axolotl regeneration and ontogeny. *J. Exp. Zool. (Mol. Dev. Evol.)* 9999B: 1–20, 2013. © 2013 Wiley Periodicals, Inc.

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Amphibian embryos once dominated the fields of developmental biology and experimental embryology, although they then temporarily fell out of favor, during the early days of developmental genetics (Gilbert, 2006). In the last decades new developmental and molecular techniques have allowed researchers to return to studying amphibian embryos and to integrate developmental studies and molecular analyses with earlier experimental findings (Gilbert, 2006). In fact, one of the model organisms that is now more commonly used in developmental

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biology laboratories is the metamorphic (biphasic development: see below) frog *Xenopus laevis*. It is therefore surprising that we know of no paper or monograph that describes the development of the fore- and hindlimb muscles of the same anuran species, because apart from their importance for developmental biology both in the past and in the present, frogs have also been crucial for evolutionary studies and are increasingly important for regenerative studies as well (Fabrezi and Alberch, '96; Gilbert, 2006; Satoh et al., 2006; Carlson, 2007; Agata and Inoue, 2012). The very few studies providing some details about the development of limb muscles in frogs refer only to a very restricted group of muscles, such as Dunlap's ('66) ontogenetic study of the pelvic/thigh and leg muscles of *Rana pipiens* or Manzano et al.'s (2013) work on the ontogeny of some hindlimb muscles in *Pleurodema borelli* and some other frogs. Moreover, these few studies mainly concern frogs with a biphasic development (which have two successive posthatching stages, an aquatic larva, and a carnivorous terrestrial adult, separated by a discrete metamorphosis) and not direct-developing frogs (which bypass the free-living larval stage and develop directly into adults; Hanken et al., 2001; Gilbert, 2006).

The main goal of this paper is therefore to provide the first detailed description, based on immunohistochemistry staining and adult dissections, of the development of the muscles of both the fore- and hindlimbs of a frog species exhibiting a direct development, *Eleutherodactylus coqui*, in order to directly compare the way in which the muscles of each limb develop and to compare our observations with the scarce information available for other frogs. Based on comparisons with non-anuran tetrapods we discuss the results obtained in a broad evolutionary and developmental context and address some major questions concerning the origin, evolution and ontogeny of the tetrapod limbs, including: (1) the evolution of the pectoral muscles and the "in-out" mechanism; (2) the evolution of the neck and the protractor pectoralis muscle and its derivatives in amniotes, the trapezius and the sternocleidomastoideus; (3) the morphogenesis and morphogenetic gradients of tetrapod limbs; (4) the fore-hindlimb enigma, the question of whether the similarity of the hind- and forelimbs is due to serial homology or to homoplasy; and (5) the peculiar anatomical features of frog limbs and their implications for the understanding of homeotic transformations and digit reduction. In a next phase of our long-term project, we will then undertake a study of the development of the fore- and hindlimb of *X. laevis*, in order to provide detailed information for a biphasic developing frog and to pave the way for further studies on this model organism and therefore for more detailed comparisons with direct developing frogs such as *E. coqui* and with other tetrapods.

## MATERIALS AND METHODS

Larvae (37 embryos/hatchlings) and adults (3 adults) from *E. coqui* are from Richard Elinson's Lab (Duquesne University, Pittsburgh, PA). The adult specimens were anesthetized in 1% MS222 and the

larvae in 0.1% MS222. When no retraction reflex after pinching the foot of the adult frogs could be observed, they were frozen. The dissection of the adults was done under low magnification with a dissecting microscope (Nikon Inc., Melville, NY, USA, SMZ-2B). We used all animals to investigate the development and morphology of the fore- and hindlimb muscles. All larval stainings were done at Elinson's Lab by JMZ and Elinson, while observation of the development and the dissection of the adults were done in RD's lab. The embryos were studied under a microscope (Nikon AZ100) and photographed with an attached camera (Nikon DS-Fi1; software NIS-Elements D4.00.03). The 37 embryos/hatchlings of *E. coqui* analyzed are from stages TS5 to TS15 to posthatch (staging after Townsend and Stewart, '85): four froglets, six TS15, four TS14, three TS12, two TS11, three TS10, five TS9, four TS8, three TS7, and two TS6 specimens and one TS5 specimen.

For immunohistochemistry of the non-adult specimens we followed the procedure described by Elinson and Fang ('98). The embryos were fixed in Dent's fixative (80% methanol and 20% dimethyl sulfoxide [DMSO]; Dent et al., '89), and stored at  $-20^{\circ}\text{C}$ . The larvae's pigment was bleached with 10%  $\text{H}_2\text{O}_2$  in 67% Dent's for 1–3 days when necessary. The larvae were stained for muscle with 12/101 antibody (Kintner and Brockes, '85). The primary antibody was visualized using an HRP-labeled goat antimouse secondary antibody (horse radish peroxidase [HRP]). In order to check the relationship between muscle fibers, tendons and skeletal structures in the larvae we additionally undertook manual microdissections.

The nomenclature of the muscles mainly follows Diogo and Abdala (2010), Diogo and Tanaka (2012), and Diogo (in press). It should be noted that this nomenclature takes into account the evolution and homologies of the limb muscles of all the major tetrapod groups. Therefore there are cases where some of the names used here do not correspond to names that are commonly used in the literature that is exclusively focused on frogs, which often includes names that are employed in human anatomy despite the fact that the anuran and human muscles designated by the same names are almost always not homologous (Diogo et al., 2013; Diogo, in press). In Tables 1–4, we thus provide a list of the names used in the present work followed by some of the names more commonly used in the literature about anurans. When we refer to the anterior, posterior, dorsal and ventral regions of the body, we 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). As noted above, apart from *E. coqui*, we have dissected, in the past, specimens from all the major tetrapod taxa, as well as numerous non-tetrapod vertebrates; a list of the dissected specimens is given in Diogo and Abdala (2010) and Diogo and Wood (2012). Regarding the nomenclature of the four digits of the hand of *E. coqui*, we follow the commonly accepted idea that the digit missing in frogs is digit 1, and thus we refer to digits 5, 4, 3, and 2

**Table 1.** Axial and appendicular pectoral and arm muscles of adults of *E. coqui*, following the nomenclature of Diogo and Abdala (2010) and Diogo and Tanaka (2012); some synonyms commonly used in the literature about anurans are given (D&T 86 means "sensu Duellman and Trueb, '86").

Axial pectoral
Serratus anterior (part of "serrati" D&T 86)
Rhomboideus ("rhomboideus posterior" D&T 86)
Rhomboideus occipitalis ("rhomboideus anterior" D&T 86)
Levator scapulae superior
Opercularis
Columellaris present or not in <i>E. coqui</i> ?
Levator clavicularae ("levator scapulae inferior" D&T 86)
Appendicular pectoral and arm
Pectoralis
Supracoracoideus (part of "coracoradialis" D&T 86)
Deltoides scapularis ("dorsalis scapulae" D&T 86)
Procoracohumeralis ("deltoideus" D&T 86)
Subcoracoscapularis ("subscapularis" D&T 86)
Latissimus dorsi
Triceps brachii
Coracoradialis (part of "coracoradialis" D&T 86)
Coracobrachialis

(plus the prepollex, which is not homologous to a true digit) from ulnar to radial and, respectively, to metacarpals V, IV, III, and II (Fabrezi and Alberch, '96; Fabrezi, 2001; Tokita and Iwai, 2010).

## RESULTS

The results of our anatomical and developmental analyses are summarized in Tables 1–4 and shown in Figures 1–7. Therefore in this Section we will provide, for each region of the forelimb and of the hindlimb, a brief description of the muscles of the *E. coqui* adults and froglets, and then of the development of these muscles until the froglet/adult stage, following the order shown in Tables 1–4. When there are significant differences between our developmental observations and the scarce information provided by other authors about the ontogeny of the forelimb or hindlimb muscles of other frogs, these will be stated in the text.

### Pectoral and Arm Muscles

In adults and froglets the *serratus anterior* and *rhomboideus* (Fig. 1A) run from vertebrae to the dorsomedial portion of the suprascapula. The *rhomboideus occipitalis* (Fig. 1A) runs from the anteromesial surface of the suprascapula to the posterior region of the skull (Table 1). The *levator scapulae superior*, *levator clavicularae*, and *opercularis* connect the opercular region of the skull to the suprascapula, lying mainly deep to head muscles such as the depressor mandibulae (we could not discern if the columellaris, which is often present in anurans and is related

**Table 2.** Ventral/flexor forearm, hand, and dorsal/extensor forearm muscles of adults of *E. coqui*, following the nomenclature of Diogo and Abdala (2010) and Diogo and Tanaka (2012); some synonyms commonly used in the literature about anurans are given (D&T 86 means "sensu Duellman and Trueb, '86").

Ventral forearm
Pronator quadratus ("abductor pollicis" D&T 86)
Contrahentium caput longum ("ulnocarpalis/intercarpalis" D&T 86)
Flexor accessorius ("palmaris profundus" D&T 86)
Flexor digitorum communis ("palmaris longus" D&T 86)
Flexor carpi ulnaris
Epitrochleoanconeus ("epitrochleocubitalis" D&T 86)
Flexor carpi radialis
Pronator teres ("flexor antibrachii medialis" D&T 86)
Hand
Flexores breves superficiales ("lumbricales breves" D&T 86)
Lumbricales ("lumbricales longi" D&T 86)
Contrahentes digitorum (might include "adductor pollicis" D&T 86)
Flexores breves profundi (probably include "opponens pollicis" D&T 86)
Flexores digitorum minimi ("flexores teretes" D&T 86) present or not in <i>E. coqui</i> ?
Interphalangei
Abductor digiti minimi ("abductor primus IV" + probably "abductor secundi IV," D&T 86)
Intermetacarpales ("transversi metacarpi" D&T 86)
Dorsal forearm
Extensor carpi radialis ("extensor carpi radialis caput inferius" D&T 86)
Brachioradialis ("flexor antibrachii lateralis superficialis + perhaps profundus" D&T 86)
Supinator ("flexor antibrachii lateralis profundus" or part of "extensor carpi radialis" D&T 86)
Extensor carpi ulnaris
Anconeus ("epicondylo-cubitalis" D&T 86)
Extensor digitorum
Extensores digitorum breves ("extensores digitorum breves superficiales and medii" D&T 86)
Dorsometacarpales ("extensores digitorum breves profundi" D&T 86)
Abductor pollicis longus ("abductor indicis longus" D&T 86)

to the three former muscles, was present or not in the *E. coqui* adults and froglets examined). The *pectoralis* (Fig. 1B) has a anterior pars epicoracoidea originating from the epicoracoid cartilage, a pars sternalis originating from the sternum and a posterior pars abdominalis originating from the rectus abdominis, the three portions inserting onto the proximal portion of the

**Table 3.** Appendicular pelvic and thigh muscles of adults of *E. coqui*, following the nomenclature of Diogo (in press); some synonyms commonly used in the literature about anurans are given (D 60 means "sensu Dunlap, '60").

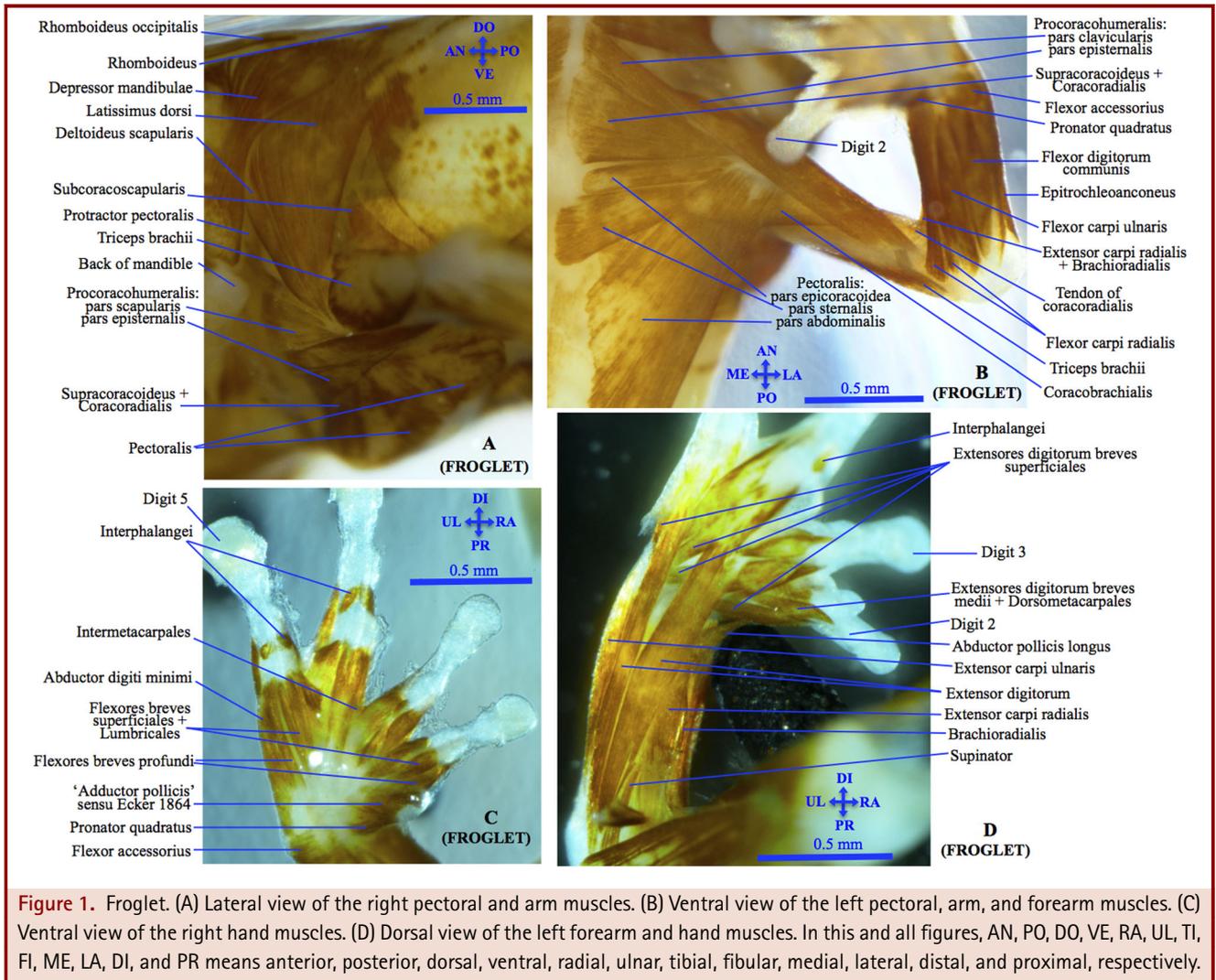
Dorsal mass
Posterior
Iliofemoralis
Tenuissimus ("iliofibularis" D 60)
Extensor ilirotibialis A ("tensor fasciae latae" D 60)
Extensor ilirotibialis B ("gluteus maximus or maximus" D 60)
Cruralis
Anterior
Puboischiofemoralis internus A ("iliacus internus" D 60)
Puboischiofemoralis internus B ("iliacus externus" D60)
Ventral mass
Adductors
Adductor femoris ("adductor magnus" D 60)
Pubotibialis A ("sartorius" D 60)
Pubotibialis B ("semitendinosus" D 60)
Gracilis major
Gracilis minor
Hamstrings
Ischioflexorius ("semimembranosus" D 60)
Caudofemoralis ("piriformis" D 60)
Ischiotrochantericus
Puboischiofemoralis externus A ("pectineus" D60)
Ischiotrochantericus A ("gemellus" D 60)
Ischiotrochantericus B ("obturator externus" D 60)
Ischiotrochantericus C ("quadratus femoris" D 60)
Ischiotrochantericus D ("obturator internus" D 60)

humerus. The *supracoracoideus* and *coracoradialis* (Fig. 1A, B) are deeply blended to each other, running from the ventromedial surface of the pectoral girdle to the proximal humerus, near the insertion of the pectoralis, and then sending a long tendon to the proximal region of the radio-ulna. The *deltoideus scapularis* (Fig. 1A) extends from the suprascapula to the proximal humerus. The *procoracohumeralis* (Fig. 1A, B) has three portions; a pars episternalis running from the omosternum to the distal humerus and a pars clavicularis and a pars scapularis running from the clavicle, scapula and precoracoid to the proximal humerus. The *subcoracoscapularis* extends from the medial portion of the pectoral girdle (the muscle thus cannot be seen in a lateral view of the pectoral girdle) to the proximal humerus. The *latissimus dorsi* (Fig. 1A) runs from vertebrae and dorsal fascia to the proximal humerus. The *triceps brachii* (Fig. 1A, B) is divided into triceps scapularis medialis, triceps humeralis lateralis and triceps humeralis medialis, which originate mainly from the scapula, lateral humerus, and medial humerus, respectively; all three

**Table 4.** Ventral/flexor leg, foot and dorsal/extensor leg muscles of adults of *E. coqui*, following the nomenclature of Diogo (in press); some synonyms commonly used in the literature about anurans are given (D 60 means "sensu Dunlap, '60").

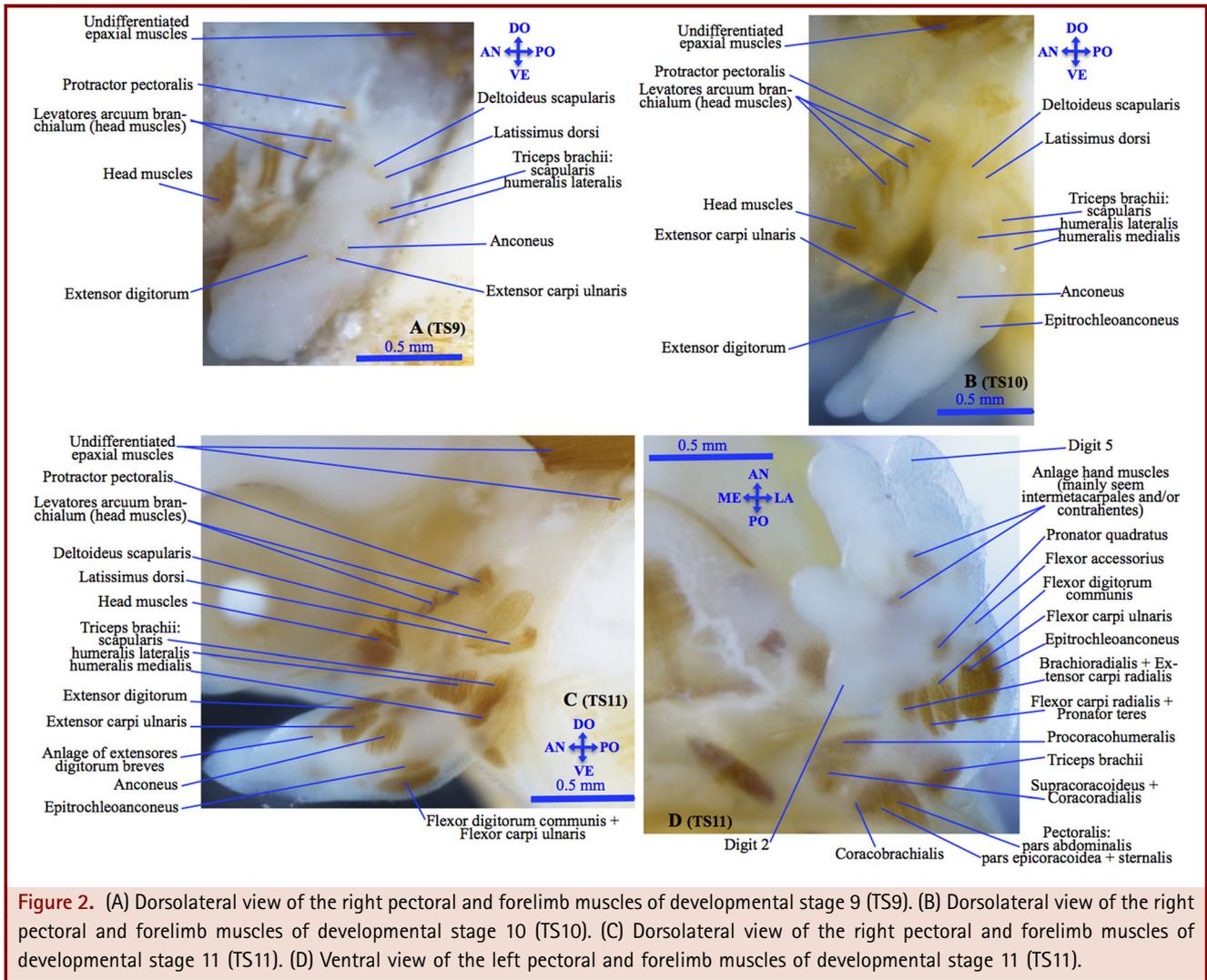
Ventral leg
Long flexors
Flexor digitorum communis ("lantar longus" D 60)
Cruroastragalus ("tibialis posticus" D 60)
Flexor accessorius ("transversus plantae proximalis et distalis" D 60) <sup>a</sup>
Contrahentium caput longum ("tarsalis posticus" D 60) <sup>a</sup>
Interosseus cruris ("intertarsalis" D 60) <sup>a</sup>
Tibialis posterior ("plantaris profundus" D 60) <sup>a</sup>
Foot
Intrinsic foot
Flexores breves superficiales ("flexor digitorum brevis superficialis" D 60)
Lumbricales ("lumbr. breves" plus "lumbr. longi" and "lumbr. longissimus IV" D 60)
Abductor prae hallucis
Abductor digiti minimi ("abductor brevis dorsalis digiti V" D 60)
Contrahentes pedis ("contrahentes digitorum" D 60)
Flexor hallucis accessorius ("opponens hallucis" D 60)
Flexores digitorum minimi ("flexores teretes digitorum" D 60)
Interphalangei
Flexores breves profundi ("flexores ossi metatarsales" D 60)
Abductor brevis plantaris digiti V
Abductor proprius digiti IV
Intermetatarsales ("transversi metatarsi" D 60)
Dorsal leg
Long extensors
Extensor digitorum longus <sup>a</sup>
Tarsalis anticus <sup>a</sup>
Tibialis anticus brevis
Extensor cruris tibialis ("extensor cruris brevis" D 60)
Peroneus
Tibialis anticus longus
Short ext.
Extensores digitorum breves ("extensores breves superficiales and medii" (D 60)
Dorsometarsales ("extensores breves profundi" D 60)
Abductor brevis dorsalis hallucis

bundles are blended distally to insert onto the proximal radio-ulna. The *coracobrachialis* (Fig. 1B) is seemingly differentiated into two bundles that originate from the posteroventral margin of the coracoid and insert onto the proximal humerus (*coracobrachialis brevis*) and onto about 1/2 of the proximo-distal length of the humerus (*coracobrachialis longus*).



Regarding the development until the adult/froglet stage, the first appearance of stained pectoral and arm muscles is at TS9 (Fig. 2A): the deltoideus scapularis, latissimus dorsi, and at least some bundles of the triceps brachii (scapularis and humeralis lateralis) are differentiated on the dorsal side of the forelimb; the procoracohumeralis, supracoracoideus + coracoradialis, and pectoralis are differentiated on the ventral side of this limb (it was not possible to discern if the deeper coracobrachialis was also differentiated, or not, at this stage). The configuration at TS10 (Fig. 2B) is similar to that at TS9, but the pars humeralis medialis of the triceps brachii is now also clearly visible. At TS11 (Fig. 2C, D) the pectoral appendicular muscles (Table 1) are clearly extending medially toward the midline of the body. The coracobrachialis is visible at this stage but it is not possible to discern if it is already differentiated into a coracobrachialis brevis and a coracobrachialis longus because it lies deep (dorsally in a ventral view) to the

pectoralis, which is now differentiated into a pars abdominalis and a pars epicoracoidea + sternalis (Fig. 2D). At TS12 (Fig. 3A, B) the appendicular pectoral muscles are even more extended medially toward the midline of the body, while the arm muscles are extended distally toward the elbow. At this stage all the bundles of the pectoralis and of the procoracohumeralis are clearly differentiated (Fig. 3B). From this stage to the froglet and adult stages the major changes of the muscles therefore concern their change in size. Interestingly, none of the axial pectoral muscles listed in Table 1 appeared as a clearly stained and differentiated muscle in our TS9, TS10, TS11, and TS12 specimens (Figs. 2 and 3). This is probably due to the fact that the axial pectoral muscles are often deep muscles that are covered superficially by the appendicular pectoral muscles (Diogo and Abdala, 2010; Valasek et al., 2011), and not to the absence of all the axial pectoral muscles in these stages of development (e.g., these muscles appear early in



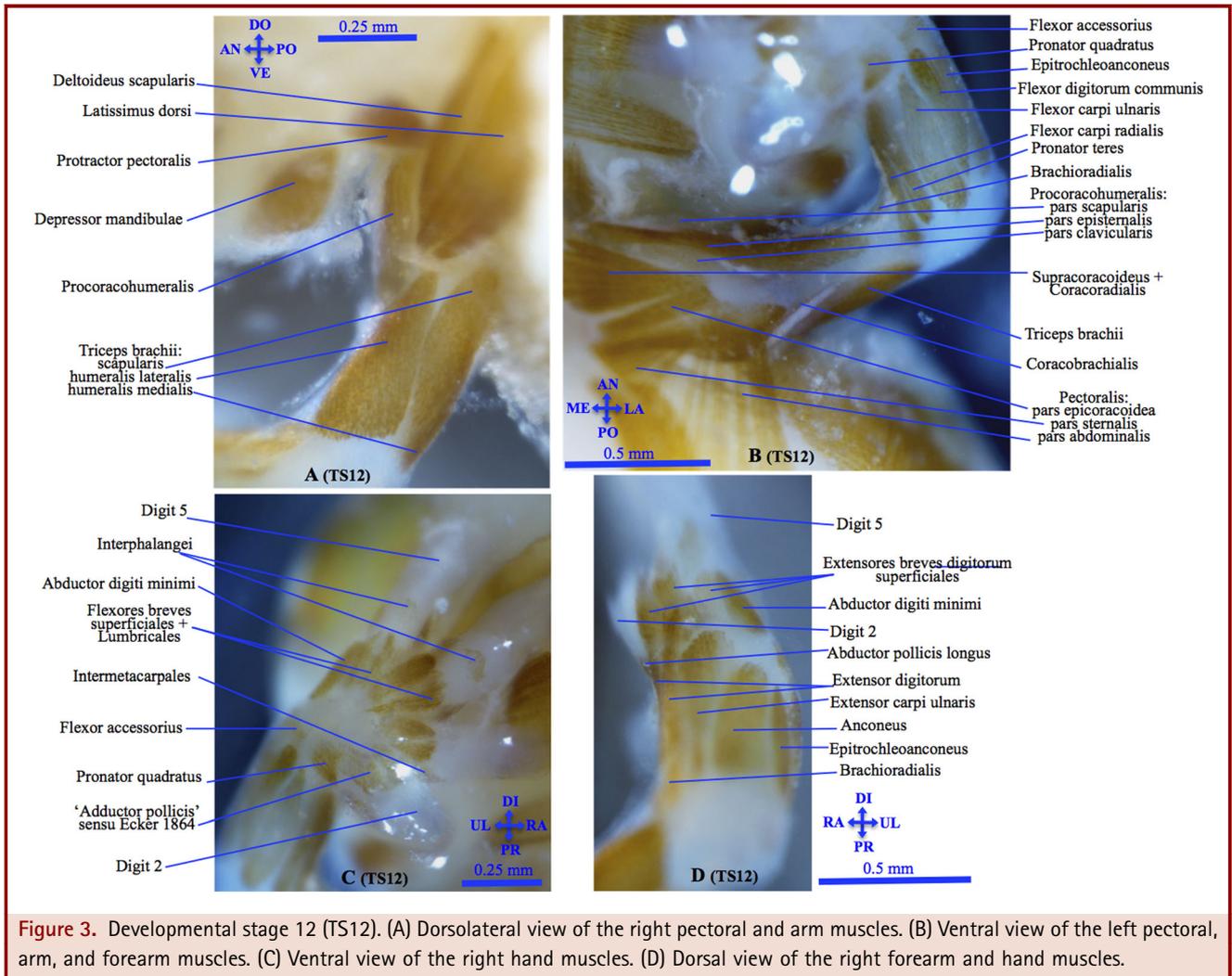
**Figure 2.** (A) Dorsolateral view of the right pectoral and forelimb muscles of developmental stage 9 (TS9). (B) Dorsolateral view of the right pectoral and forelimb muscles of developmental stage 10 (TS10). (C) Dorsolateral view of the right pectoral and forelimb muscles of developmental stage 11 (TS11). (D) Ventral view of the left pectoral and forelimb muscles of developmental stage 11 (TS11).

the ontogeny of the urodele amphibian *Necturus*: Chen, '35; see Discussion). It should also be noted that since TS9 the protractor pectoralis is present; this muscle clearly develops in the head region, together with the levatores arcuum branchialium (Fig. 1), confirming the idea that anatomically this muscle develops as a head branchial, and not a true pectoral muscle (see Discussion section).

#### Ventral/Flexor Forearm Muscles

In adults and froglets the *pronator quadratus* (Fig. 1B, C) connects the ventral surface of the carpal region/radio-ulna to the “prepollex” and/or metacarpal II (Table 2). The *contrahentium caput longum* lies deep (dorsal) to the long flexors of the forearm and runs from the distal radio-ulna to the ulnare. The *flexor accessorius* (Fig. 1B, C) peculiarly lies mainly radial to

the flexor digitorum communis and connects the distal radio-ulna to the palmar aponeurosis, being deep (dorsal) to the tendons of the flexor digitorum communis. The *flexor digitorum communis* (Fig. 1B) originates from the medial surface of the distal humerus and elbow joint to digits 3, 4, and 5 and seemingly also to digit 2. The *flexor carpi ulnaris* (Fig. 1B) runs from the medial surface of the distal humerus to the carpal region. The *epitrochleoanconeus* (Fig. 1B) lies between the flexor digitorum communis and the dorsal muscle extensor carpi ulnaris, running from the proximal humerus to the distal radio-ulna. The *flexor carpi radialis* (Fig. 1B) runs from the distal humerus to the carpal region. The *pronator teres* runs from the medial surface of the distal humerus to the radial side of the radio-ulna, the landmark separating the forearm extensors, including the brachioradialis, and the forearm flexors, including

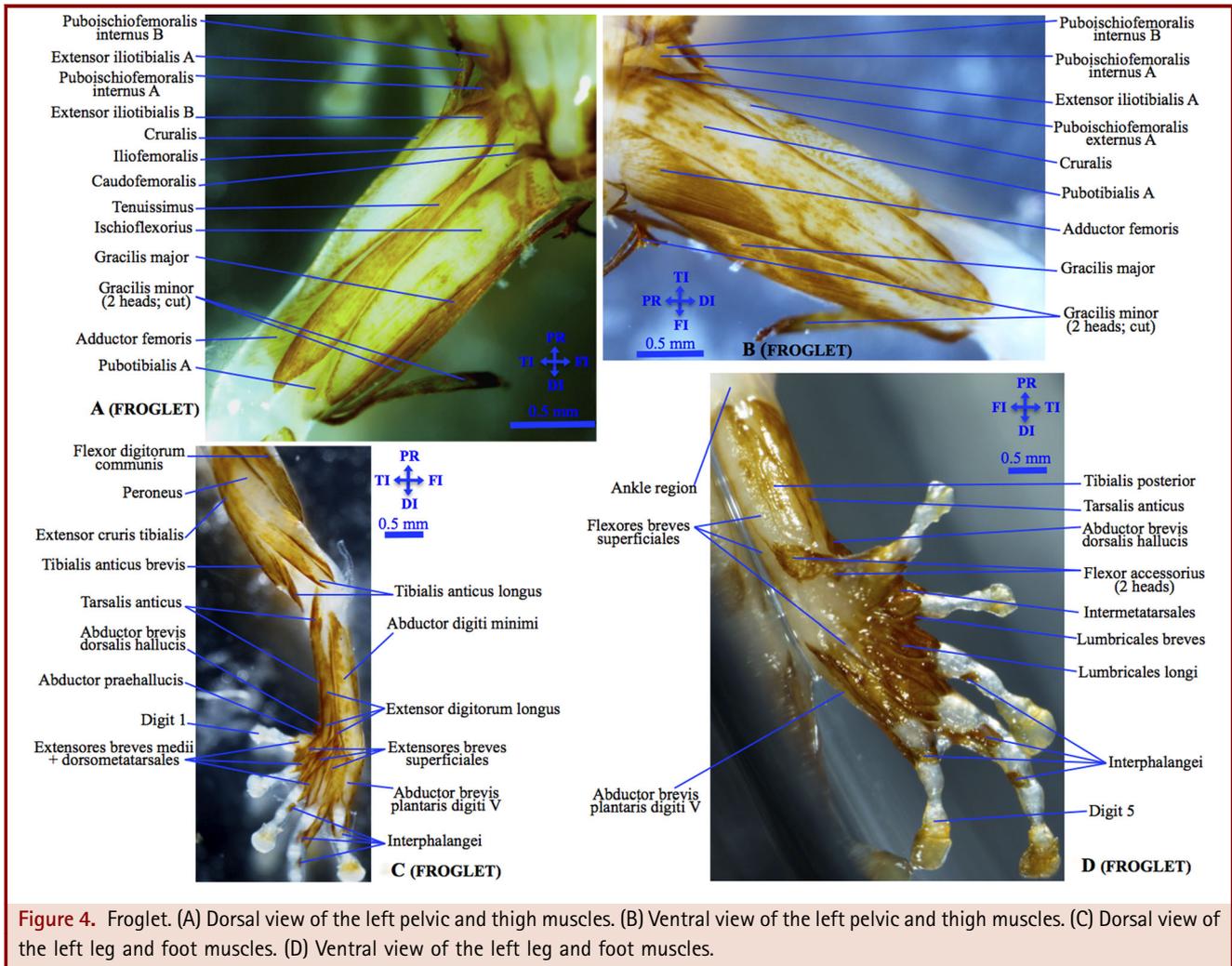


the flexor carpi radialis and pronator teres, being the tendon of the coracoradialis (Fig. 1B).

Concerning the development until the adult/froglet stage, it was difficult to discern if the ventral forearm muscles were already present as distinct structures at TS9, with exception to the epitrochleoanconeus, which seemingly started to form at this stage. At TS10 and TS11 (Fig. 1B–D) all the ventral forearm muscles are clearly differentiated, with two exceptions: the pronator teres, which seems to be still undifferentiated from the flexor carpi radialis, and the *contrahentium caput longum*, which might be present but is difficult to visualize because of its deep position within the ventral forearm musculature. All the ventral forearm muscles are present and more clearly separated from each other at TS12 (Fig. 3B–D), where they are more elongated proximodistally and their shape more closely resembles the configuration found in later stages, including froglets and adults.

### Intrinsic Hand Muscles

The *flexores breves superficiales* and the *lumbricales* (Fig. 1C) are the most superficial (ventral) intrinsic muscles of the hand, running from the carpal region to the digits, each digit receiving one or more of these muscles (Table 2). The *contrahentes digitorum* constitute the second layer of intrinsic hand muscles and run from the carpal region to the proximal region of digits 2, 3, 4, and 5. The “*adductor pollicis*” (Fig. 1C) sensu Ecker (1889), Gaupp (1896) and Duellman and Trueb ('86) might correspond to part or the totality of the *contrahens* to digit 2; however, because this “*adductor pollicis*” extends very radially and reaches the prepollex, it might well be actually part of the pronator quadratus instead. The *flexores breves profundi* (Fig. 1C) form the third layer, running from the carpal region to the radial and ulnar sides of the each digit, being therefore eight muscles in total, the most radial one probably corresponding to the “*opponens pollicis*” sensu sensu



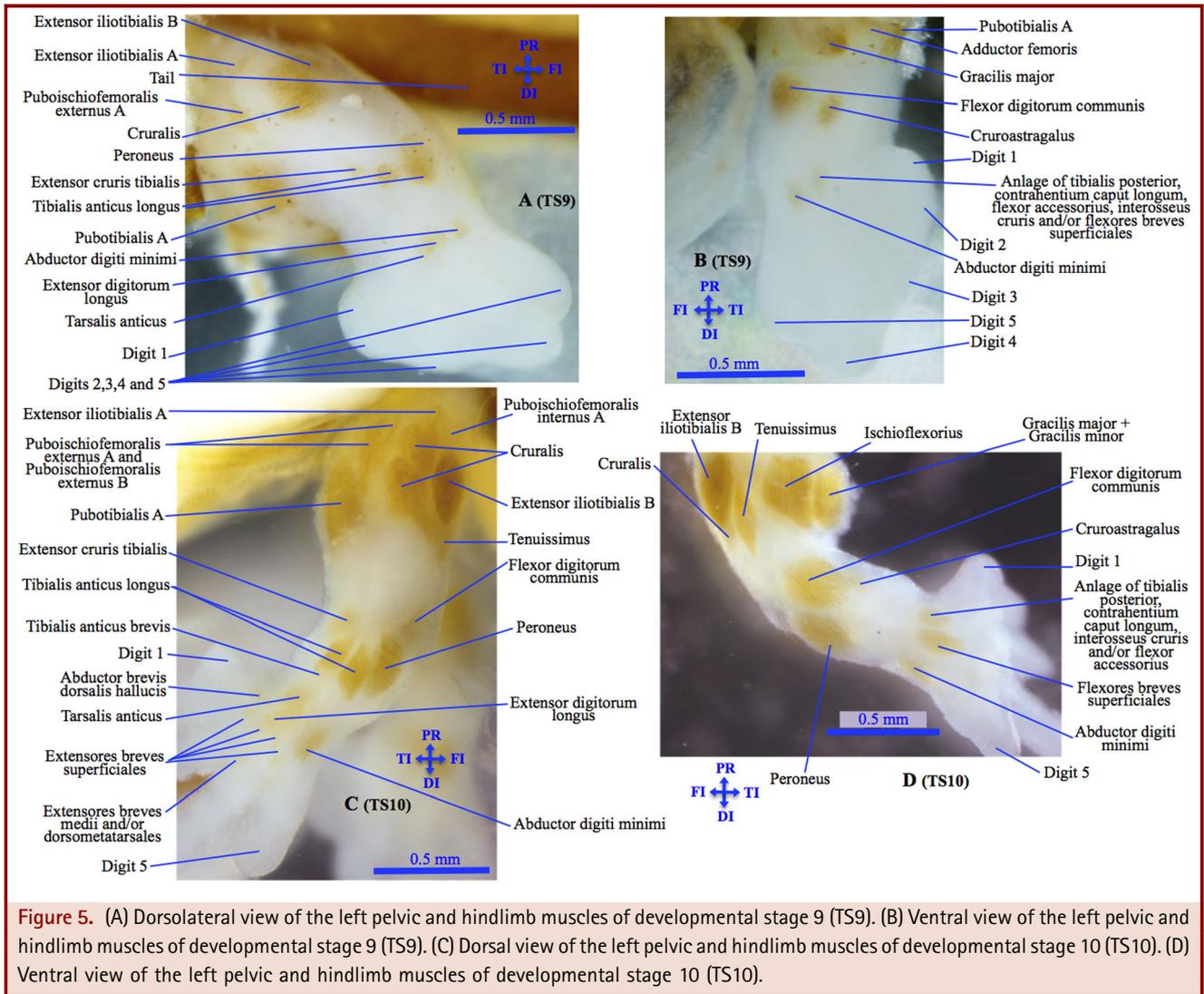
Ecker (1889) and Duellman and Trueb ('86). The *flexores digitorum minimi* of other frogs are short, thin and very deep muscles that lie in the central part of the ventral surface of the digits; we could not find these muscles in the adults and froglets examined, either because they are very deep and/or deeply blended with other hand muscles, or because they are absent. The *interphalangei* (Fig. 1C, D) run from the proximal phalanx to the middle phalanx of digits 4 (interphalangeus IV) and 5 (interphalangeus V). The *abductor digiti minimi* (Fig. 1C) extends from the ulnar portion of the carpal region to the ulnar side of the proximal surface of digit 5. There are three *intermetacarpales* (Fig. 1C) connecting the four digits/metacarpals.

During the development until the adult/froglet stage, the first appearance of intrinsic hand muscles is at TS11, in which the contrahens of digit 4 and the intermetacarpalis connecting digits 3 and 4, and seemingly also the intermetacarpalis connecting digits

4 and 5, are starting to form (Fig. 2D). That the muscles associated with digit 4 are the first to form is somewhat expected by what is known about the skeletal development of the hand in *E. coqui*: the phalanges form in the order digit 4, 5, 3, and only then 2, that is, the more central/ulnar digits form before the more radial ones (Hanken et al., 2001; see Discussion section). At TS12 all the hand muscles are present, but some are clearly just starting to form, as for instance the interphalangeus V (Fig. 3C, D). At later stages the configuration of the hand muscles is essentially similar to that seen in froglets and adults.

#### Dorsal/Extensor Forearm Muscles

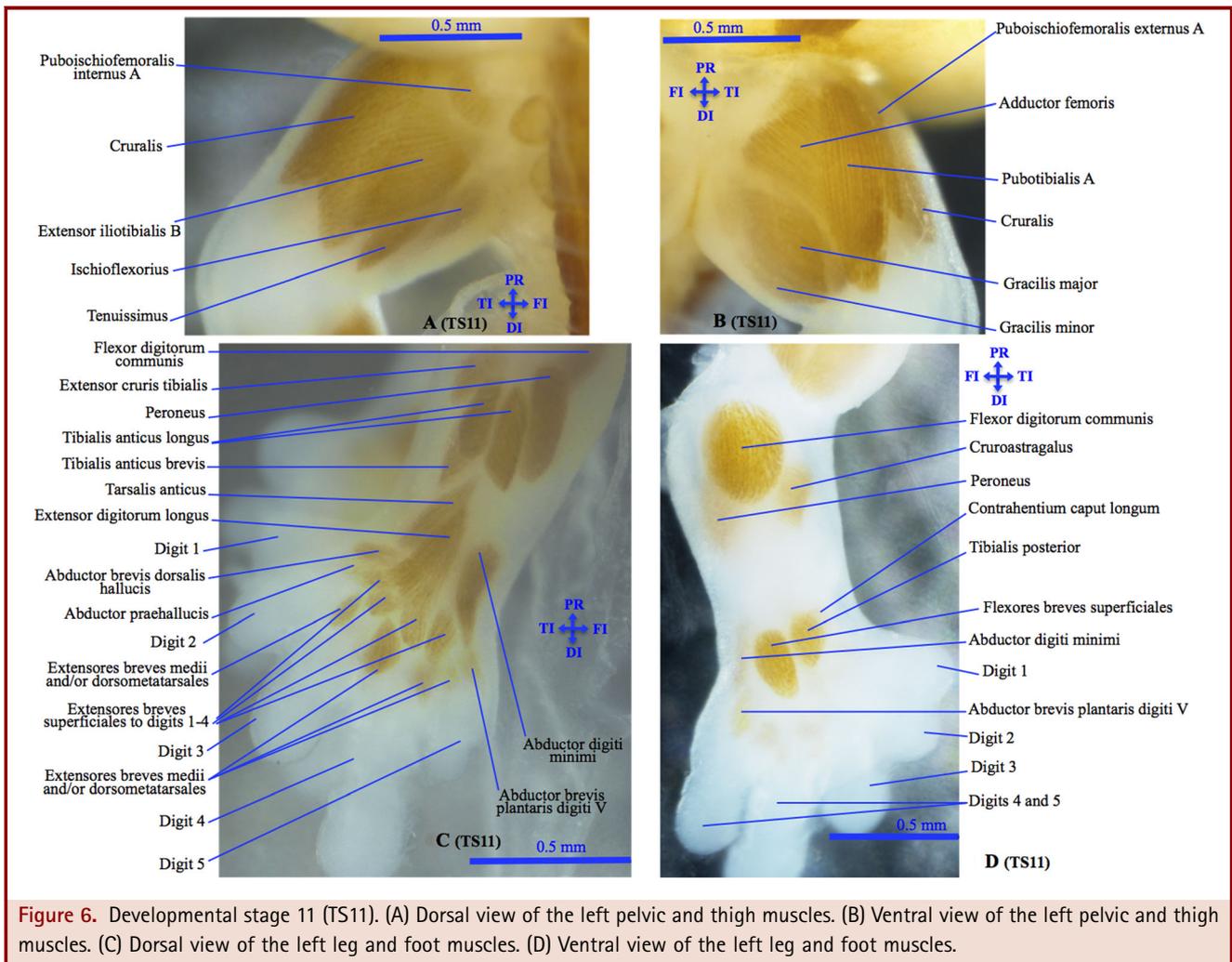
In adults and froglets the *extensor carpi radialis* (Fig. 1B, D) is well differentiated from the supinator and from the brachioradialis, running from the distal humerus and elbow joint to the carpal region, passing deep (ventrally) to the abductor pollicis longus



(Table 2). The *brachioradialis* (Fig. 1B, D) is radial to the extensor carpi radialis, running from the radial surface of the distal humerus to the radial side of the carpal region. The *supinator* (Fig. 1D) is a short muscle lying on the ulnar side of the extensor carpi radialis and connecting the distal humerus to the proximal radio-ulna. The *extensor carpi ulnaris* (Fig. 1D) extends from the lateral surface of the distal humerus to the ulnar side of the carpus. The *anconeus* lies just ulnar and somewhat deep to the extensor carpi ulnaris, connecting the proximal humerus to the distal radio-ulna. The *extensor digitorum* (Fig. 1D) is one of the most superficial (dorsal) of the dorsal forearm muscles, originating from the lateral portion of the distal humerus and going to digits 3, 4, and 5. There are four *extensores digitorum breves superficiales* (Fig. 1D) running from the ulnar side of the carpal region to digits 2, 3,

4, and 5. There are also four *extensores digitorum breves medii* and *dorsometacarpales* (Fig. 1D), which are deep to the *extensores digitorum breves superficiales* and lie close to each other, running from the carpal region to all four digits. The *abductor pollicis longus* (Fig. 1D) runs from the radio-ulna to metacarpal II, passing superficially (dorsal) to the extensor carpi radialis.

Within the development until the adult/froglet stage, the first appearance of dorsal forearm muscles is at TS9, in which the extensor digitorum, extensor carpi ulnaris and anconeus are differentiated (Fig. 2A). The configuration at TS10 is similar (Fig. 2B) but at TS11 these muscles are clearly more developed and the anlage of the *extensores breves digitorum* is present; the *brachioradialis* and *extensor carpi radialis* are however seemingly still undifferentiated from each other, and the *supinator* seems to

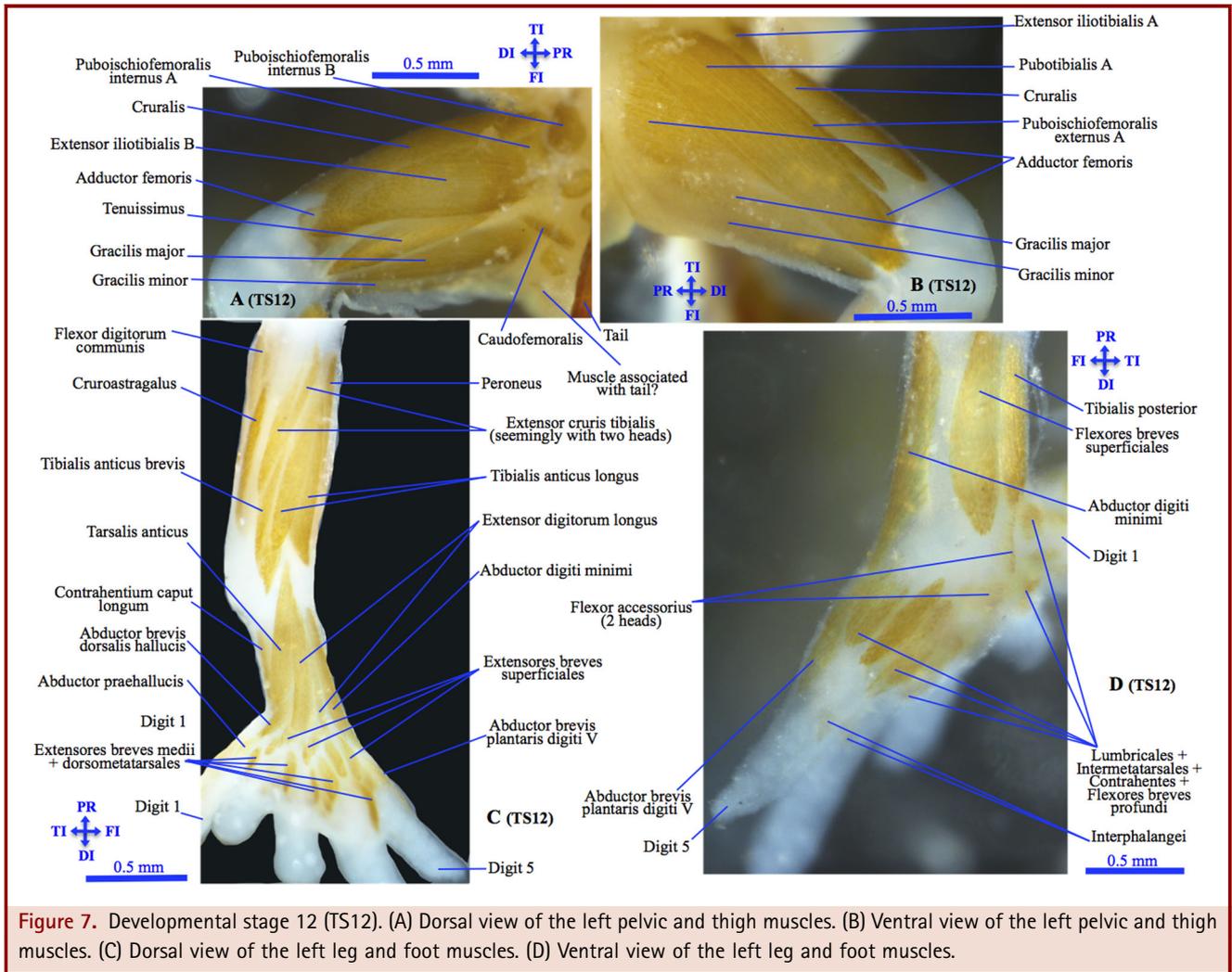


be somewhat differentiated but is still very small (Fig. 2C, D). At TS12 all the dorsal forearm muscles are present, but some are clearly just starting to form, as for example some of the short extensors of the digits (Fig. 3B, D). At later stages the configuration of the hand muscles is essentially similar to that seen in froglets and adults.

#### Pelvic and Thigh Muscles

In the froglets and adults the *iliofemorales* (Fig. 4A) runs from the ilium to the dorsomedial border of the femur (Table 3). The *tenuissimus* (Fig. 4A) connects the ilium to the aponeurosis of the knee. The *extensor iliotibialis A* (Fig. 4A, B) originates from the ilium and then blends with the extensor iliotibialis B and the cruralis. The *extensor iliotibialis B* (Fig. 4A) originates from the ilium and blends with the cruralis. The *cruralis* (Fig. 4A, B) connects the hip joint capsule to the distal femur and proximal

cruris. The *puboischiofemorales internus A* (Fig. 4A, B) runs from the ilium to the dorsal and medial region of the femur, while the *puboischiofemorales internus B* (Fig. 4A, B) connects the ilium to the posterodorsal surface of the caput femoris. The *adductor femoris* (Fig. 4A, B) originates by two heads from the pelvis and runs to the femur, the ventral head fusing to the dorsal head and with an accessory head of the pubotibialis B. The *pubotibialis A* (Fig. 4A, B) originates from the ilium and pubis and then blends with muscles such as the cruralis and pubotibialis B. The *pubotibialis B* is a deep muscle connecting the pelvic rim to the proximal cruris. The *gracilis major* (Fig. 4A, B) runs from the pelvis (one head) and skin (other head) to the knee aponeurosis and the proximal cruris. The *gracilis minor* (Fig. 4A, B) runs from the pelvis (one head) and skin (other head) to the knee aponeurosis and the proximal cruris together with the gracilis major. The *ischioflexorius* (Fig. 4A) connects the ischium to the distal femur and proximal cruris. The *caudofemorales*



**Figure 7.** Developmental stage 12 (TS12). (A) Dorsal view of the left pelvic and thigh muscles. (B) Ventral view of the left pelvic and thigh muscles. (C) Dorsal view of the left leg and foot muscles. (D) Ventral view of the left leg and foot muscles.

(Fig. 4A) runs from the coccyx to the proximal femur. The *puboischiofemoralis externus A* (Fig. 4B) runs from the ischium and pubis to the femur; as noted by Dunlap ('60) the *puboischiofemoralis externus B*, or “adductor longus,” is not present as a distinct muscle in *E. coqui*, being probably fused with the *puboischiofemoralis A*. The *ischiotrochantericus A* and *ischiotrochantericus C* are deep pelvic muscle running from the ischium to the proximal femur. The *ischiotrochantericus B* and *ischiotrochantericus D* are also deep pelvic muscles, running from the pubis and from the entire surface of the pelvic rim, respectively, to the femur.

During development until the adult/froglet stage, the first appearance of pelvic and thigh muscles is seen at TS9, where all the superficial muscles seen in froglets (Fig. 4A, B) are already differentiated (Fig. 5A, B). As in froglets and adults, the *puboischiofemoralis externus B* is not differentiated, being

probably included in the anlage of the *puboischiofemoralis externus A*. Interestingly, these two muscles seem to be somewhat differentiated at TS10 (Fig. 5C), to then become seemingly fused again at later stages, as they are in froglets and adults. In frogs such as *R. pipiens* the *puboischiofemoralis externus A* and *puboischiofemoralis externus B* are also incorporated in a single anlage at earlier developmental stages and then become separated muscles at later stages, but contrary to *E. coqui* they remain separate in adults (Dunlap, '66). Manzano et al. (2013) reported that at early developmental stages of frogs such as *P. borelli* there is a *puboischiofemoralis externus A* and *B* + *puboischiofemoralis internus A* and *B* complex. Dunlap ('66) also reported that at early stages of *R. pipiens* these muscles form a continuous sheet despite the fact that they form from different anlagen. At early developmental stages of *E. coqui* the *puboischiofemoralis internus* is in close proximity of the

puboischiofemoralis A and B, but these structures do not seem to form a continuous sheet (e.g., Fig. 5A). Also interestingly, a main difference between both TS9 and TS10 and later stages in *E. coqui*, including froglets and adults, is that at TS9 and TS10 the extensor iliobtibialis B is not yet fused with the cruralis and is actually superficial (dorsal) to all the other dorsal pelvic/thigh muscles (Figs. 5A, C, D, compare with, e.g., Fig. 6C; see Table 3). At TS11 all the pelvic/thigh muscles are present, including the gracilis major and gracilis minor, which, contrary to previous stages, now seem to be more differentiated. However, the overall configuration of the pelvic/thigh muscles is still markedly different from that seen at later stages, for instance the muscles are in general less elongated proximodistally, and the fibers of the pubotibialis A have a marked tibio-fibular direction (Fig. 6B, compare with Figs. 7B and 4B). On both sides of at least one of the TS12 specimens examined there is an additional muscle originating just posteriorly to the caudofemoralis from the region of the tail and running anterolaterally to the pelvis/thigh (Fig. 7A). This additional muscle seems to be a muscle associated with the tail, such as are for instance the muscles of the caudofemoralis group, and it is not present at later stages of development, perhaps due to the disappearance of the tail. It does not seem to correspond to the compressor cloacae or circumflex arteriae of Figure 11 of Dunlap ('66), which however are also derived from the anlage of the caudofemoralis according to that author. At later stages of development the configuration of the muscles is essentially similar to that seen in froglets and adults.

#### Ventral/Flexor Leg Muscles

In froglets and adults the *flexor digitorum communis* (Fig. 4C) runs from the knee region to the distal cruris and proximal tarsus and also to the palmar aponeurosis and therefore indirectly to digits 1 and 2 (Table 4). The *cruroastragalus* runs from the cruris to the tibiale. The *flexor accessorius* (Fig. 4D) is divided into distal and proximal bundles and runs from the fibulare to the dorsal surface of the plantar aponeurosis and the tibiale. The *contrahentium caput longum* is a deep muscle running from the ligamentum calcanei, together with the tibialis posterior, to the tibiale. The *interosseus cruris* is also a deep muscle running from the fibulare and tibiale to the os centrale. The *tibialis posterior* (Fig. 4D) runs from the ligamentum calcanei to the dorsal surface of the aponeurosis plantaris.

Regarding the development until the adult/froglet stage, the first appearance of ventral leg muscles is seen at TS9, in which the flexor digitorum communis and the cruroastragalus, as well as an anlage that seemingly includes the flexor accessorius, contrahentium caput longum, interosseus cruris, and tibialis posterior and probably also the foot muscles flexores breves superficiales, are present (Fig. 5B). Interestingly, this anlage lies very distant, proximodistally, to the two former muscles, lying almost as distally as the intrinsic foot muscle abductor digiti minimi (Fig. 5B). In this respect the development of *E. coqui* is very similar

to that of *R. pipiens*, in which at early stages the ventral leg muscles consist of a separate flexor digitorum communis, a separate cruroastragalus, and an anlage that includes ventral leg muscles such as the interosseus cruris, tibialis posterior, and contrahentium caput longum but also the flexores breves superficiales of the foot (Dunlap, '66). Our observations thus also support Manzano et al.'s (2013) statement that, at early developmental stages of frogs such as *P. borelli*, the tibialis posterior and flexores breves superficiales derive from the same anlage. At TS10 the flexor digitorum communis and cruroastragalus are much more developed, and what seems to be the anlage of the foot muscles flexores breves superficiales can now be seen just next to what seems to be the anlage of the flexor accessorius, contrahentium caput longum, interosseus cruris, and tibialis posterior (Fig. 5D). At TS11 the latter anlage is already differentiated into at least some individual muscles such as the contrahentium caput longum and the tibialis posterior, which lie on the tarsal region and markedly far from the cruroastragalus and flexor digitorum communis (Fig. 6D). At TS12 all the ventral leg muscles are seemingly differentiated (the interosseus cruris cannot be seen but this is a very deep muscle) and their overall configuration is similar to that seen in froglets and adults, including the division of the flexor accessorius into two heads (Fig. 7D).

#### Intrinsic Foot Muscles

In adults and froglets the *flexores breves superficiales* (Fig. 4D) run from the ligamentum calcanei to the plantar aponeurosis, then giving rise to the superficial flexor tendons of digits 3, 4, and 5 (Table 4). The *lumbricales breves* (Fig. 4D) run from the aponeurosis plantaris to the metatarsophalangeal joint of digits 1–5, by lateral and medial muscles to digit 1, to digit 4 and to digit 5, and by a single muscle to digit 2, and a single muscle to digit 3. The *lumbricales longi* (Fig. 4D) run from the aponeurosis plantaris to the second phalanx of digit 3, the interphalangeal joint capsule of digit 4, the second phalanx of digit 4 (this is usually considered to be a separate “lumbricalis longissimus digiti IV”) and the proximal interphalangeal joint of digit 5. The *abductor prae hallucis* (Fig. 4C) runs from the aponeurosis plantaris to the prehallux. The *abductor digiti minimi* (Fig. 4C) runs mainly from the fibulare to the proximal portion of metatarsal V. The single *contrahens* muscle runs from the tarsal region to the metatarsophalangeal joint of digit 1. The *flexor hallucis accessorius* runs from the tarsal region to metatarsal I. The *flexores digitorum minimi* are deep muscles running mainly from the ventral margin of the metatarsals to the ventral base of the proximal phalanges of digits 2, 3, 4, and 5. There are four *interphalangei* (Fig. 4C, D): the interphalangeus of digit 3, one of the interphalangei of digit 4, and the interphalangeus of digit 5 connect the proximal and second phalanges of digit 3, 4, and 5, respectively; the other interphalangei of digit 4 connects the second and third phalanges of that digit. The *flexores breves profundi* are deep foot muscles

running mainly from the fibulare to the metatarsals II, III, and IV. The *abductor brevis plantaris digiti V* (Fig. 4C, D) runs from the fibulare to the lateroventral border of metatarsal V. The *abductor proprius digiti IV* is a deep muscle that connects the metatarsals IV and V, while the four *intermetatarsales* (Fig. 4D) connect the metatarsals I and II, II and III, III and IV, and IV and V.

Concerning the development until the adult/froglet stage, the first appearance of intrinsic foot muscles seems to be at TS9, in which the *abductor digiti minimi* is present and in which there is an anlage that apparently includes some leg muscles plus the *flexores breves superficiales*, as noted above (Fig. 5B). At TS10 there is a separate anlage of the *flexores breves superficiales* lying near the *abductor digiti minimi* (Fig. 5D). At TS11 other foot muscles such as the *abductor prae hallucis* and the *abductor brevis plantaris digiti V* can also be seen (Fig. 6C, D). At TS12 all the foot muscles seem to be present, including the *interphalangei*, which are however still starting to develop. The configuration at later stages is essentially similar to that found in froglets and adults.

#### Dorsal/Extensor Leg Muscles (Table 4)

In the froglets and adults the *extensor digitorum longus* (Fig. 4C) runs from the distal extremity of the cruris to the short extensors of digits 3 and 4 (and sometimes also of digit 5, in some *E. coqui* specimens dissected by Dunlap, '60). The *tarsalis anticus* and *tibialis anticus brevis* (Fig. 4C, D) connect the cruris to the tibiale. The *extensor cruris tibialis* (Fig. 4C) runs from the medial condyle of the femur to the cruris. The *peroneus* (Fig. 4C) runs from the region of the knee joint to the distal cruris and fibulare. The *tibialis anticus longus* (Fig. 4C) runs from the medial condyle of the femur to the fibulare (its medial head) and to the tibiale (its lateral head), the muscle being often fused with the *peroneus*. The *extensores digitorum breves superficiales* (Fig. 4C) mainly run from the fibulare to the proximal phalanges of digits 1–4, while the *extensores digitorum breves medii* (Fig. 4C) mainly run from the fused distal extremities of the tibiale and fibulare to the proximal phalanges of digits 1–4. The *dorsometacarpales* (Fig. 4C) mainly connect the metatarsals to the distal phalanges of digits 1–5. The *abductor brevis dorsalis hallucis* (Fig. 4C, D) runs from the prehallux and os centrale to the dorsomedial border of metatarsal I, being blended with the *extensor brevis superficialis* of digit 1.

With respect to the development until the adult/froglet stage, the first appearance of dorsal leg muscles is at TS9, in which the *extensor digitorum longus* and *tarsalis anticus* are situated markedly distal to the *extensor cruris tibialis*, *peroneus* and *tibialis anticus longus* (Fig. 5A). Also interestingly, the *tibialis anticus longus* is already divided into two bundles, which seems to indicate it might actually derive from two different leg anlagen (Fig. 5A). At TS10 and TS11 all these muscles are more developed, and the *tibialis anticus brevis*, the *abductor brevis dorsalis hallucis*, the *extensores digitorum breves* and at least some *dorsometatarsales* (e.g., that of digit 4) are also present (Fig. 5C). From TS12 on all the dorsal leg muscles seem to be present and

their configuration essentially resembles that found in the froglets and adults.

## DISCUSSION

### The Pectoral Muscles, the "In-Out" Mechanism, and the Protractor Pectoralis

According to Valasek et al. (2011) the superficial girdle muscles ("appendicular pectoral muscles" sensu Table 1) 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 Table 1) are induced by the forelimb field that promotes myotomal extension directly from the somites. According to these authors the appearance of the forelimb is followed by pectoral girdle development that braces the proximal limb to the axial skeleton; the limb program was therefore able to induce and recruit axial structures for its anchorage, such as the medial scapular border in mammals, the scapular blade in birds, and the axial pectoral muscles in all tetrapods. As explained in the Results, the fact that the axial pectoral muscles are often deep girdle muscles probably explains why none of these muscles appeared as a clearly stained and differentiated muscle at the TS9, TS10, TS11 and TS12 specimens examined by us (Figs. 2 and 3). Our observations and comparisons also support Valasek et al.'s (2011) "in-out" mechanism because in earlier ontogenetic stages the appendicular pectoral muscles clearly start to develop very far from the midline at the level of the proximal region of the arm and only later in development extend medially to cover a substantial part of the ventral and dorsal regions of the thoracic region (compare Figs. 1 with 3).

A major evolutionary issue regarding the pectoral region of tetrapods that attracts much attention concerns the muscle protractor pectoralis, mainly due to its implications for the origin and evolution of the neck in vertebrates (Diogo and Abdala, 2010; Ericsson et al., 2012). Edgeworth ('35) defended that the protractor pectoralis is a head muscle derived in both ontogeny and phylogeny from the levatores arcuum branchialium group that has markedly extended posteriorly during tetrapod evolution to cover a substantial part of the neck, pectoral girdle, and back regions, as seen for instance in adult humans (where the muscle gave rise to the trapezius and sternocleidomastoideus). The results of more recent developmental and molecular studies however indicate that the protractor pectoralis of salamanders and the trapezius of chickens and mice (which derives from the protractor pectoralis) are at least partially derived from somites (Matsuoka et al., 2005; Noden and Francis-West, 2006; Piekarski and Olsson, 2007). These studies have also shown that during the ontogeny of mice some of the cells of the trapezius that are originated from the somites pass the lateral somitic frontier in order to develop within lateral plate-derived connective tissue of the forelimb (Shearman and Burke, 2009). That is, according to these studies

the trapezius is a rather peculiar muscle that is seemingly directly associated with three different types of connective tissue: derived from branchial arch crest cells, somite-derived, and lateral plate-derived (forelimb). Therefore, authors have questioned whether the protractor pectoralis and its amniote derivatives trapezius and sternocleidomastoideus are primarily derived from the paraxial mesoderm, as suggested by Edgeworth ('35), and only later became ontogenetically associated with the cranialmost somites and even with lateral plate-derived connective tissue of the forelimb, or are instead primarily derived from somites (Ericsson et al., 2012).

However, recent works have shown that apart from the protractor pectoralis and laryngeal muscles such as the constrictor laryngis and dilatator laryngis, even branchial muscles *sensu stricto* such as the levatores arcuum branchialum and hyoid muscles such as the interhyoideus are also partially derived from somites in tetrapods such as amphibians (Piekarski and Olsson, 2007). Thus, the fact that muscles such as the protractor pectoralis have a partial somitic origin does not necessarily mean that they cannot be considered to be part of the branchial musculature. In fact, Matsuoka et al. (2005) recognize that the amniote trapezius is partially derived from somites, but also argue that the sum of the data available (i.e., innervation, topology, development and phylogeny) provides more support for grouping this muscle, and thus the protractor pectoralis of non-amniote vertebrates, with the branchial musculature. In fact, lineage tracing analyses in transgenic mice provide some support for the idea that the trapezius is effectively a branchial muscle, because they reveal that neural crest cells from a caudal pharyngeal arch travel with the trapezius myoblasts and form tendinous and skeletal cells within the spine of the scapula (Noden and Schneider, 2006). According to Noden and Schneider (2006) this excursion seemingly recapitulates movements established ancestrally, when parts of the pectoral girdle abutted caudal portions of the skull. The innervation of the trapezius by the accessory nerve (CNXI) and, in many cases, by C3 and C4 spinal cord segments supports the idea that the muscle has a branchial component due to the position of the accessory nucleus in the ventral horn of the spinal cord, which is in line with the more cranial branchiomotor nuclei (Wilson-Pauwels et al., 2002). Importantly, the recent study of Theis et al. (2010) strongly suggests that the trapezius and sternocleidomastoideus of birds and mice are essentially head muscles with just a little contribution (about 3% according to them) from somites and that these muscles use genetic pathways of head muscles, develop like head muscles, and attach through connective tissues derived from neural crests as do the head muscles. The results of the present work strongly support the idea that, at least in what concerns its anatomical development, the protractor pectoralis is clearly a head, and namely a branchial, muscle, because at its first appearance this muscle lies just next to, and in line with, the branchial head muscles levatores arcuum branchialum (Fig. 1A). Then, during development, the muscle

extends posteriorly toward the region of the pectoral girdle (Figs. 1A, 2C, and 3A). Our observations of frog development and comparisons with the ontogeny of urodeles clearly indicate that the protractor pectoralis is essentially a posterior member—usually the most posterior—of the levatores arcuum branchialum group (see also Ziermann and Diogo, 2013).

### The Morphogenesis of Frog Limbs

In general, our observations of the development of the fore- and hindlimb muscles in the direct developing *E. coqui* are similar to the scarce detailed information provided in the literature about the ontogeny of the fore- or hindlimb musculature of frogs with a biphasic development (e.g., *R. pipiens*: Dunlap, '66; *P. borelli* and some other frogs: Manzano et al., 2013; the few, minor differences are noted in the Results section). In a recent study Diogo et al. (in press) reported that apart from the known radio-ulnar and proximo-distal morphogenetic gradients (Grim and Carlson, '74), there is also a ventro-dorsal gradient regarding the differentiation and elongation of the forearm and hand muscles in axolotl forelimb regeneration. We use the term “morphogenetic gradient” to refer for instance to a proximo-distal morphogenetic gradient we mean a gradient in the progress of formation and differentiation where the proximal muscles develop and differentiate earlier than the distal elements, with a gradient of progress of differentiation between proximal and distal elements. The radio-ulnar/tibio-fibular and proximo-distal gradients have also been observed in ontogenetic studies of both the fore- and hindlimb muscles of axolotls (Grim and Carlson, '74; Diogo et al., unpublished data). A ventro-dorsal gradient had however not been reported in previous studies of limb muscle regeneration or ontogeny in any tetrapod taxon; in fact, an opposite, dorsoventral gradient was reported in Kardon's ('98) study of the ontogeny of the hindlimb of chickens.

Regarding our present study of the *E. coqui* frog, the proximo-distal gradient is evident in both limbs, with for instance the hand and foot muscles developing later than the other limb muscles (Figs. 2, 3, 5–7). The ventro-dorsal gradient observed in the regeneration of the axolotl forelimb muscles was not observed within the development of the frog forelimb musculature. Interestingly, we observed the opposite gradient, that is, a dorsoventral gradient, within the development of the frog hindlimb muscles, which was also reported in Kardon's ('98) study of the hindlimb musculature of chickens. This is seen for instance at TS9, where five anlagen/muscles of the long extensors of the leg are visible, against only three anlagen/muscles of the long flexors of the forearm (Fig. 5A, B; see Table 4). It is particularly seen at T11, in which, in a dorsal view of the leg and foot, the muscles are already almost all present and well developed (Fig. 6C), in marked contrast with the few muscles that can be seen in a ventral view of the leg and foot (Fig. 6D). Also, instead of a radio-ulnar gradient as seen in the musculature of both limbs of axolotls, there is mainly a ulno-radial/fibulo-tibial gradient in the

musculature of the fore- and hindlimbs of frogs. Such a gradient is seen for instance in the forearm, where the ulnar muscles extensor carpi ulnaris and flexor carpi ulnaris differentiate before, and then expand faster, than the radial muscles extensor carpi radialis and flexor carpi radialis (see Results section). It is also seen for example in the hand, where the contrahentes and intermetatarsales associated with digits 4 and 5 differentiate before the muscles associated with digit 2 (see Results section). This makes sense in view of what is known about the ontogeny of the skeletal structures of the hand of *E. coqui*: the first phalanges to form are those of digit 4, then 5, then 3, and only then 2, that is, the more central/ulnar digits form before the more radial ones (Hanken et al., 2001). 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: Carlson, 2007).

#### The Fore-Hindlimb Enigma: Serial Homology Versus Homoplasy

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. This is for instance reflected in the phylogenetically older muscles of the girdles, which are present in fish: the muscles of the pectoral girdle are extremely different from the muscles of the pelvic girdle in not only tetrapods but also in gnathostome fish. The only muscles of the pectoral and pelvic appendages that are actually similar are the muscles of the forearm/hand and leg/foot, which are only present in tetrapods and are therefore phylogenetically much more recent. According to this theory, the similarity of the forearm/hand and leg/foot muscles is therefore due to derived homoplastic events and not to serial homology (Diogo et al., 2013). That is, this similarity was acquired during the “fins-limbs transition” due to 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; Pavlicev and Wagner, 2012). The *derived* cooption of a few genes does not represent a true case of forelimb-hindlimb serial homology under the developmental concept of homology. This was recognized by Tabin ('92), who stated that the structures of the fore- and hindlimb of tetrapods evolved independently from the pectoral and pelvic appendages of fish and that despite their general similarity there are significant differences between the fore- and hindlimb patterns that can be seen in fish prior to the evolution of the limb, as noted by Rackoff ('80). According to Tabin ('92) the similarities of the tetrapod fore- and hindlimbs may be a direct consequence of the fact that although the buds of these limbs evolved independently, each

evolved by reorienting the expression of similar genes (e.g., *Hox-1* and *Hox-4*) along orthogonal axes, which have an effect on downstream target genes.

Within the results of the present study there is effectively a marked similarity, both in the larvae and the froglets/adults of *E. coqui*, between the flexores breves superficiales, lumbricales, abductor digiti minimi, contrahentes, interphalangei, flexores breves profundi, and intermetacarpales/intermetatarsales of the hand and foot (compare Figs. 1C, D and 4C, D). Also, the forearm muscle flexor digitorum communis clearly seems to correspond, topologically, to the leg muscle flexor digitorum communis, and there are also similarities between the leg muscle cruroastragalus and the forearm muscles flexor carpi radialis + pronator teres (compare Figs. 5B with 2D; see also Diogo, in press). And, despite the peculiar distal position of the leg muscles flexor accessorius, contrahentium caput longum and interosseus cruris (see below), these muscles share some clear developmental similarities with the forearm muscles flexor accessorius, contrahentium caput longum and pronator quadratus (e.g., all these muscles lie distal, and often deep, to leg/forearm muscles such as the flexor digitorum communis: compare Fig. 5B with 2D). Regarding the dorsal forearm and leg muscles, there is a clear similarity between the extensores breves superficiales, the dorsometacarpales/dorsometatarsales and the abductor pollicis longus/abductor brevis dorsalis hallucis (compare Figs. 3D with 6C).

However, concerning the musculature of the girdles there is in fact no clear similarity or correspondence between any pelvic and any pectoral muscle of *E. coqui*, at any developmental stage including the earlier ones (compare Figs. 2 with 5), as there is not in any other tetrapod taxon analyzed so far. One possible explanation, according to the serial homology hypothesis, would be that the pelvic and pectoral muscles have been so markedly modified during evolution that their similarities were lost. However, both in phylogenetically plesiomorphic gnathostome fish and in anatomically plesiomorphic tetrapods such as salamanders the muscles of the two girdles are extremely different from each other as well (Diogo et al., 2013). Another 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 (e.g., Figs. 2 and 5), as well as in an ongoing study of axolotl limb development using antibody staining and GFP transgenic animals (Diogo and Tanaka, unpublished data), from early ontogenetic stages the muscles of the two girdles are also markedly different from each other. Faced with these facts, a defender of the serial homology hypothesis can still argue that the facts may be explained by a change of the developmental mechanisms during evolutionary history, that is, that serial homologous muscles now develop in totally different ways. There are of course known examples of homologous

structures that exhibit very different developmental mechanisms in different taxa (Gilbert, 2006). However, one may wonder if a hypothesis that has to assume that the ontogeny of each and every pelvic and pectoral muscle had to be dramatically changed during evolution is still a scientific hypothesis that can be fully tested and eventually contradicted. That is, by trying to explain everything, such an hypothesis may actually end by not explain anything at all. This is because there is, for instance, a huge anatomical divergence among taxa in the adult forelimb of tetrapods, from the wings of reptiles such as birds to the pectoral fins of mammals such as dolphins. However, 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 making some muscles developing before others do, in different taxa), but there is still a clearly recognizable pattern/bauplan of the forelimb in all tetrapods (Diogo and Abdala, 2010). 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, in more than two centuries authors have tried without success to find clear resemblances between not only the adult configuration but also the ontogeny of any single pectoral and pelvic muscle. Moreover, we were also unable to find such resemblances in our studies of the development of the hind and forelimbs of the very same species, that is, *E. coqui* (present work), and also of *Ambystoma mexicanum* (Diogo and Tanaka, unpublished data), although a direct comparison of the structures of the girdles in a same species should in theory be particularly appropriate to reveal resemblances between the muscles of these two girdles. Therefore, one can say that our studies, as well as the data provided by other comparative morphologists and comparative developmental anatomists, do provide at least strong evidence against the serial homology hypothesis. It is of course not a definitive refutation of this hypothesis, but it is clearly further anatomical and developmental evidence (together with other lines of evidence, such as paleontological, functional and genetic evidence: see review in Diogo et al., 2013) against it.

#### The Peculiar Anatomical Features of Frog Limbs, Homeotic Transformations, and Digit Reduction

As explained above, one of the reasons leading to the marked topological difference between the anuran dorsal leg and forearm muscles concerns the fact that in the hindlimb of these amphibians the highly elongated tarsal bones tibiale and fibulare somewhat replace functionally the tibia and fibula of other tetrapods. Consequently, in anurans various leg muscles migrated distally to the tarsal region where lie the tibiale and fibulare. These muscles are the ventral leg muscles flexor accessorius, contrahentium caput longum, interosseus cruris, and tibialis posterior and the dorsal leg muscles extensor digitorum longus and tarsalis anticus; the normal position of these muscles, or their homologs, in

tetrapods is to lie on the leg (zeugopodial) region (see Table 4; Note: it is not peculiar that short extensor muscles such as the extensores breves digitorum and dorsometatarsales lie in the foot region because these muscles usually lie, in other tetrapods, in the region of the digits). Interestingly, there is developmental evidence supporting the idea that the transformation of the anuran tibiale and fibulare represents a distal shift in the zeugo-autopodial border, that is, that homeotically these bones have a zeugopodial (leg) identity (Blanco et al., '98; Wagner and Chiu, 2002).

The present study provides support for the idea that the tibiale and fibulare have zeugopodial identity, because from the first appearance of the leg muscles in *E. coqui* those leg muscles that will peculiarly lie in the tarsal region in the froglets and adults are already situated very far from and distal to the remaining leg muscles (e.g., Fig. 5A, B). This is in clear contrast with what happens with the forearm muscles, as can be seen for instance if one compares the distal position of the leg muscle extensor digitorum longus and its marked proximodistal distance to muscles such as the extensor cruris tibialis (Fig. 5A) with the position of the forearm muscle extensor digitorum and its short proximodistal distance to muscles such as the extensor carpi ulnaris (Fig. 2A), in TS9 specimens. The peculiar position of the leg muscles that lie in the tarsal region of frogs, not only in adults but from the earlier stages of development, and the marked contrast to what happens in the forelimb of frogs and in the hindlimb of other tetrapods including the urodele amphibians (Diogo and Tanaka, unpublished data), do support the idea of a homeotic transformation of the tibiale and fibulare elements into zeugopodial (leg) structures. Importantly, our results suggest that the peculiar position and development of these leg muscles is probably due to the fact that the muscles are following the clues provided by the hard tissues, rather than the muscles themselves being also the subject of a homeotic transformation of foot structures into leg structures. This is because these muscle peculiarities concern the distal displacement of leg muscles to the tarsal region, and not the proximal displacement of intrinsic foot muscles to the leg. That is, all the muscles that lie normally in the proximal ankle region of the foot in tetrapods are still present and lie in the foot region of *E. coqui*, having a clear autopodial identity. So there is no homeotic transformation of foot muscles to a zeugopodial identity, the autopodial muscles remain the same and the zeugopodia muscles remain the same, there is no change in the identity. What seems to be happening is simply that instead of lying in the region of tibia/fibula, as they do in other tetrapods, the most distal leg muscles lie in the region of the tibiale/fibulare, because these bones (not the muscles) changed their identity and are now homeotically zeugopodial bones. However, more mechanistic studies, involving for instance gene expression analyses, are needed to test this hypothesis and to clarify what the interactions are between soft and hard tissues in such cases involving homeotic transformations (see below).

Another peculiarity of the limbs of frogs is that the hand usually has four, and not five, digits. Regarding this digit reduction, if one

compares the muscles of the hand of *E. coqui*, with four digits, and the muscles of the foot of the same species, with five digits, as well as with the hand of tetrapods with five digits, one would think that the most radial digit of the hand of *E. coqui* is digit 1. This is because this species has a muscle that goes to digit 1 in most tetrapods: the abductor pollicis longus (Fig. 1D), which develops exactly as it develops in connection with digit 1 in other tetrapods, that is, lying radial and somewhat deep to the extensor digitorum and running distoradially to attach onto metacarpal I/digit 1 (Fig. 3D; see Diogo and Abdala, 2010; Diogo and Tanaka, unpublished data). Apart from this muscle, there is also an “opponens pollicis” and an “adductor pollicis” sensu Ecker (1889). These muscles are not homologous to the opponens pollicis and the adductor pollicis of tetrapods such as humans, because the last common ancestor of amphibians and amniotes did not have these muscles. However, the two frog muscles seem to derive from the same groups (flexores breves profundi and contrahentes, respectively) that gave rise to, and do share some anatomical similarities with, the opponens pollicis and adductor pollicis of other tetrapods (see Diogo and Abdala, 2010). Therefore, the presence of these two muscles in frogs also seems to support the idea that these muscles are the result of an homoplasy between frogs and other tetrapods that is due to the specialization of digit 1, and thus the idea that the most radial digit of the frog hand would correspond to digit 1 of other tetrapods (tetrapods almost never have specialized “opponens” or “adductor” muscles to digit 2). There is also a muscle in the frog hand that normally goes to digit 5 in other tetrapods, the abductor digiti minimi (Fig. 1C). This would seem to point out that the most ulnar hand digit of frogs is digit 5. Also, in the *E. coqui* hand there are interphalangei in the two most ulnar digits (one interphalangeus in each digit), while in the *E. coqui* foot there are interphalangei in the three most fibular digits (one interphalangeus in digit 3, two in digit 4 and one in digit 5). Based on what is known about the similarity of the developmental mechanisms involved in the ontogeny of the hand and foot and the resulting striking topological resemblance of the muscles of these autopodia found principally in anatomically plesiomorphic tetrapods (due to homoplasy and not to serial homology: Diogo et al., 2013; Diogo, in press), the overall analysis of the hand muscles and comparison with the foot muscles of *E. coqui* would thus appear to point out that in this species the hand is formed by digits 1, 2, 3, and 5 or 1, 2, 4, and 5 of other tetrapods.

However, although there is still no consensus about the identity of the anuran hand digits, there is some evidence, both from ontogenetic (Fabrezi and Alberch, '96; Fabrezi, 2001) and gene expression studies (e.g., of *hoxd11*: Satoh et al., 2006) that the digit missing is digit 1. Moreover, assuming that the *E. coqui* hand digits correspond to digits 1, 2, 3, and 5 or 1, 2, 4, and 5 of other tetrapods would also seem counterintuitive in face of the fact that in this species the first phalanges to form are those of the two most ulnar digits (Hanken et al., 2001; see above). This is because the last digits to form are normally the first to disappear in both normal

and abnormal phenotypes (e.g., Gilbert, 2006); for instance in salamanders the first of the four hand digits to form are the radial digits and there is a consensus that the missing digit is that from the opposite side, that is, digit 5 (Francis, '34; Duellman and Trueb, '86; Walthall and Ashley-Ross, 2006). But despite the absence of digit 5 in the hand of urodeles such as axolotls, these urodeles also have an abductor digiti minimi. Also, although digits 3 and 4 are present in the axolotl hand, only one of these digits has an interphalangeus (digit 3), while in the axolotl foot both digits 3 and 4 have interphalangei (Diogo, in press). That is, having more digits in the foot with interphalangei muscles than in the hand does not necessarily mean that one of those digits of the foot having these muscles is lacking in the hand.

In fact, a theory that seems to resolve the apparent contradictions listed above concerning the muscle configuration and the digit identity in the frog hand (e.g., absence of digit 1 but presence of abductor pollicis longus) and also in the axolotl hand (e.g., absence of digit 5 but presence of abductor digiti minimi) was recently proposed by Diogo and Abdala (2010) and further developed by Diogo (in press). According to this theory, the presence and configuration of the hand and foot muscles in tetrapods is mainly related to the physical position, and not the number of the anlage or even the homeotic identity, of the digits to which they are attached. It is however important to clarify what we mean by “topological position” versus “number of anlage” versus “homeotic identity” of the digits. Topological position refers to the *adult* relationship with other structures and to *adult* spatial data, and not necessarily to the position of the developmental anlagen. For instance, it is now commonly accepted that the digits of the *adult* bird wing derive from the second, third and fourth developmental anlagen, but that homeotically and morphologically these digits correspond to digits 1, 2, and 3 of other tetrapods (for a recent review about the controversies around this issue, see Bever et al., 2011). For Diogo and Abdala's theory, the *topological position* of the *adult* bird digit that derives from the second developmental anlage is *digit 1*, because this is the most radial digit in the *adult*; that is, in this case the topological position (digit 1) and homeotic identity (digit 1) are the same and are different from the developmental anlage from which the digit develops (the second anlage). Accordingly, birds such as chicken do usually have an abductor pollicis brevis going to this most radial digit, despite the fact that in pentadactyl taxa this muscle is always inserted to the digit that derives from the first, and not the second, anlage.

The study of the relationship between hard and soft tissues in non-pentadactyl tetrapods affects not only the knowledge about major evolutionary subjects such as evolutionary reversions, macroevolutionary patterns and limb integration, but also about human evolution and human medicine (Galis et al., 2001; Diogo and Wood, 2012). This is because changes in the number of digits are among most common anomalies of humans at birth (e.g., the presence of an extra toe and/or finger has a 0.2% incidence, i.e., 1 in 500 births), although the information about the soft tissue

changes occurring in these anomalies remains extremely scarce (Castilla et al., '96). However, the scarce medical data about these changes does provide support for Diogo and Abdala's (2010) theory that the configuration of the hand and foot muscles is mainly related to the position of the digits. This is because in preaxial polydactyly, which is one of the most common congenital anomalies of the human hand, the duplication of the thumb (leading to the two most radial digits having an homeotic identity of digit 1) is not accompanied by a duplication of the muscles that normally go to the thumb (Lighth, '92). Instead, the muscle abductor pollicis brevis, which is the most radial thumb muscle in the normal phenotype, goes to the most radial of the two thumbs, while the adductor pollicis, which is the most ulnar thumb muscle in the normal phenotype, goes to the most ulnar of the two thumbs. That is, the muscles are not simply duplicated as are the thumb bones, but instead go to each respective thumb according to the adult topological position of each of the duplicated digits.

Diogo and Abdala (2010) and Diogo et al. (2013) have provided numerous examples from other tetrapod groups in which the loss of digit 1 and/or 5 in the hindlimb or forelimb is related to a muscle change in which a muscle similar to the short abductor of digit 1 and/or 5 becomes inserted onto digit 2 or 4, respectively (as is the case in salamanders, and seemingly in frogs if we accept that the frog hand digits are digits 2–5: see above). If future studies confirm that the homeotic identity of the frog hand digits is 2–3–4–5 (see below), then the topological position of the most radial digit is digit 1, while the homeotic identity and the developmental anlage are those of a digit 2. If this is so, this would further support Diogo and Abdala's theory, because despite the homeotic identity of the most radial digit being that of a digit 2, the digit is associated with muscles that usually go to digit 1 in other tetrapods, such as the abductor pollicis longus. It should however be noted that there are some exceptions to the rule postulated by that theory. For instance, the absence of the tendon of some long forearm muscles that normally insert to digit 1 has been reported in some tetradactyl species of tetrapods (Haines, '39). Also, Heiss ('57) described a peculiar case in which a human subject had two pentadactyl hands that had no thumbs and in which, contrary to the cases referred to above, there were no major topological changes of the muscles resulting in, for example, the attachment of normal thumb muscles to the most radial digit of that human subject. Instead, in both hands of this human subject the normal thumb muscles were all reported as missing. In general, this configuration seems to be characteristic of the rare human disorder named "tri-phalangeal thumb," which is a malformation of digit I including a perfect homeotic transformation of the thumb into an index finger and in which the muscles that are normally associated with the thumb are absent (e.g., abductor opponens/adductor pollicis; Young and Wagner, 2011).

Apart from these few exceptions, most of the data obtained so far on the relationships between the hard and soft tissues of the fore- and hindlimbs do support the idea that the configuration of

the hand and foot muscles is mainly related to the position of the bones to which they attach (Diogo, in press). This idea makes evolutionary sense because the most extreme digits, that is, digits 1 and 5, are often specialized anatomically, have an increased mobility and/or are moved by peculiar muscles, such as the abductors (e.g., abductor pollicis longus, abductor digiti minimi). Therefore, on the one hand it makes sense that the loss of, for example, digit 1 in the forelimb of taxa such as birds is accompanied by a homeotic transformation where the most radial digit of the wing (which derives from the anlage of digit 2) recovers the identity of digit 1. On the other hand, it also makes sense that even in those cases where there are no such homeotic transformations there is a developmental mechanism (configuration/identity of muscles related to position, and not identity, of digits) assuring that despite the digit reduction and lack of homeotic transformation, the digits of the extremities still keep the peculiar muscles that are related to the specialized functions of digits 1 and/or 5. This could also explain the occurrence of those exceptions to Diogo and Abdala's (2010) model that were listed above, that is this model may be mainly due to adaptive constraints and forces, rather than to a hard rule caused by a strong developmental constraint. And this is also probably why the "muscle configuration/identity–bone position" relationship seems to mainly apply to the autopodium and particularly to the digits (including their phalanges and metatarsals/metacarpals), and not to the other regions of the limbs. One example illustrating this statement is precisely the fact, noted above, that the peculiar distal placement of various leg muscles of frogs is apparently not explained by a change of position of the tibiale and fibulare bones, which despite being elongated remain in the tarsal region, but instead to a homeotic change in which these bones assume the identity of leg bones.

It is also important to note that the discussion provided in above paragraphs does not necessarily mean that we consider that the issues concerning the homeotic identity of the digits of the anuran hand and of the tibiale and fibulare of the anuran hindlimb are settled. On the contrary, we consider that more mechanistic studies about forelimb digit reduction and hindlimb tarsal changes in frogs and the potential homeotic transformations that may have been, or not, involved in these changes are clearly needed. For instance, a potential problem with studies of *hoxd11* expression suggesting that the frog hand digits are 2–3–4–5, such as that of Satoh et al. (2006), is that *hoxd11* expression is somewhat dynamic and one could sample the wrong time and erroneously conclude that the identity is 2–3–4–5; more developmental stages should thus be analyzed in gene expression studies in order to address this question in a more comprehensive way (Gunter Wagner, personal communication; see also the recent review of Woltering and Duboule, 2010, and references therein). Together with other colleagues, we plan to undertake such gene expression studies in the near future. One of the aims of the present paper is precisely to stimulate, and pave the way, for such mechanistic studies and for

developmental, genetic and comparative studies of limb muscle development in not only frogs but in other tetrapods in general.

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