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PATTERNS & PHENOTYPES

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Comparative development of limb musculature in phylogenetically and ecologically divergent lizards

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Abstract

Background: Squamate reptiles (lizards, snakes, and amphisbaenians) exhibit incredible diversity in their locomotion, behavior, morphology, and ecological breadth. Although they often are used as models of locomotor diversity, surprisingly little attention has been given to muscle development in squamate reptiles. In fact, the most detailed examination was conducted almost 80 years ago and solely focused on the proximal limb regions. Herein, we present forelimb and hindlimb muscle morphogenesis data for three lizard species with different locomotion and feeding strategies: the desert grassland whiptail lizard, the central bearded dragon, and the veiled chameleon. This study fills critical gaps in our understanding of muscle morphogenesis in squamate reptiles and presents a comparative and temporospatial analysis of muscle development.

Results: Our results reveal a conserved pattern of early muscle development among lizards with different adult morphologies and ecologies. The variations that exist are concentrated in distal regions, particularly the specialized autopodia of chameleons, where differentiation of muscles associated with the digits is delayed.

Conclusions: The chameleon autopod provides an example of major evolutionary modifications to the skeleton with only minor disruption of the conserved order and pattern of limb muscle development. This robustness of muscle patterning facilitates the evolution of extreme yet functional phenotypes.

KEYWORDS

chameleon, locomotion, morphogenesis, muscle development, reptile, squamate

1 | INTRODUCTION

Squamate reptiles (lizards, snakes, and amphisbaenians) are an incredibly diverse assemblage of amniotes with respect to behavior, morphology, and ecological breadth,[1] making up approximately 38% of all living amniotes and 96% of nonavian reptiles (11 052 recognized species).[2-4] Squamate reptiles have a rich history of descriptive osteology and limb myology together with studies of their kinematics and locomotion; yet few muscle developmental studies have been conducted for this lineage.[5-10] This relative lack of attention to muscle development is primarily due to the challenge of obtaining sufficient embryos for research in the field along with the

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constraint that most squamate embryos initiate development within the oviduct or are viviparous, which would necessitate euthanasia of the female to obtain early stage embryos.[11-13] Early work examining specializations in limb-reduced reptiles was conducted by Raynaud,[14,15] while Romer,[16,17] through thorough histological examination of lizard embryos belonging to a species with a generalized terrestrial body plan, described the development and differentiation of skeletal and soft tissues of the proximal forelimb and hindlimb in the common wall lizard *Podarcis muralis* (Lacertidae). These works by Romer[16,17] contain one of the earliest descriptions of what was only recently coined the "in-out" model of muscle development,[18,19] in which migratory muscle precursors (MMPs) travel laterally from the ventrolateral lip of the dermomyotome[19,20] into the limb bud and subsequently migrate medially back into the trunk to form superficial muscles associated with the girdle. Later stages of muscle morphogenesis were described by Sewertzoff[21] in a gekkonid species belonging to the genus *Tarentola*. Recent work has focused on the development of the appendicular skeleton and developmental variation of distal limb anatomy[22,23] and limb reduction/loss in snakes,[24-28] while a few studies have examined squamate somitogenesis,[29,30] somite differentiation,[31] and primary myogenesis in the limb bud.[20,32]

Despite modification of the appendicular plan related to function in diverse aerial, aquatic, arboreal, and terrestrial habitats,[33-36] the early development and patterning of soft and hard tissues across tetrapod limbs remains highly conserved.[19,37] Limb buds begin as bilateral pairs of swellings composed of an ectodermal epithelial outer covering with an inner core of mesenchymal cells derived from the lateral plate mesoderm.[38,39] Endothelial and MMP cells differentiate from the ventrolateral lip of the somitic dermomyotome (paraxial mesoderm) and subsequently migrate into the limb mesenchymal core, though modifications to this plan have been seen across vertebrates.[19,40-42] MMPs express Pax3, Lbx1, and c-Met, which prevent the differentiation of cells and allow for proper migration.[43-46] MMPs express receptors for ligands expressed in the limb bud, which serve as chemoattractants, such as the ligands hepatocyte growth factor/scatter factor and stromal cell derived factor 1, also known as CXCL12, that bind to MMP c-MET and CXCR4 receptors, respectively.[43,44,46-51] Sonic hedgehog (Shh), fibroblast growth factor (Fgf), and Wnt signaling pathways have been found to not only pattern the limb bud but also regulate the downstream production of MMP chemoattractant ligands,[43] once in the limb bud MMPs proliferate and differentiate in response to local signals.[52-55]

While our understanding of the logic behind amniote muscle cell differentiation in vivo and in vitro continues to grow,[56] few studies have examined the patterns of limb muscle morphogenesis at a macroscopic scale in non-avian reptiles.[19,20,37,57] This contrasts with detailed studies of limb muscle development in avians, mice, frogs, and salamanders.[16,17,58-68] Recent methods for clearing and imaging have dramatically advanced our ability to trace development and differentiation of musculoskeletal tissues in situ through antibody staining and have even been applied to human limbs.[69,70] In addition, advancements in contrast-enhanced microcomputed tomography (DiceCT) allow three-dimensional visualization of embryonic and adult soft tissues.[71-74]

Herein, we present forelimb and hindlimb data on limb muscle morphogenesis in three lizard species with different limb morphologies and phylogenetic histories. The desert grassland whiptail lizard (*Aspidoscelis uniparens*, Teiidae) and the central bearded dragon (*Pogona vitticeps*, Agamidae) are terrestrial species with plesiomorphic limb morphologies, whereas the veiled chameleon (*Chamaeleo calyptratus*, Chamaeleonidae) has limbs and autopodia specialized for grasping and moving slowly in a fine-branch niche.[75,76] Our work fills gaps in our knowledge of distal limb muscle morphogenesis (picking up where Romer[16,17] left off) and also presents a comparative and temporospatial analysis of muscle development from precursor muscle masses in the dorsal and ventral aspects of the embryonic forelimbs and hindlimbs. Early works by Romer[16,17] utilized wax sections in specimens, whose only measurements were the size of the embryo with no knowledge of the overall morphogenesis of the limbs, while more recent studies have not examined overall muscle development in limbs. As the first detailed study comparing the development of the limb muscles of chameleons and other lizards, our work provides a foundation for future studies to examine how tetrapod limb muscle development varies across clades and limb morphologies. In addition, this is the first study to examine the dorsal and ventral muscle mass growth and differentiation into distinct muscles through ontogeny for both forelimb and hindlimbs as well as within a comparative framework between species of squamate lizards.

While non-chameleon lizards showed few differences in the number, attachments, or architecture of limb musculature, a number of differences were apparent in adult chameleons, particularly in the shoulder and autopodium.[23,72] Therefore, due to grasping with their autopodia and adduction of limbs toward the body during climbing,[77,78] chameleons would be expected to exhibit muscle patterning distinct from generalized terrestrial lizards, which present sprawling limbs and show propulsion primarily derived from the hindlimbs.[57] In particular, we expect to find changes in the timing of appearance and muscle mass splitting in muscles that are

important for climbing slowly on narrow substrates in chameleons when compared to the phylogenetically, ecologically and kinematically distinct central bearded dragon (*P vitticeps*; Agamidae) and the desert grassland whiptail lizard (*Aspidoscelis uniparens*; Teiidae).[79,80]

2 | RESULTS

The results of our work are summarized in Figures 8,9 and Tables 2,3. For a detailed examination of adult lizard limb anatomy for taxa reviewed in this project (*A uniparens* and *C calyptratus*), see Reference 72. The current study makes the use of three species representing three different families (Chamaeleonidae, Agamidae, and Teiidae[81]) showing different phenotypes and locomotor behaviors. A comparative approach was taken herein to examine whether differences in the timing and order of muscle differentiation during embryogenesis were conserved despite differences in specialized autopodial morphology, as previous work by Diaz and Trainor[23] highlighting skeletal differences in the distal limb (autopodia) of chameleons relative to generalized lizard autopodia.

Our results build upon and largely confirm the muscle cleavage patterns reported in the shoulder and thigh of *Podarcis* ("*Lacerta*") *muralis* (Lacertidae).[16,17] The latter author used the traditional method of serial sectioning, which provided good visualization of deeper structures but was limited in terms of the stages and regions described. In contrast, while we were not able to visualize some of the deeper and more proximal muscles (due to both antibody penetrance and lack of visibility due to overlying musculature), our method of antibody staining on whole embryos allowed us to describe the order of muscle cleavage in more detail and to observe the muscles of the distal limb regions as well. This distinction is important because different methodologies and criteria for separation may lead to different interpretations of the timing and sequence of muscle differentiation among developmental studies.[64]

2.1 | Detailed description of embryonic muscle development

2.1.1 | Stage 29: Whole embryos

Aspidoscelis uniparens is shown at early stage 29 (Figure 1A): the forelimb begins at approximately the 12th somite and the hindlimb at the 21st. The forelimb bud is more prominent than the hindlimb bud (organismal embryonic morphogenesis proceeds in an anterior-

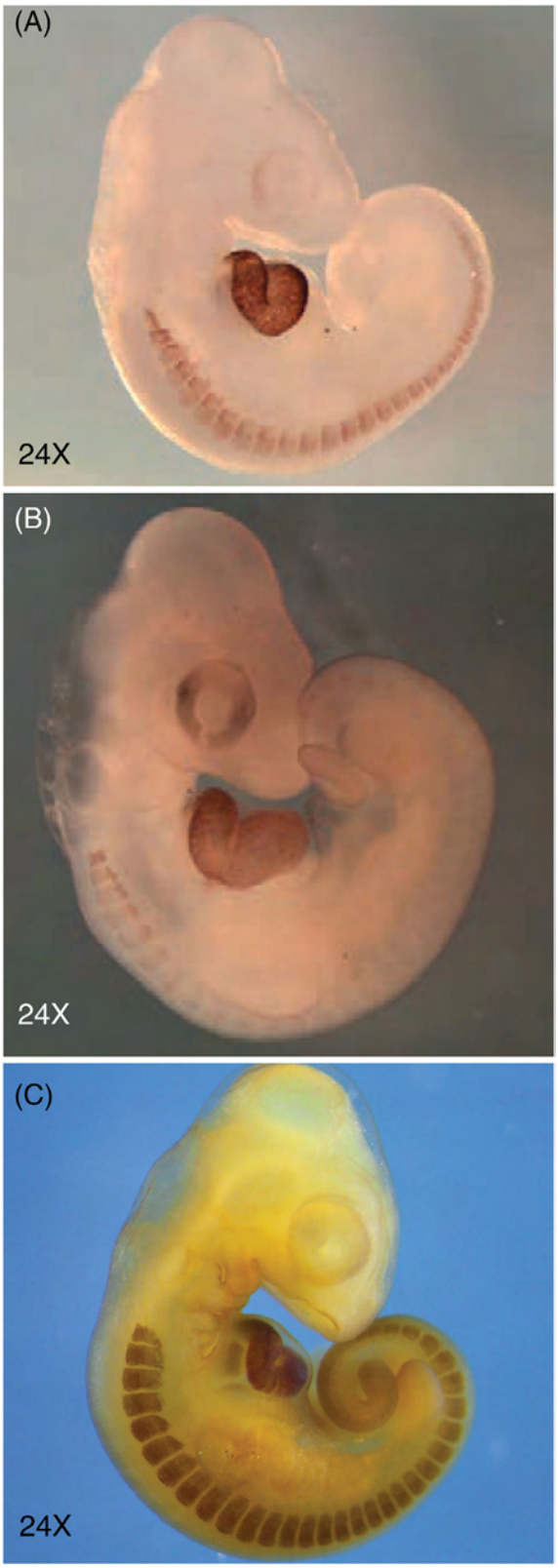

FIGURE 1 Comparison of stage 29 embryos of *Aspidoscelis uniparens* (A; 33 somites), *Pogona vitticeps* (B; 38 somites), and *Chamaeleo calyptratus* (C; 39 somites) stained with MF20 antibody and showcasing the early stage differentiation of myocytes within the somites and no MF20 positive cells in the early stage limb buds. Asterisks denote the placement of limb buds

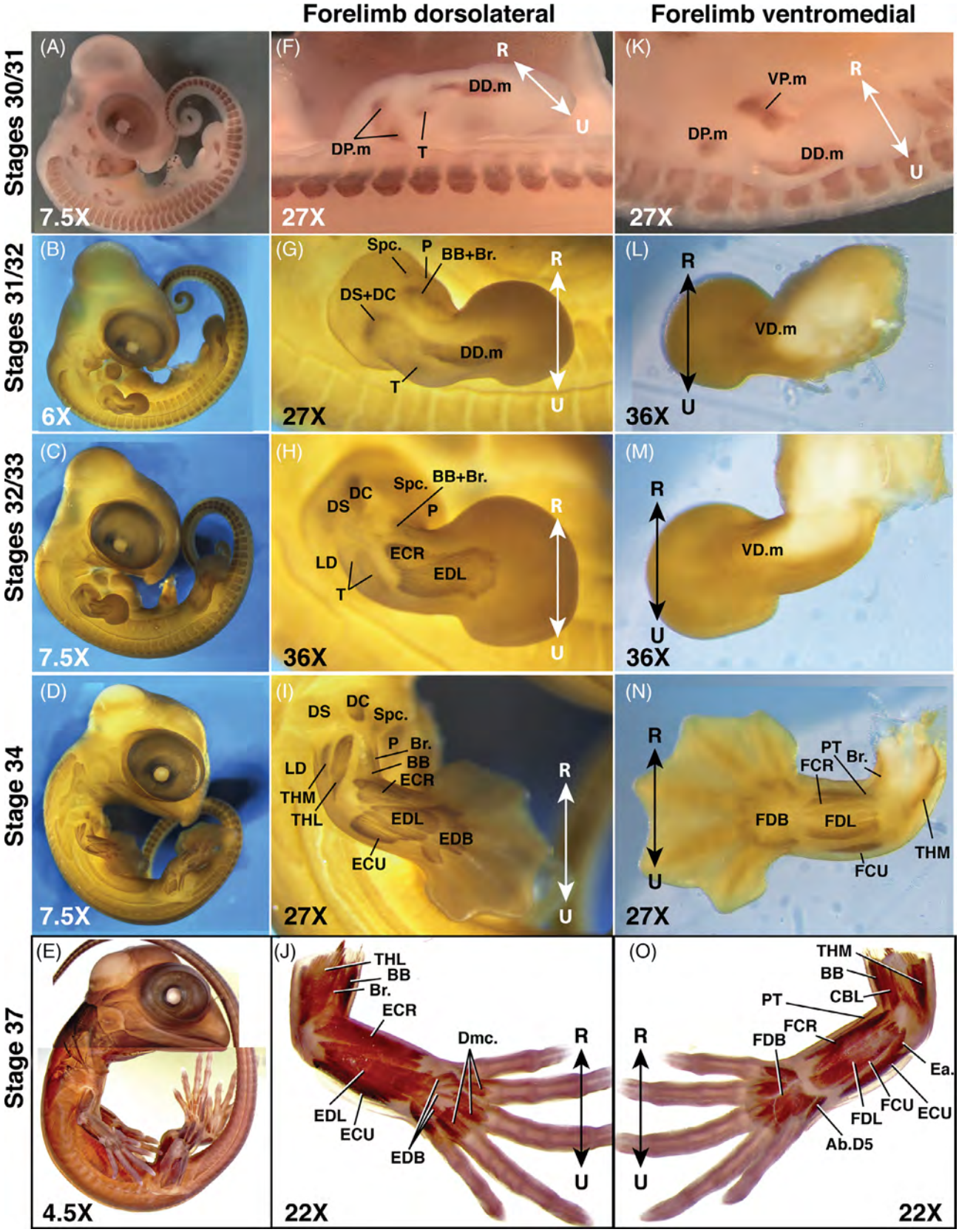

FIGURE 2 Forelimb muscles of *Aspidoscelis uniparens* at five stages of development, visualized by antibody staining. Left column shows whole embryos in right lateral view at stages 30/31 (A), 31/32 (B), 32/33 (C), 34 (D), and 37 (E). Middle column shows dorsolateral views of the forelimb at stages 30/31 (F), 31/32 (G), 32/33 (H), 34 (I), and 37 (J). Right column shows ventromedial views of the forelimb at stages 30/31 (K), 31/32 (L), 32/33 (M), 34 (N), and 37 (O). Arrow indicates radio-ulnar axis. See Table 4 for muscle abbreviations

posterior direction). Approximately 33 somites are visible. The tail has almost completed its first coil. While staged equivalently with respect to facial morphogenesis,[82] *A uniparens* appears to be at an earlier stage of axial morphogenesis in comparison to stage 29 *P vitticeps* (Figure 1B) and stage 29 *C calyptratus* (Figure 1C). Somitic myocytes in stage 29 *A uniparens* stain darker than *P vitticeps* and less so than in *C calyptratus* at this stage.

P vitticeps (Figure 1B): Approximately 38 somites are visible in this embryo. The forelimb and hindlimb outgrowths project laterally; the forelimb is distally broader than hindlimb and more pronated. The tail coils counterclockwise.

C calyptratus (Figure 1C): Approximately 39 somites are visible. Tail coils counterclockwise while cardiac and somitic myocytes stain for MF20.

2.1.2 | Stages 30-31: Whole embryos

At stage 30/31, *A uniparens* (Figure 2A) forelimbs extend posteriorly and in parallel with the primary body axis. Forelimb length at this stage (from putative shoulder to distal limb tip) is approximately eight myotome lengths. The hindlimb projects posterolaterally, with the hindlimb autopodium less round/paddle shaped than the forelimb. At this stage, the forelimbs are pronated so the zeugopodium and autopodium are parallel to the lateral surface of the trunk (Figure 2,3).

At stage 30 *P vitticeps* (Figure 4A), the hindlimb is primarily lateral in its projection, whereas the forelimb is posterolaterally oriented with slight pronation and a more distinct autopodium (also becoming rounder/paddle shaped).

2.1.3 | Stages 31-32: Whole embryos

At late stage 31/early stage 32 in *A uniparens* (Figure 2B), the forelimb has a more pronounced curvature at the elbow, showing a distinct stylopod, zeugopod, and autopod, with two proximal domains subequal in length. The hindlimb is still projected posterolaterally with slight pronation and initiation of bending at the incipient knee. The distal forelimb autopodium remains round, while the hindlimb has begun to develop a distinct triangular expansion along its posterior border. The hypaxial region between the limbs is not ventrally tapered but rather square in general with more equal dorsoventral staining. The forelimb is more pronated than at the previous stage, and the autopodium is parallel to the trunk. The forelimb now extends across eight myotomes (Figure 2).

At stage 32 in *C calyptratus* (Figure 6A), the anteriormost myotomes in the putative cervical region are differentiating and fusing. Interlimb myotomes are extending into the abdominal domain to form the hypaxial portion of the axial musculature. The forelimb is more developmentally advanced and larger than the hindlimb, and the distal flattening characteristic of chameleon autopodia is more obvious.[23] The forelimb has a more distinct curvature at the putative elbow and greater pronation than in the previous stage (29). The hindlimb has become more ventrally directed and slightly pronated. *P vitticeps* at this stage was not available.

2.1.4 | Stages 32-33: Whole embryos

At late stage 32/early stage 33 in *A uniparens* (Figure 2C), digital rays II-V are at early stages of endochondral ossification and are cartilaginous as described in figure 13 of Reference .[23] The forelimb extends posteriorly approximately 10-11 myotome spaces, though the anterior four myotomes have begun to differentiate into merged epaxial muscles, so borders are difficult to distinguish. The hindlimb projects posteriorly approximately nine myotome lengths.

In *P vitticeps* at stage 33 (Figure 4B), the forelimb and hindlimb have distinct stylopodial, zeugopodial, and autopodial domains. Both limbs have significant pronation, with both autopodia now parallel to the lateral surface of the trunk and tail. The autopodia have distinct digital condensations with maximum interdigital mesenchymal tissue (webbing) extending to the tips of the digits.

While most myotomes remain segmented at stage 33 in *C calyptratus* (Figure 6B), they continue to differentiate in an anterior to posterior direction with adjacent myotomes. The forelimbs have a more sharply angled elbow and the autopodium has expanded anteroposteriorly with the distal cleft beginning to form. The hindlimb is not as pronated as the forelimb and is also smaller overall.

2.1.5 | Stage 34: Whole embryos

Figure 2D shows a stage 34 embryo for *A uniparens*, stage matched using Wise et al[82] for *Eublepharis macularius*, with distinct condensed digital rays (as putative endochondral elements at this stage) and full interdigital webbing thinner than the adjacent digital rays. Posterior trunk myotomes remain visible as distinct MF20 positive myocyte blocks, while the more anterior trunk (thoracic) and cervical myotomes are no longer distinct due to

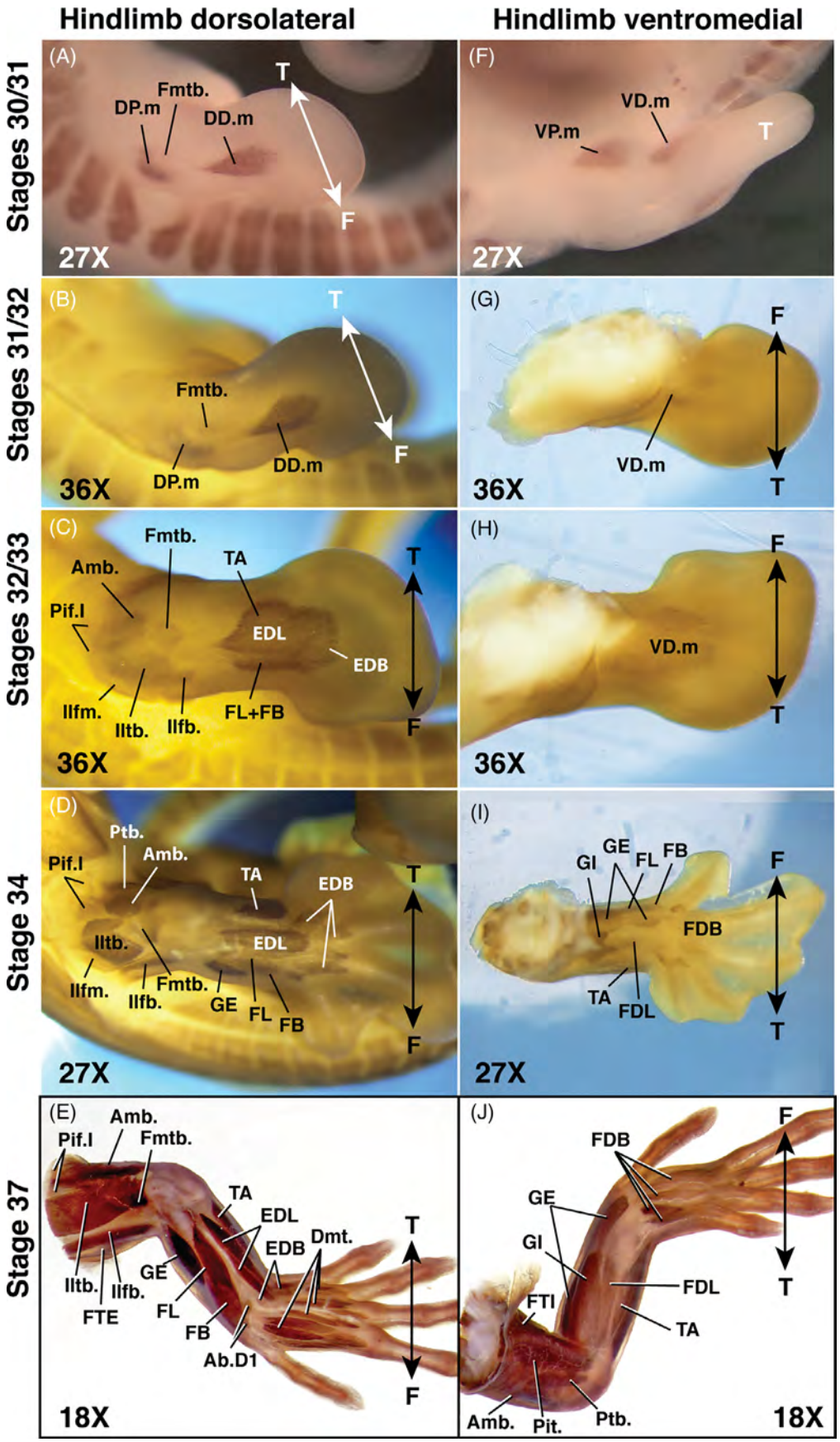

FIGURE 3 Hindlimb muscles of *Aspidoscelis uniparens* at five stages of development, visualized by antibody staining. Left column shows dorsolateral views of the hindlimb at stages 30/31 (A), 31/32 (B), 32/33 (C), 34 (D), and 37 (E). Right column shows ventromedial views of the hindlimb at stages 30/31 (F), 31/32 (G), 32/33 (H), 34 (I), and 37 (J). Arrow indicates tibiofibular axis. See Figure 2 for images of whole embryos and Table 5 for muscle abbreviations

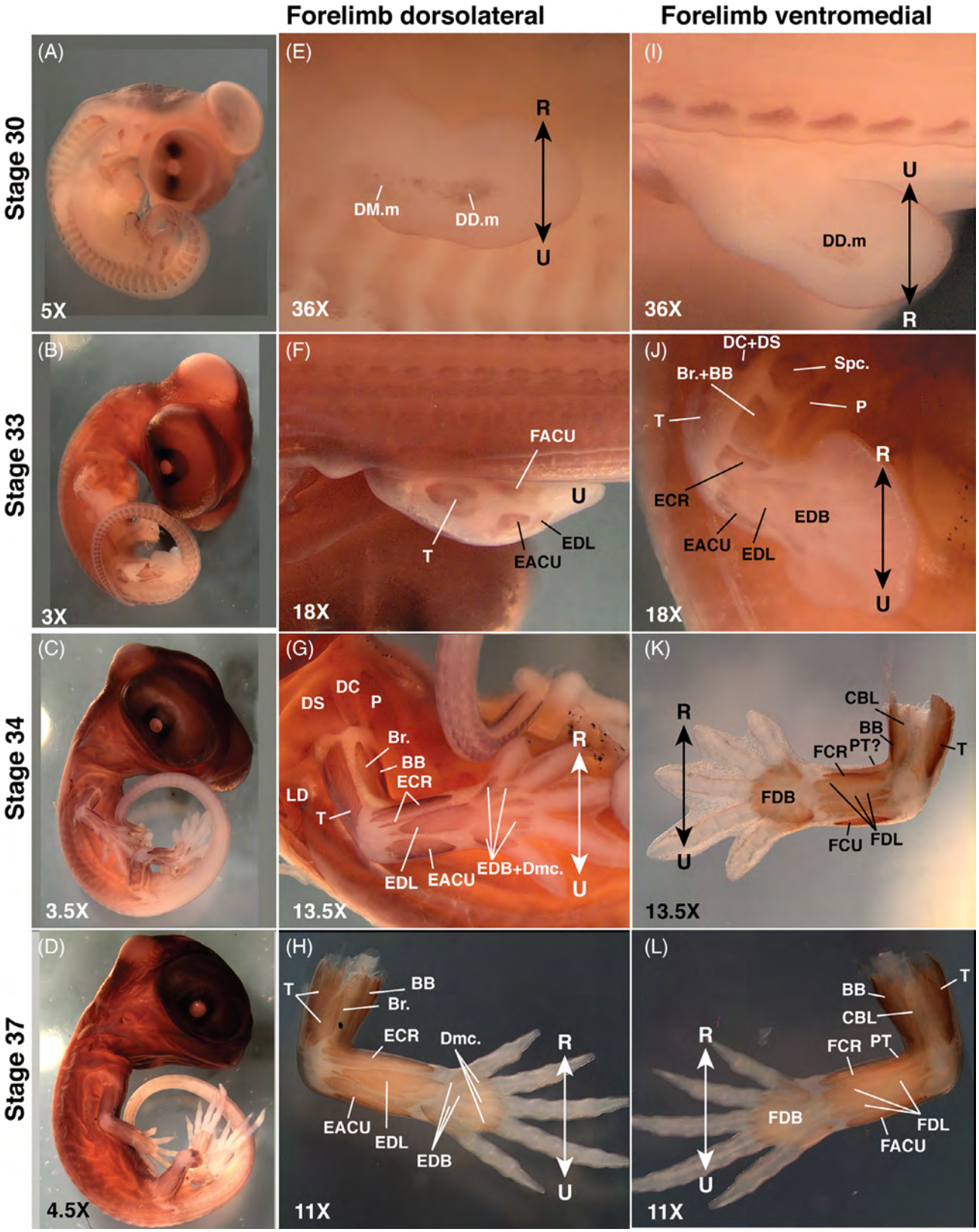

FIGURE 4 Forelimb muscles of *Pogona vitticeps* at four stages of development, visualized by antibody staining. Left column shows whole embryos in right lateral view at stages 30 (A), 33 (B), 34 (C) and 37 (D). Middle column shows dorsolateral views of the forelimb at stages 30 (E), 32 (F), 34 (G), and 37 (H). Right column shows ventromedial views of the forelimb at stages 30 (I), 32 (J), 34 (K), and 37 (L). Arrow indicates radio-ulnar axis. See Table 4 for muscle abbreviations

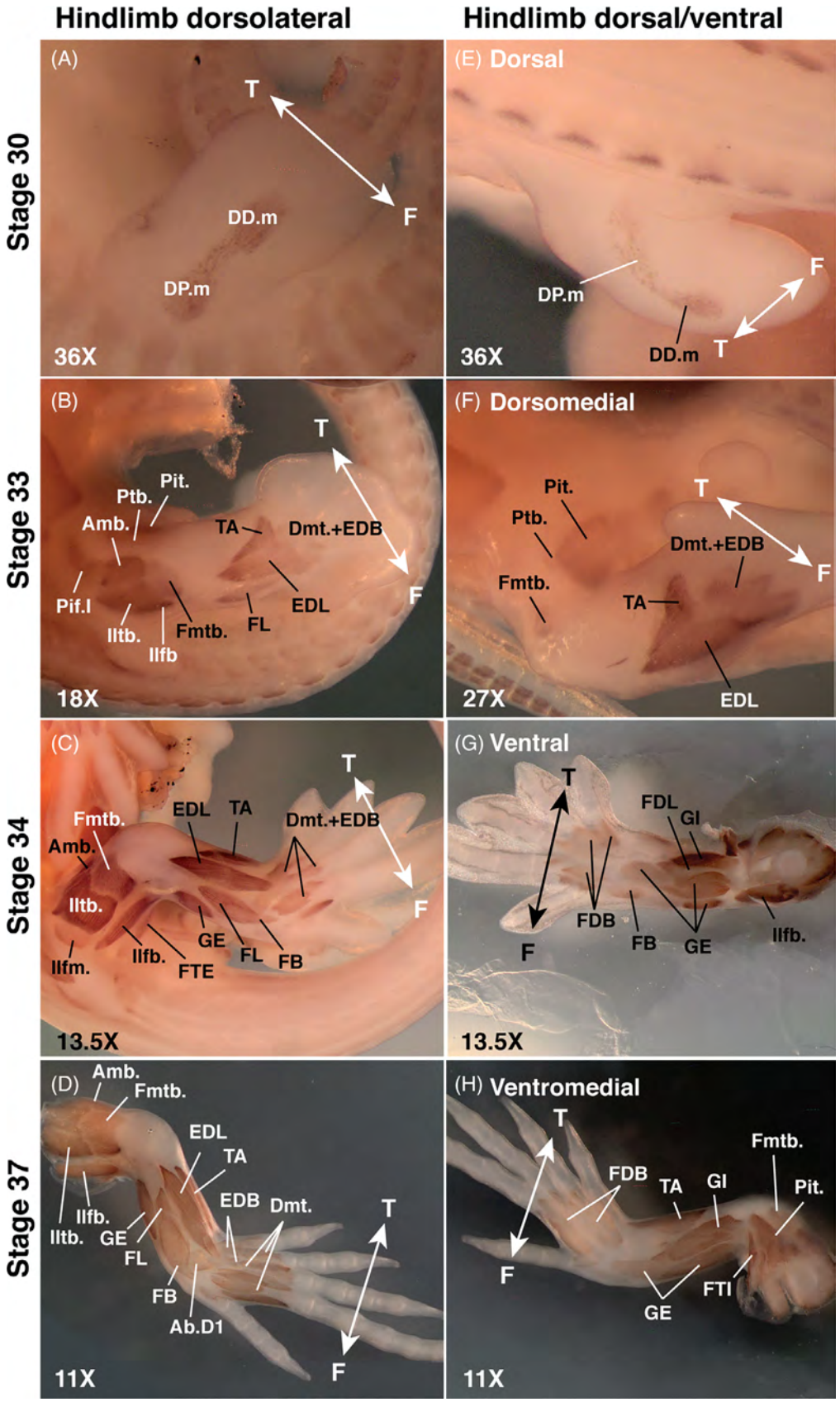

FIGURE 5 Hindlimb muscles of *Pogona vitticeps* at four stages of development, visualized by antibody staining. Left column shows dorsolateral views of the hindlimb at stages 30 (A), 32 (B), 34 (C), and 37 (D). Right column shows ventromedial views of the hindlimb at stages 30 (E), 32 (F), 34 (G), and 37 (H). Arrow indicates tibiofibular axis. See Figure 4 for images of whole embryos and Table 5 for muscle abbreviations

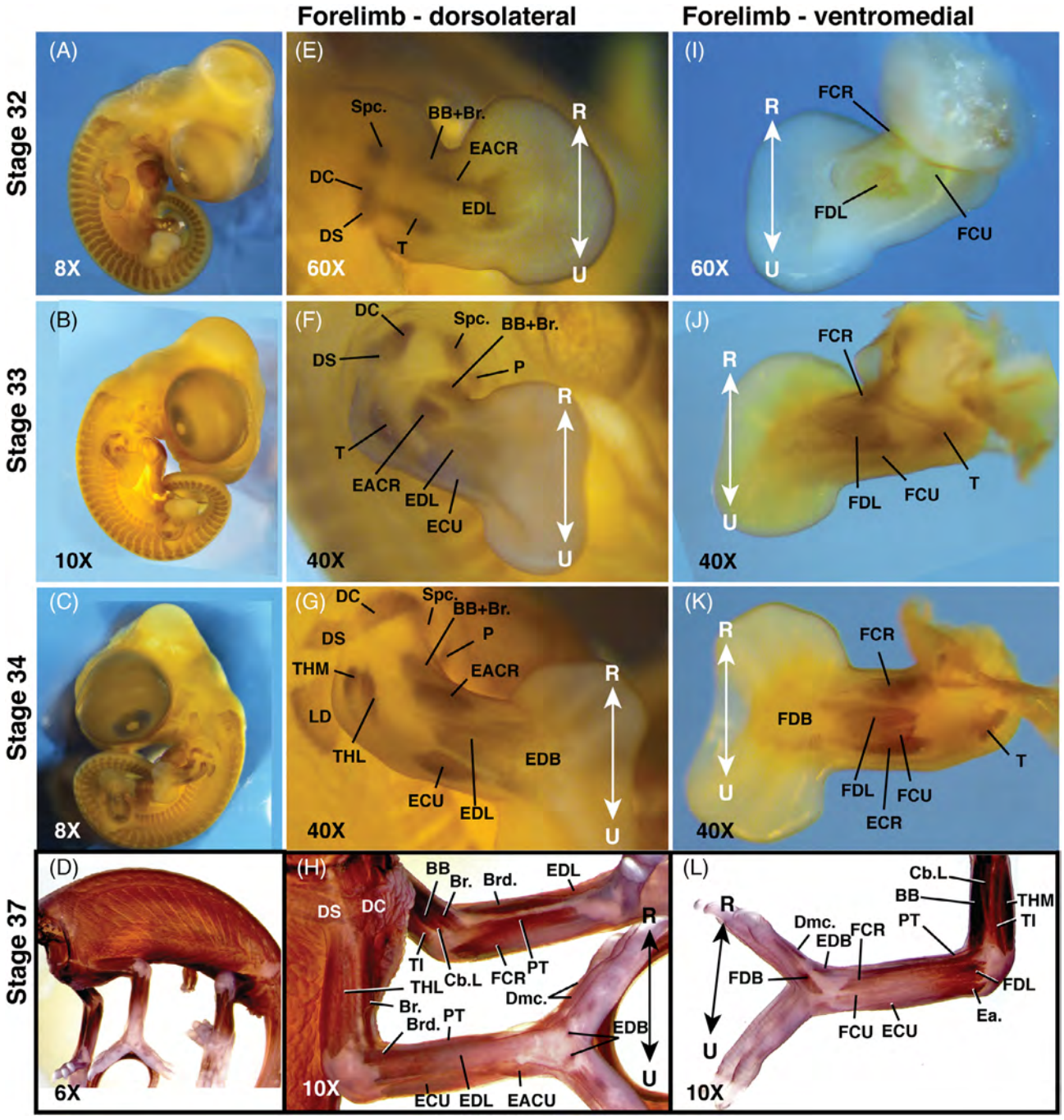

FIGURE 6 Forelimb muscles of *Chamaeleo calyptratus* at four stages of development, visualized by antibody staining. Left column shows whole embryos in right lateral view at stages 32 (A), 33 (B), 34 (C), and 37 (D). Middle column shows dorsolateral views of the forelimb at stages 32 (E), 33 (F), 34 (G) and 37 (H). Right column shows ventromedial views of the forelimb at stages 32 (I), 33 (J), 34 (K), and 37 (L). Arrow indicates radio-ulnar axis. See Table 4 for muscle abbreviations

differentiation and fusion into separate muscles. At this stage, the latissimus dorsi (LD), trapezius, and episternocleidomastoideus are visible surrounding the shoulder and cervical region adjacent to the proximal forelimb. Abdominal myotomes (hypaxial muscle precursors) have lost their distinction as individual blocks as neighboring myotomes differentiate into abdominal muscles. Axial muscles of the lumbar, sacral, and anterior caudal domain have begun to differentiate (through intermyotomal fusion).

At stage 34 in *P vitticeps* (Figure 4C), forelimb growth has extended to beyond the proximal hindlimb if forelimb is adpressed to body wall caudally. Interdigital tissue in fore- and hindlimbs has begun to reduce distally and

has become thinner throughout when compared to the digital rays. Phalanges (and thus, joints) are now visible when illuminated ventrally.

At stage 34 *C calyptratus* (Figure 6C), forelimb pronation places the palmar surface of the hand in parallel with the lateral wall of the body though the distal limb (autopodium and stylopod) are orienting ventrally due to more pronounced bending/flexion at the elbow. The forelimb skeleton is cartilaginous at this stage, described in figure 11 in Reference 23. The hindlimb is also pronated, with bending at the knee much weaker than seen at the forelimb elbow, thus the distal limb is almost straight (overall slightly posteroventrally oriented). Skeletogenesis at this stage is described in figure 12 of Reference 23 highlighting the tarsals undergoing chondrogenesis with the digital rays remaining primarily as mesenchymal condensations not staining for Alcian.

2.1.6 | Stage 37: Whole embryos

At stage 37 in *A uniparens* (Figure 2E), claws are present on all digits with complete loss of all interdigital mesenchyme. Muscle masses appear to have reached their adult complement and are primarily enlarging. Areas free of MF20+ reactivity represent bone and tendinous/ligamentous connective tissue. Pronation of the forelimbs is at maximum. Pronation along with continued growth of the triceps muscles places the lateral head of the triceps (TLH) anteriorly close to the brachialis (BR). The latissimus dorsi (LD) and trapezius (TR) have expanded significantly.

At stage 37, *P vitticeps* forelimbs and hindlimbs no longer retain interdigital mesenchyme, joints between phalanges are distinct through the translucent skin while claws have also differentiated (Figure 4D). The hindlimb total length is longer than that of the forelimbs in gross comparison.

2.2 | Detailed description of forelimb muscle development

2.2.1 | Stages 30-31: Dorsal forelimb

At stage 30/31 in dorsal and dorsolateral views (Figure 2F,K, respectively), *A uniparens* limbs present three seemingly distinct major muscle masses arranged along the proximal-distal axis. The most proximal dorsal muscle mass (DP.m) is composed of a larger and more darkly MF20+ stained pool of anterior and posterior MMPs with a deeper and more proximal (arch-like) population of MMPs bridging between both of these more superficial masses. Distally, an intermediate and posteriorly placed muscle mass is present superficially, which will ultimately differentiate into the triceps. Anterior to the incipient triceps, a deeper stained population of MMPs (appearing to bridge the proximal and distalmost populations) is visible as a very narrow strip. The distal most population of dorsal MMPs (DD.m) is visible as a broad, distally rounded sheet-like population of MMPs with a proximo-anterior branch of MMPs (slightly overlapping with the anterior-most level of the triceps posteriorly). The DD.m has a clear distal limit proximal to the future autopodial domain.

At stage 30 in *P vitticeps* (Figure 4E), MMPs are present as a continuous series of MMP clusters along the proximodistal dorsal midline, and the distalmost region (DD.m) has a greater number of MMPs and is more compact. The proximal MMPs (DP.m) are less numerous and primarily run along the anterior margin of the gradient (relative to the more anteroposteriorly expanded distal population).

2.2.2 | Stages 30-31: Ventral forelimb

In *A uniparens* at stage 30/31 (Figure 2K), the limbs were not excised to expose ventral musculature to preserve the specimen; yet, some ventral muscles were visible in ventromedial view. A large proximal muscle mass (VP.m) is present, appearing to consist of a larger and more darkly stained proximal and distal pair of masses with a lighter stained (and deeper) posterior population of MMPs. This ventral mass extends distally from the base of the limb to overlap the proximal branch of the most distal dorsal muscle mass (DD.m) following the curvature of the limb (the developing elbow). The ventral aspect of the forelimb was not visible in *P vitticeps* embryos at this stage (Figure 4I).

2.2.3 | Stages 31-32: Dorsal forelimb

At late stage 31/early stage 32 in *A uniparens* (Figure 2G), the dorsal muscle masses have increased in size with the growth of the limb. The most proximal dorsal mass is less diffuse between its most superficial anterior and posterior masses (most likely due to an increase in tissue thickness in the limb making deeper masses more difficult to see). Some of the MMPs derived from the ventral muscle mass are visible in this view but will be described in the section 2.2.4.

At stage 32 in *C calyptratus* (Figure 6E), the dorsal muscles consist of two large irregularly shaped masses, with each already showing distinct domains for forming musculature. Proximally and closer to the posterior

margin of the developing forelimb is a "Y"-shaped muscle mass, whose apex extends distally. The proximal bifurcation corresponds to the more posteriorly forming deltoideus scapularis (DS) and the adjacent deltoideus clavicularis (DC, also called deltoideus acromialis et clavicularis). Distally, these two muscles are still continuous with the elongated triceps anlage, which extends distally along the posterior margin of the forelimb. Along the distal and anterior border of the limb is an "L"-shaped MMP aggregate. The longer arm of the "L" lies along the anterior border and the shorter arm of the "L" extends anteroposteriorly in the distal forelimb. The long arm corresponds to the future extensor antebrachii et carpi radialis (EACR) and the shorter arm to the extensor digitorum longus (EDL, also called extensor digitorum). A diffuse MMP population extends distally from the short arm of the "L" in a broad fan shape.

2.2.4 | Stages 31-32: Ventral forelimb

Also from dorsolateral view at stages 31/32 in *A uniparens* (Figure 2G), the most proximal portion of the ventral forelimb presents a new pair of MMP populations, the supracoracoideus (SPC) and the pectoralis (P), which appear to have expanded from the proximal-most ventral mass (VP.m). Excision of the forelimb (Figure 2L) presents the more distal ventral mass (VD.m) with loss of more proximal muscle masses (F).

As in *A uniparens*, the proximal ventral muscle masses at stage 32 in *C calyptratus* are best viewed from dorsolateral view in the intact embryo, whereas the distal masses are best viewed in the excised limb. Proximally, an MMP aggregate is present anterior to the deltoideus clavicularis (DC) (Figure 6E). This aggregate is the SPC, which is derived from the ventral proximal mass (DP.m) and has expanded dorsally along with the more distal mass adjacent to the ECR, which will give rise to the biceps brachii (BB) and brachialis (BR). The ventral aspect (Figure 6I) presents a distal muscle mass (DD.m), which has differentiated into three parts: the flexor carpi radialis (FCR) along the anterior aspect, the flexor carpi ulnaris (FCU) along the posterior margin, and the flexor digitorum longus (FDL) distal to and between the FCR and FCU.

2.2.5 | Stages 32-33: Dorsal forelimb

At late stage 32/early stage 33 in *A uniparens* (Figure 2H), the most proximal anterior and posterior MMP populations are expanding further proximally into the trunk from the limb as the deltoideus clavicularis (DC) anteriorly and deltoideus scapularis (DS) posteriorly. The previously distal most muscle mass (DD.m) has now differentiated into the larger anterior and proximolateral muscle extensor digitorum longus (EDL) and the smaller antero-proximal muscle extensor carpi radialis (ECR). The T and ECR extend across the limb curvature in the region of the elbow. A diffuse boundary at the zeugopodium/autopodium remains with no distinct MMPs in the autopodium. Pronation at this stage is more obvious. In conjunction with the anteroposterior growth of the muscles, pronation of the limb brings the deltoideus clavicularis (DC) to an anterior position in the proximal limb (and in closer proximity to the expanding SPC).

At stage 33 in *P vitticeps* (Figure 4J), a distinct doublet of muscle masses is differentiating from the proximal dorsal mass, with the deltoideus clavicularis (DC) and deltoideus scapularis (DS) in the antero-proximal region. What was previously a narrow band of MMPs extending along the anterior margin and bridging the proximal and distal masses has now differentiated into the triceps muscle mass and divided into the more superficial medial head of the triceps (TMH) and the deeper lateral head (TLH). The dorsal distal mass (DD.m) has now differentiated into three distinct sheets of MMPs: radial, ulnar, and intermediate. The largest dorsal midline fan-shaped population has differentiated into the extensor digitorum longus (EDL) proximally and the extensores digitorum breves (EDB) distally with distal projections corresponding to digits II-IV. The anterior portion of the distal mass becomes the extensor antebrachii et carpi radialis (EACR), and the posterior portion becomes the extensor antebrachii et carpi ulnaris (EACU).

At stage 33 in *C calyptratus* (Figure 6F) proximally, forelimb pronation along with expansion of individual muscles has shifted the SPC (of ventral muscle mass origin) anteriorly into a more ventral position into closer proximity with the pectoralis (P). On the dorsum, the deltoideus complex has shifted anteriorly on the proximal arm and separated distally from the triceps (T), which remains along the posterolateral margin of the arm near the developing elbow region. Distally, the extensor digitorum longus has grown from a narrow band (short leg of an "L") to a large rectangular sheet. The extensor antebrachii et carpi ulnaris (EACU) cleaves from the posterior margin of the EDL. Along the anterior margin and proximal to the EDL, the extensor antebrachii et carpi radialis (EACR) has separated completely from the EDL. Pronation has shifted the BB + BR mass ventrally.

2.2.6 | Stages 32-33: Ventral forelimb

In dorsolateral view at late stage 32/early stage 33 in *A uniparens* (Figure 2H), the most proximal ventral muscle

masses, the SPC and pectoralis (P), have continued to expand proximally into the ventral trunk while the more distal brachialis (BR) has become more prominent and distinct. Ventral distal musculature is not clear from Figure 2H, and upon excision of the limb proximal muscle masses are lost with only the ventral distal muscle mass (VD.m) visible with an anterior boundary at the putative wrist (Figure 2M).

From the limited views presented in whole specimens due to limb pronation (Figure 4B), at stage 33 in *P vitticeps*, the distal most of the ventral masses has differentiated into the more posterior flexor antebrachii et carpi ulnaris (FACU) (Figure 4F). Ventromedially, the SPC and pectoralis (P) are now visible (Figure 4J). The more proximal ventral mass has differentiated into three distinct masses; the lateral-most in the stylopod will give rise to the biceps brachii (BB) and the brachialis (BR).

At stage 33 in *C calyptratus* (Figure 6J), the distal ventral mass has expanded into a large sub-rectangular shape along the midline, framed along its proximolateral margins by the flexor carpi radialis (FCR) and flexor carpi ulnaris (FCU). Along the curvature (proximal to FCU) that will give rise to the elbow, the postero-dorsally located triceps (T) muscle mass is visible. From a dorsal view, pronation has shifted the BB + BR mass ventrally (compare Figure 6E and F).

2.2.7 | Stage 34: Dorsal forelimb

At stage 34 in *A uniparens* (Figure 2D,I), further pronation of the forelimb is apparent, with the deltoideus clavicularis (DC) moving to a more ventral position adjacent to the SPC. The deltoideus scapularis (DS) is broader and more vertically oriented (perpendicular to the primary body axis and approximately 90° from the deltoideus clavicularis). The triceps (T) has bifurcated into the lateral (THL) and medial humeral (THM) heads. The extensor carpi radialis (ECR) has a bilobed proximal origin from the medial distal humerus, while the proximolateral margin of the previous extensor digitorum longus (EDL) muscle mass has differentiated into a separate extensor carpi ulnaris (ECU, also called extensor antebrachii et carpi ulnaris). Thus, the ECR, EDL, and ECU now share a common origin from the distal humerus. From the distalmost margin of the previous extensor digitorum longus (EDL), muscle mass emerges a sheet composing precursors of five separate extensores digitorum breves (EDB). The five muscles are indistinguishable in the wrist region, but divisions between them are visible in the region of the metacarpals.

At stage 34 in *P vitticeps* in dorsolateral and dorsomedial aspect (Figure 4C,G), the dorsal proximal aspect of the brachium is covered by the lateral and medial head of the triceps (THM, THL). Dorsal muscles of the antebrachium consist of the extensor antebrachii et carpi radialis (EACR) proximo-anteriorly and the more posterior extensor antebrachii et carpi ulnaris (EACU), both framing the lateral margins of the extensor digitorum longus (EDL) which extends further distally than the ECR and EDL. From the distal margins of the EDL arise five extensores digitorum breves (EDBs) from wrist to proximal digits.

In lateral view at stage 34 in *C calyptratus* (Figure 6G), the latissimus dorsi (LD) can be seen deep to the proximal antebrachium and extending to the body wall. Proximally, the deltoideus clavicularis (DC) now has an anteroventrally directed position while sharing its original insertion with the more posteriorly oriented deltoideus scapularis (DS). The DC now overlaps the SPC, as in the adult condition. Distally along the posterior margin of the brachium, the triceps now has two bellies, the medial head (TMH) and lateral head (TLH) of the triceps. Increased antebrachial pronation along with growth of the extensor antebrachii et carpi radialis (EACR) has placed this muscle superficially over the distolateral margin of the muscle mass consisting of the biceps brachii (BB) and brachialis (BR) anlagen. Along the dorsal distal midline, the expanded extensor digitorum longus (EDL) has given rise to the deeper population of extensor digitorum brevis (EDB) MMPs, corresponding to the appearance of distinct digital rays and thinner interdigital mesenchyme. The extensor antebrachii et carpi ulnaris (EACU) is prominent along the posterior margin of the antebrachium.

2.2.8 | Stage 34: Ventral forelimb

Compared to stages 32-33, the SPC and the pectoralis (P) at stage 34 in *A uniparens* are more ventromedially placed and present a broader, more fan-shaped appearance (Figure 2I). Also in dorsolateral view, the biceps brachii (BB) is now a distinct mass along the anterior margin of the brachium and placed between the more posterior lateral head of the triceps (TLH) and the anteroventrally located brachialis (BR). The BB inserts deep and radial to the ECR. Upon excision of the forelimb (Figure 2N), the more distal ventral muscle mass (VD.m) previously seen in Figure 2M has differentiated into a parallel series of dorsal muscle masses including the posteroventral muscle flexor carpi ulnaris (FCU), the anteroventral muscles flexor carpi radialis (FCR) and pronator teres (PT), and the larger ventromedial muscle flexor digitorum longus (FDL). A broad, distal mass proximal to the metacarpals is the flexor digitorum brevis (FDB), which separated from the VD.m.

In ventral view at stage 34 in *P vitticeps*, superficial muscles were lost after limb excision (Figure 4K). This loss of muscles exposed the deeper coracobrachialis longus (CBL) medial to the biceps brachii (BB). Along the posterior surface of the brachium is the medial head of the triceps (THM). The flexor digitorum longus (FDL) covers most of the midline surface of the ventral antebrachium and is bordered anteriorly by the flexor carpi radialis (FCR) and posteriorly by the flexor carpi ulnaris (FCU). Proximally, nested between the FCR and the BB, is the pronator teres (PT). Distal in the palmar region is a broad sheet of the flexores digitorum breves (FDB, also called flexores breves superficiales).

The major change between stages 33 and 34 in *C calyptratus* is the distal expansion of the flexor digitorum longus (FDL) MMPs that have now given rise to a broad diffuse array of MMPs in the palmar region, which will become the flexores digitorum breves (FDB) (Figure 6K).

2.2.9 | Stage 37: Dorsal forelimb

At stage 37 in *A uniparens* (Figure 2J), the primary change along the forelimb is further muscle splitting in the distal limb, where the extensor digiti brevis (EDB) has differentiated from a sheet along the proximal autopodium (Figure 2F-I) to become a series of five well-spaced proximal extensores digitorum breves (EDB) and five distal (and larger) muscles, the dorsometacarpales (DMCs).

By stage 37 in *P vitticeps* (Figure 4H), the muscles have grown and are in closer proximity with adjacent muscles. The lateral head of the triceps (THL) is now in contact with the lateral margin of the brachialis (Br). Medial to the Br. is the biceps brachii (BB), and the two muscles are now subequal in size with clear muscle fiber directions. The antebrachium also has larger muscles with clearer muscle fibers. The ECR and EACU are now in contact with the lateral margins of the EDL. The extensores digitorum breves (EDB) are distinct proximally, and the dorsometacarpales (DMCs) extend distally toward the base of digits II-V.

At stage 37 in *C calyptratus* (Figure 6H), muscles are denser with more distinct fibers and have all expanded anteroposteriorly to "fill in" their region of the limb (less MF20 negative space superficially). In the proximal brachium, the lateral head of the triceps (THL) occupies most of the posterolateral brachium, while the brachialis (Br.) occupies the more medial portion of the brachium. Along the anterior margin of the antebrachium the brachioradialis (Brd.) is visible (superficial to the no-longer-visible extensor antebrachii et carpi radialis [EACR]), differentiating from the medial margin of the extensor digitorum longus (EDL) with which it remains fused distally. Brachioradialis ("supinator longus" sensu[57]) is present as a separate muscle in chameleons but is largely fused with ECR in other lizards.[57,72] Distally on the dorsal surface of the manus are now visible distinct extensores digitorum breves (EDB) and dorsometacarpales (DMCs).

2.2.10 | Stage 37: Ventral forelimb

In dorsal view of the limb of *A uniparens* (Figure 2J), no ventral muscles are visible due to completion of forelimb pronation. Upon excision of the forelimb (Figure 2O), the differentiated muscles of the brachium and antebrachium appear to have reached their adult complement, with the appearance of a distinct coracobrachialis longus (Cb.L) ventral to the brachialis (Br.) and anterior to the medial head of the triceps (THM). As in the dorsal forelimb, the most significant change has taken place distally where the sheet-like flexor digitorum brevis (FDB) has differentiated into five flexores digitorum breves (also called flexores breves superficiales), each of which bifurcates distally around the base of the ventral metacarpals. Additionally, abductor digiti minimi (Ab. D5) is visible postero-ventrally.

Ventromedially in *P vitticeps* at stage 37 (Figure 4L), the medial head of the triceps has enlarged and meets the anteromedial muscle coracobrachialis (Cb.L), which separates it from the brachialis (Br). The FCR, FDL and FCU leave little space superficially in the antebrachium due to their lateral and anteroposterior expansion and tight parallel organization. The distal flexores digitorum brevis (FDB) have differentiated distally and insert on the radial and ulnar sides of the proximal phalanges, with abductor digiti minimi (Ab.D5) inserting on digit V.

At stage 37 in *C calyptratus* (Figure 6L), ventral muscles have expanded and little space is present between adjacent structures. The ventral brachium presents an anterior margin dominated by the biceps brachii (BB) ventrally and the brachialis (Br.) more laterally. The coracobrachialis longus (Cb.L) and triceps internus (TI) are now visible. Distally, the dorsometacarpales (DMCs) and flexores digitorum breves (FDB) are more distinct as separate muscles.

2.3 | Detailed description of hindlimb muscle development

2.3.1 | Stages 30-31: Dorsal hindlimb

In the dorsal aspect at this very early stage in *A uniparens* (Figure 3A), the hindlimb presents with three muscle masses: a large, proximal mass (DP.m) corresponding to the thigh, a proximally tapering tear-drop shaped distal mass (DD.m) corresponding to the crus, and a smaller

and deeper population of MMPs between these two larger masses. The larger proximal population presents a bilobed shape with a smaller anterior lobe relative to the more proximodistally elongated posterior lobe. The distal autopodial region is free of MMPs.

At stage 30 in *P vitticeps* (Figure 5A,E), the hindlimb is rather straight with no signs of an incipient knee region. Two distinct larger populations of MMPs are visible distally (VD.m) and proximally (VP.m), connected by a continuous band of MMPs joining the anterior margins of the two larger masses. *C calyptratus* were not available at this stage for comparison.

2.3.2 | Stages 30-31: Ventral hindlimb

In ventromedial view in *A uniparens* (Figure 3F), two MMP populations are visible, corresponding the thigh (VP.m) and crus (VD.m).

2.3.3 | Stages 31/32: Dorsal hindlimb

At a slightly more advanced stage in *A uniparens* (Figure 3B), the two main MMP populations are still visible, with the proximal mass unevenly bilobed as described above. The middle population of MMPs appears to have expanded proximodistally between the larger proximal and distal muscle masses and remains in a deeper and more anterior position within the incipient thigh region.

At stage 32 in *C calyptratus* (Figure 7A), the previously continuous proximodistal MMPs has now begun to differentiate into more proximal (DP.m) and distal (DD.m) muscle masses with only a few scattered MMPs between these two larger masses.

2.3.4 | Stages 31/32: Ventral hindlimb

At stage 31/32 in *A uniparens* after excision of the hindlimb (Figure 3G), in ventral view only a distal diffuse MMP population was visible (VD.m) as the proximal population was damaged during processing.

Similar to the dorsum, in *C calyptratus* at stage 32 (Figure 7E), MMP populations in the ventral hindlimb have separated into a distinct distal and proximal series.

2.3.5 | Stages 32-33: Dorsal hindlimb

In *A uniparens* at stage 32/33 (Figure 3C), the hindlimb has grown in diameter and length, increasing from approximately eight myotomal blocks (sacral + proximal caudal) to nine myotomal blocks (with the anterior trunks now beginning to differentiate dorsoventrally). The hindlimb has now become pronated, bringing the dorsoventral axis of the autopodium parallel with that of the trunk and tail. Growth in girth of the limb has also led to expansion of the muscle masses with subsequent differentiation into distinct masses. The proximal dorsal muscle mass (DP.m) is now partitioned into distinct anterior and posterior portions. The superficial part of the anterior proximal mass will differentiate into ambiens (Amb, also called sartorius), while the deeper MMP populations will form and the proximal and lateral heads of the puboischiofemoralis internus (PIF.I). The more posterior of the proximal masses will give rise to the iliotibialis (Iltb) superficially and the iliofibularis (Ilfb, also called tenuissimus) laterally. Deep within this mass is the more proximal iliofemoralis (Ilfm). Distal to the midline between these two larger proximal masses is the deeper population of MMPs, which will differentiate into the femorotibialis (Fmtb), which lies deep to the iliotibialis (Iltb) and distal to the iliofemoralis (Ilfm). The distal dorsal muscle mass (DD.m) is now visible as two superimposed sheets. The anterodorsal-most mass will give rise to the tibialis anterior (TA), which at this stage is still attached to the more dorso-medially placed extensor digitorum longus (EDL). The deeper sheet is composed of a distal component that will form the extensores digitorum brevis (EDB) and a more proximal mass (lateral to the EDL) that will form the fibularis longus (FL) proximally and the fibularis brevis (FB) distally.

Stage 33 in *P vitticeps* (Figure 5B) presents a much more advanced condition from the undifferentiated continuous mass of MMPs in stage 30. The larger proximal dorsal mass has differentiated into a series of superficial and deep muscles. The most distal of the deep muscles, which is derived from the thinner anterior MMP band that connected the larger distal and proximal masses in stage 30, gives rise to the femorotibialis (Fmtb). In dorsolateral view, the most proximal muscle is the puboischiofemoralis internus (Pif.I), whose distal portion is covered by the more superficial ambiens (Amb) that occupies the anterior thigh. Posterior to the ambiens (Amb) is the large iliotibialis (Iltb), whose distal portion covers the femorotibialis (Fmtb) and whose proximal portion covers the deeper iliofemoralis (Ilfm, not visible; see stage 34). A darker staining mass projecting posteroventrally from the posterior margin of the iliotibialis will become the iliofibularis (Ilfb). The distal portion of the lower limb (the crus) has also differentiated into various large masses. The largest of these lies along the dorsal midline and will become the extensor digitorum longus (EDL), from the distalmost margin of which a thinner and broad mass projects to give rise to the precursors of

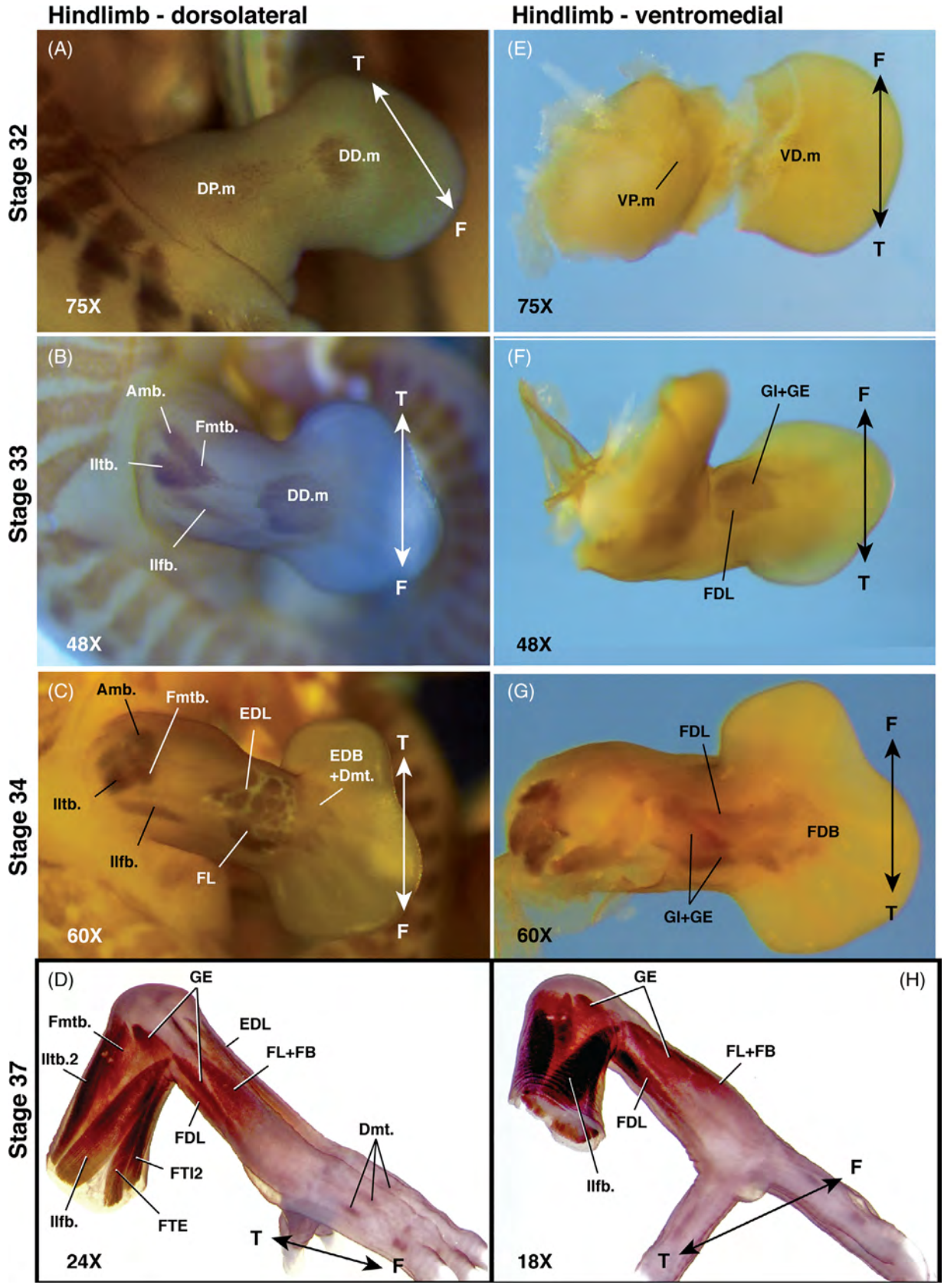

FIGURE 7 Hindlimb muscles of *Chamaeleo calyptratus* at four stages of development, visualized by antibody staining. Left column shows dorsolateral views of the hindlimb at stages 32 (A), 33 (B), 34 (C)and 37 (D). Right column shows ventromedial views of the hindlimb at stages 32 (E), 33 (F), 34 (G), and 37 (H). Arrow indicates tibiofibular axis. See Figure 2 for images of whole embryos and Table 5 for muscle abbreviations

the dorsometatarsales (DMTs) and the extensores digitorum breves (EDB). This large midline series of muscle masses is bordered anteriorly by the tibialis anterior (TA) and posteriorly by the fibularis longus (FL).

At stage 33 in *C calyptratus* (Figure 7B), the proximal dorsal mass has undergone both proximodistal and anteroposterior differentiation. Superficially, the ambiens (Amb) lies along the anterior margin of the thigh, followed posteriorly by the iliotibialis (Iltb). The Iltb lies superficially over the developing (and distal) femorotibialis (Fmtb). From the posterior margin of the proximal muscle mass, the iliofibularis (Ilfb) has differentiated and appears to be the longest muscle in the thigh (proximodistally). The distal dorsal mass (DD.m) has expanded from a smaller subcircular mass to a larger squarish mass with no distinct muscles.

2.3.6 | Stages 32-33: Ventral hindlimb

At stage 32/33 in *A uniparens*, ventral hindlimb musculature is not visible in lateral view due to pronation of the limb (Figure 3C). Upon excision of the limb, only a distal sheet-like muscle mass is visible (VD.m) (Figure 3H), lacking the differentiation that can be seen in the dorsal distal mass at this stage.

In dorsomedial view at stage 33 in *P vitticeps* (Figure 5F), only the most proximal mass can be seen on the ventral side. Two large masses can be seen: the most superficial and closest to the ventral midline is the large puboischiotibialis (Pit, also called gracilis), whose anterior margin sits above the deeper pubotibialis (PTB).

In ventral view at stage 33 in *C calyptratus* (Figure 7F), the distal muscle mass has two distinct domains of dark staining, the most anterior being the precursor muscle mass of the gastrocnemius muscles (GI and GE), while the more proximal and posterior mass will become the flexor digitorum longus (FDL). The proximal mass is not visible due to limb excision from the trunk.

2.3.7 | Stage 34: Dorsal hindlimb

At stage 34 in *A uniparens* (Figure 3D), muscle masses have split to approach the adult complement of separate muscles while also expanding in size within the limbs. Pronation of the limb is slightly more advanced. The proximal mass now has a disk-shaped ambiens (Amb.) superficially and anterodorsally. Proximal and deep to the anterior and posterior margins of the ambiens lie two heads of the puboischiofemoralis internus (PIF.i). The ambiens has expanded posteriorly and abuts the anterior margin of the superficial muscle iliotibialis (Iltb). In dorsal aspect, deep and distal to the iliotibialis is the distal aspect of the femorotibialis (Fmtb), while proximal and deep to the iliotibialis (iltb) is the proximal aspect of the iliofemoralis (Ilfm). From the lateral aspect of the posterior proximal mass visible in at stage 32/33 (Figure 3C), we can now identify the separation of the iliofibularis (ilfb) from the iliotibialis (iltb). At this stage, the iliofibularis is the longest muscle in the thigh region. The distal-most mass has undergone major changes as well, with the more anterior and superficial sheet seen at stage 32/33 splitting into the anterior-most tibialis anterior (TA) and the dorsally and medially placed extensor digitorum longus (EDL). Posteriorly, the previously distinct fibularis longus + brevis from Figure 3C has now divided into the fibularis longus proper (FL) and a distal fibularis brevis (FB). The ventral muscle gastrocnemius externus (GE) can be seen adjacent to FL. The distal sheet is now partitioned into four separate extensores digitorum breves (EDB) that extend to the base of digits II-V.

In a dorsolateral view at stage 34 in *P vitticeps* (Figure 5C), the proximal muscles of the dorsal thigh are larger and darker stained. The deeper and more proximal iliofemoralis (Ilfm), whose distal region is covered by the overlapping iliotibialis (Iltb), is now visible. The iliofibularis (Ilfb) has separated from the posterior margin of the iliotibialis and is the longest muscle derived from the dorsal proximal muscle mass, though much slenderer than the iliotibialis. The distal muscle femorotibialis is larger, darker and appears to sit more superficially in the thigh than in previous stages. Derivatives of the distal dorsal mass have continued to expand with the growing embryo. In particular, the fibularis brevis (FB) appears to be separating from the distolateral margin of the fibularis longus (FL). The ventral crural muscle gastrocnemius externus (GE) can be seen in the lateral aspect of the crus adjacent to the FL. Distally, dorsiflexion of the pes brings the proximal portion of the extensor digitorum brevis (EDB) deeper and the distal portion, closer to the metatarsals where the dorsometatarsales (DMTs) differentiate and are associated with each digit, more superficial.

At stage 34 in *C calyptratus* (Figure 7C), the proximal muscles already visible in dorsal view at stage 33 have primarily grown in size, while the distal muscle mass has expanded into a fan-shaped group of MMPs from which the extensores digitorum breves (EDB) and dorsometatarsales (DMTs) will differentiate. From the posterior margin of the distal mass, the fibularis longus (FL) is now distinct.

2.3.8 | Stage 34: Ventral hindlimb

Compared to stage 32/33, the ventral distal mass at stage 34 in *A uniparens* (Figure 3I) has a greater number of distinct muscles and has also expanded further

anteroposteriorly. Muscles present derived from the broad ventral distal muscle mass (VD.m; Figure 3F,H) are the laterally placed gastrocnemius internus and externus, the midline flexor digitorum longus (FDL) and the more distal arch in the proximal autopodium composed of the flexores digitorum brevis (FDB), whose MF20 staining is darker proximally between adjacent digits.

In ventral view at stage 34 in *P vitticeps* (Figure 5G), the more distal mass remains visible with a complex arrangement of large muscles. The distal ventral mass has differentiated into the more proximal (and larger) muscles, with the flexor digitorum longus (FDL) present deepest along the ventral midline. Superficially, a large gastrocnemius internus (GI) is present along the anterior ventral midline. The gastrocnemius externus (GE) has differentiated from the posterior margin of the proximal muscle mass. GE, which is a rather large muscle at this early stage, is superficial along its more proximal length and deep at its distal extremity. The posterior/lateral expansion of the proximal GE places this part of the muscle in close proximity with the posterolateral margin of the fibularis longus (FL). This position makes the GE visible in dorsal and ventral views. Anterior to the GE (ie, ventromedial in its placement in the crus) is the gastrocnemius "medial head" of the GE which will ultimately fuse with the more posterior component. This ventral view allows for the identification of other dorsal-derived muscles, such as the fibularis brevis distally and fibularis longus along the posterior margin of the crus. In the plantar region of the pes, the flexor digitorum brevis (FDB) has begun to differentiate from a broad sheet of MMPs to individual muscles.

At stage 34 in *C calyptratus* (Figure 7G), the distal ventral muscle mass now has distinct masses for the gastrocnemius internus (GI) and externus (GE) along the proximal and posterior margin of the crus. Distally, a broad mass of MMPs fills the plantar region of the pes as the flexores digitorum breves (FDB) precursors, while along the anteroventral region of the crus the flexor digitorum longus (FDL) dominates.

2.3.9 | Stage 37: Dorsal hindlimb

The final arrangement of mature muscles has been established by this stage in *A uniparens* (Figure 3E), primarily differing from the previous stage in the size and associated increased staining with MF20, making muscles more distinct (giving more resolution to deeper muscles). The proximal dorsal muscles of the thigh have expanded the most in their width and also anteroposteriorly, where the posterior derivative (iliofibularis) is visible just lateral to the ventral muscle flexor tibialis externus (FTE). Additionally, the iliotibialis has grown as a broad and thick sheet of muscle to superiorly overlap the femorotibialis (Fmtb) visible ventrolaterally. The dorsomedial aspect of the thigh is mostly covered by the ambiens, with the two heads of the puboischiofemoralis internus (PIF.I) present proximally. The distal (crus) muscles have differentiated and expanded anteroposteriorly, with the tibialis anterior (TA) visible ventrally. In this view, the abductor hallucis longus (Abd.D1) ("adductor et extensor hallucis et indicus sensu Russell and Bauer[57]) can be seen deep to the extensor digitorum longus (EDL) in the ankle. Distally, the extensores digitorum breves (EDB) extend across the metatarsals, while the dorsometatarsales (Dmt's) reach the bases of the digits.

Relative to stage 34, at stage 37 in *P vitticeps* (Figure 5D), the dorsal hindlimb muscles have primarily enlarged in size and have developed more visible fibers. In the distal dorsum of the pes, the dorsometatarsales (DMTs) have increased in number. Differentiation and growth of the Dmt's appear to have proceeded in an anterior to posterior direction, while also appearing to grow distally along with the lengthening of the metatarsals.

In postero-dorsal view at stage 37 in *C calyptratus* (Figure 7D), the hindlimb muscles appear to have reached their adult complement with larger, more distinct fiber striations. The iliotibialis (Iltb.) sits superficially over the femorotibialis (Fmtb). Posteroventral to the femorotibialis is a proximal head of the ventral crural muscle gastrocnemius externus (GE). Fibularis longus (FL) and brevis (FB) remain undivided at this stage, as in adult chameleons.[72,83] The dorsometatarsales (Dmt's) are now distinctly associated with individual digits.

2.3.10 | Stage 37: Ventral hindlimb

As in dorsal view at stage 37 in *A uniparens*, individual muscles have increased in size and degree of staining, due to their growth and anteroposterior expansion (Figure 3J). The ventromedial aspect of the thigh is characterized by elongated and broad superficial muscles, primarily the pubotibialis (Ptb) present between the ambiens dorsally and the flexor tibialis internus (FTI) ventrally. In ventromedial view, the ventral-most muscle of the thigh is the flexor tibialis internus (FTI) (the two parts of FTI could not be distinguished in this view). Distally in the crus, the gastrocnemius externus and internus (GE, GI) comprise a large portion of the superficial ventral musculature, bracketing the deeper flexor digitorum longus (FDL) medially. Distally in the ankle, the broad sheet of the flexor digitorum brevis (FDB) has differentiated into separate flexores digitorum brevis muscles spanning distally across the metatarsals.

The most obvious feature at stage 37 in *P vitticeps* (Figure 5H) is more distinct fibers. The flexor digitorum longus (FDL) has elongated distally toward the ankle, and the flexores digitorum breves (FDB) have increased in their staining while also lengthening distally with the elongating metatarsals. The distal portion of femorotibialis (Fmtb) can be seen medial and proximal to the knee. This muscle is derived from the proximal dorsal muscle mass (specifically, the MMP population bridging the proximal and distal larger masses). In the ventral thigh, the expanded puboischiotibialis (Pit) and flexor tibialis internus (FTI) can be identified.

In lateral view at stage 37 in *C calyptratus* (Figure 7D), flexor tibialis externus (FTE) can be seen ventral to the iliofibularis and posterior to the now visible flexor tibialis internus 2 (FTI2). The gastrocnemius externus (GE) can be seen spanning across the posterolateral knee joint into the distal crus. The GE is visible between the anterolateral FL and FB and the more ventral flexor digitorum longus (FDL). In ventrolateral view (Figure 7H), the FL and FB demarcate the lateral boundary of the crus while the flexor digitorum longus (FDL) is visible along the posterior midline of the crus.

3 | DISCUSSION

3.1 | Comparison among squamate reptiles

3.1.1 | Forelimb vs hindlimb development

At the earliest stage (29), the limb buds are distinct with forelimb buds larger than hindlimb buds (Figure 1). During stages 30-32, the forelimb becomes pronated and develops a pronounced curvature at the future elbow region, while the hindlimb remains more paddle-like. Forelimb muscle development is more advanced in *A uniparens* at stage 30/31, with three distinct muscle masses visible in the dorsal aspect of the forelimb and only two in the hindlimb (the ventral aspect of the limb could not be observed at this stage without damaging the embryo). Similarly, in *P vitticeps* at stages 29-31, the forelimb is more pronated and distally expanded than the hindlimb (Figure 1). In *C calyptratus* at stages 32-33, the hindlimb is smaller than the forelimb.

3.1.2 | Stages of forelimb muscle development

After the initial formation of ventral and dorsal muscle masses, these masses split into proximal ("VP.m" and "DP.m") and distal ("VD.m" and "DD.m") portions that will give rise to the shoulder/arm and forearm/hand muscles, respectively (Table 1, Figure 8). In the dorsal proximal forelimb of *A uniparens*, we observed a distinct triceps division at stage 30/31 (Figure 2F) and a split between the deltoid and latissimus dorsi divisions at stage 32/33 (Figure 2H). This result is similar to stage 30 [II] in Romer's[17] description of *P muralis*, when the dorsal mass splits into three divisions: proximo-medial (latissimus dorsi, subcoracoscapularis), proximo-lateral (scapular and clavicular deltoids, scapulohumeralis anterior), and triceps. We were not able to observe the deep, proximal muscles subcoracoscapularis or scapulohumeralis anterior in our specimens. Early development of the triceps division varied among taxa: in *P vitticeps*, the triceps was not visible at stage 30 (Figure 4E) (stages 31-32 were not visualized), and in *C calyptratus* it was visible by the first stage (32) but remained connected to the deltoideus division until stage 33 (Figure 6F). In the shoulder and brachium of all three lizards, divisions between the medial and lateral heads of triceps were the last to become visible (at stage 34 [V] in *P muralis*, *A uniparens*, and *C calyptratus* and stage 33 in *P vitticeps*). At the final stage (37) in *C calyptratus*, the derived chameleon muscle triceps internus split from triceps medialis (Figure 5J).

In the ventral proximal forelimb of *A uniparens*, we observed a three-way split at stage 30/31 between pectoralis, SPC, and ventral brachial divisions (Figure 8). Stage 31 was not represented in *P. vitticeps* or *C calyptratus*, but these three muscle groups were visible at the next available stage. Similarly, at stage 30 [II] in *P muralis*, the ventral mass splits into four groups: pectoralis, supracoracoideus, deep (coracobrachialis longus and brevis, biceps short head), and lateral distal (biceps long head, brachialis).[17] However, the muscles of Romer's[17] "deep" group, coracobrachialis longus + brevis and biceps short head, did not appear in our images of *A uniparens* until stage 34 (this portion of the limb was not visible until stage 37 in the other two lizards), and we were not able to trace their origins confidently. The last parts of the ventral shoulder-brachial musculature to differentiate were biceps brachii, brachialis, and coracobrachialis, at stages 33-34 in *A uniparens* (Figure 2H, I) and *P vitticeps* (Figure 4G,J) but apparently not until the final stage (37) in *C calyptratus* (Figure 6H,L). In *P muralis*, these muscles differentiated at stage 32 [III-IV].[17]

The three lizards we studied share a common pattern of development in the antebrachium: somewhat after cleavage begins in the proximal forelimb, the distal mass begins to separate proximodistally into radial, ulnar, and intermediate divisions, with the radial portion cleaving slightly earlier (stages 32-33 vs 33-34) (Figure 8). The radial portion (anlage of extensor carpi radialis) is identifiable at the earliest stages of limb development as a proximal, anterior extension of the distal mass (Figures 2F,G, and 6E,F). In all three lizards, the last muscles to

TABLE 1 Comparative development of the forelimb

Region	*Aspidoscelis uniparens*	*Pogona vitticeps*	*Chamaeleo calyptratus*
Stages 30/31			
Dorsal FL: proximal	Three masses arranged along proximal-distal axis; proximal dorsal muscle mass (DP.m) composed of large, superficial, darkly staining MMP population bridged by deeper, proximal population	(stage 30): Continuous series of MMP clusters along proximodistal dorsal midline; MMPs primarily along anterior margin of limb bud	NV
Dorsal FL: distal	Distal muscle mass (DD.m) is broad, distally rounded, sheet-like and ends proximal to future autopodial domain; anlage of T appears superficially and posteriorly between DP.m and (DD.m)	Distalmost region (DD.m) larger and denser than DP.m	
Ventral FL: proximal	Large proximal mass (VP.m) containing two darkly staining regions (proximal and distal); deeper, lighter staining mass	NV	
Ventral FL: distal	NV		
Stages 31/32			
Dorsal FL: proximal	DP.m less diffuse between superficial anterior and posterior masses; some MMPs from ventral visible in this view	NV	(Stage 32): two large, irregular masses show distinct muscle domains; proximal, posterior "Y"-shaped muscle mass (anlagen of DS and DC) is continuous with elongated T anlage
Dorsal FL: distal	No change		"L"-shaped MMP aggregate w/longer anterior arm (anlage of EACR) and shorter posterior arm (anlage of EDL); diffuse MMP population fans out distally from short arm of "L" (anlagen of EDB)
Ventral FL: proximal	Two new MMP populations from VP.m appear proximally (anlagen of Spc. and P)		Spc. And DC anlagen appear; BB and BR anlagen appear adjacent to ECR
Ventral FL: distal	Single mass (VD.m)		VD.m cleaves proximodistally into FCR, FDL, and FCU
Stages 32/33			
Dorsal FL: proximal	Anlagen of DC and DS expand proximally into trunk; muscle growth and limb pronation bring DC closer to Spc.	(Stage 33): anlagen of DC and DS appear; anlagen of TMH (superficially) TLH (deep) differentiate from MMPs bridging DP.m and DD.m	(Stage 33): muscle growth and limb pronation bring Spc. closer to ventral muscles (P) and shift anlagen of BB+ Br. ventrally; DS and DC shift anteriorly and separate from T
Dorsal FL: distal	DD.m cleaves into EDL and ECR; T and ECR extend to elbow region; MMPs extend toward autopodium	DD.m cleaves proximodistally into EACR, EACU and anlagen of EDL + EDB; EDB forms distal projections to digits II-IV	EDL expands into large rectangular sheet; EACU and ECR cleave from EDL

(Continues)

TABLE 1 (Continued)

Region	*Aspidoscelis uniparens*	*Pogona vitticeps*	*Chamaeleo calyptratus*
Ventral FL: proximal	Spc. and P expand proximally; Br. distinct	Spc. and P visible; VP.m differentiated into three masses, including anlagen of BB and Br. (laterally)	Pronation shifts BB + Br. mass ventrally
Ventral FL: distal	Distinct anterior boundary of VD.m at incipient wrist	FACU cleaves from posterior margin of VD.m; more anterior muscles not visible in this view	FDL anlage expands into large subrectangular midline mass
Stage 34			
Dorsal FL: proximal	DS broader and more vertically oriented (~90° from DC); T bifurcates into THL and THM	THM and THL expand to cover dorsal proximal aspect of brachium	LD appears; DC oriented anteroventrally while DS is oriented posteriorly; THM and THL differentiate
Dorsal FL: distal	ECU cleaves from EDL; ECR, EDL, and ECU converge toward common origin; anlage of EDBs cleaves from EDL	EDL extends further distally; anlage of EDBs cleaves from EDL as 5 almost completely distinct muscles	Anlagen of EDB arise deep to EDL
Ventral FL: proximal	Spc. and P broader and more ventromedial	Due to removal of superficial muscles, CBL is visible	No change
Ventral FL: distal	BB and Br. appear; VD.m cleaves proximodistally into FCU, FCR, PT, and FDL; anlage of FDBs appears	FDL expands; PT appears; anlage of FDBs appears	FDL expands distally; anlage of FDBs appears
Stage 37			
Dorsal FL: proximal		THM expands to contact lateral margin of Br.	THL expands to occupy most of the posterolateral brachium
Dorsal FL: distal	EDB anlage differentiates into five well-spaced proximal EDBs and five distal (and larger) DMCs	ECR and EACU expand; EDB distinct proximally, and Dmc's extend distally to base of digits	Brd. (in chameleons only) cleaves proximally from EDL (remaining fused distally); distinct EDBs and DMCs appear
Ventral FL: proximal	Cb.L appears	THM enlarges to contact Cb.L	Cb.L and TI (in chameleons only) are now visible
Ventral FL: distal	FDB anlage cleaves intofive FDBS, Ab. D5 appears	BB and Br. now subequal in size w/ clear fiber directions; FCR, FDL and FCU expand; FDB fully differentiated; Ab.D5 appears	BB and Br. expand; FDB and DMC distinctly separate

Note: NV indicates a stage/region that was not available/visible. See Table 3 for muscle abbreviations
Abbreviations: Fl, forelimb; MMPs, migratory muscle precursors.

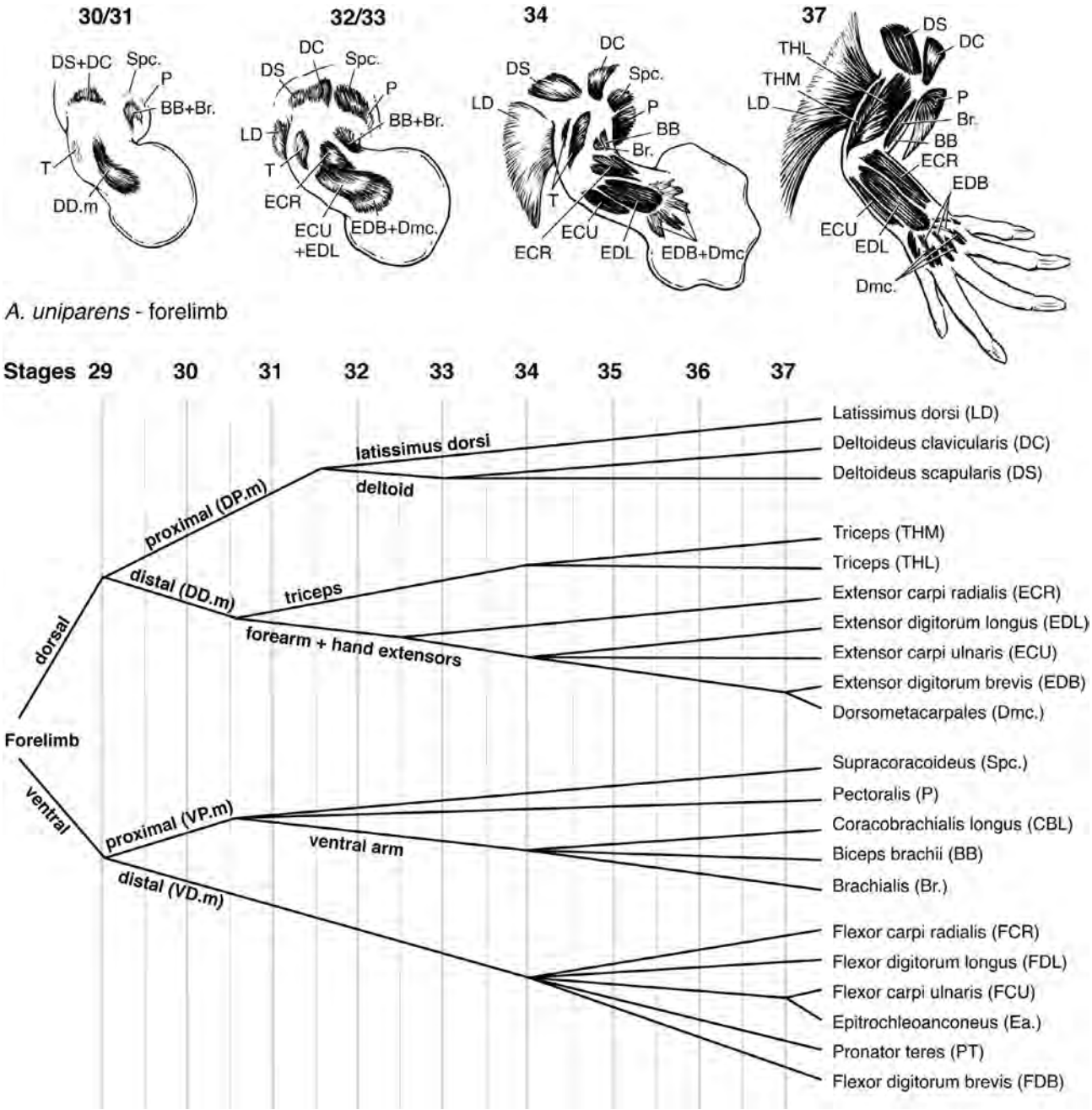

FIGURE 8 Branching diagram showing timing of appearance of forelimb muscles of *Aspidoscelis uniparens*. At the top are schematic drawings of the forelimb in dorsolateral view at stages 30/31, 32/33, 34, and 37. At stage 29, only dorsal and ventral muscle masses are present. At stage 30/31, the dorsal and ventral masses are divided into distinct proximal and distal portions. In addition, several muscle groups are faintly visible in the proximal limb region: the triceps group from the proximal dorsal muscle mass, and the SPC and pectoralis from the proximal ventral muscle mass. At stage 32/33, latissimus dorsi is differentiated and separations become visible between muscle groups in the shoulder and forearm, including proximal-distal (eg, ECU + EDL from EDB + Dmc.) and medial-lateral (ECU + EDL from ECR). At stage 34, the shoulder, arm, and forearm muscles are differentiated. Between stages 34 and 37 the dorsal muscles of the manus become differentiated and the epitrochleoanconeus splits from the flexor carpi ulnaris

differentiate were extensor digitorum brevis from the dorsometacarpales and the flexor carpi ulnaris from the epitrochleoanconeus (stage 37; Figure 8). In *A uniparens* and *P vitticeps*, the dorsal muscles of the manus were visibly distinct at stage 32-33, and at stage 34 the digital extensors were fully separated from the antebrachial extensors and beginning to form individual muscles. In contrast, at stage 34 in *C calyptratus*, the digital extensor muscle mass was barely distinguishable (Figure 6G). Similarly, muscle development is markedly delayed in the

manus of the chick, with individual muscles differentiating 5-10 stages later than the first shoulder muscles.[62] In contrast, development of the distal limb muscles in (more distantly related) salamanders follows the general tetrapod pattern.[59] This convergence between chicks and chameleons suggests that delayed muscle differentiation may be related to extreme specialization of the autopodia in both taxa.

3.1.3 | Stages of hindlimb muscle development

As in the forelimb, initial dorsal/ventral segregation of MMPs is followed by proximodistal separation into hip/thigh ("DP.m" and "VP.m") and crus/pes populations ("DD.m" and "VD.m") (Table 3, Figure 9). In the dorsal proximal hindlimb of *A uniparens*, the first division is a

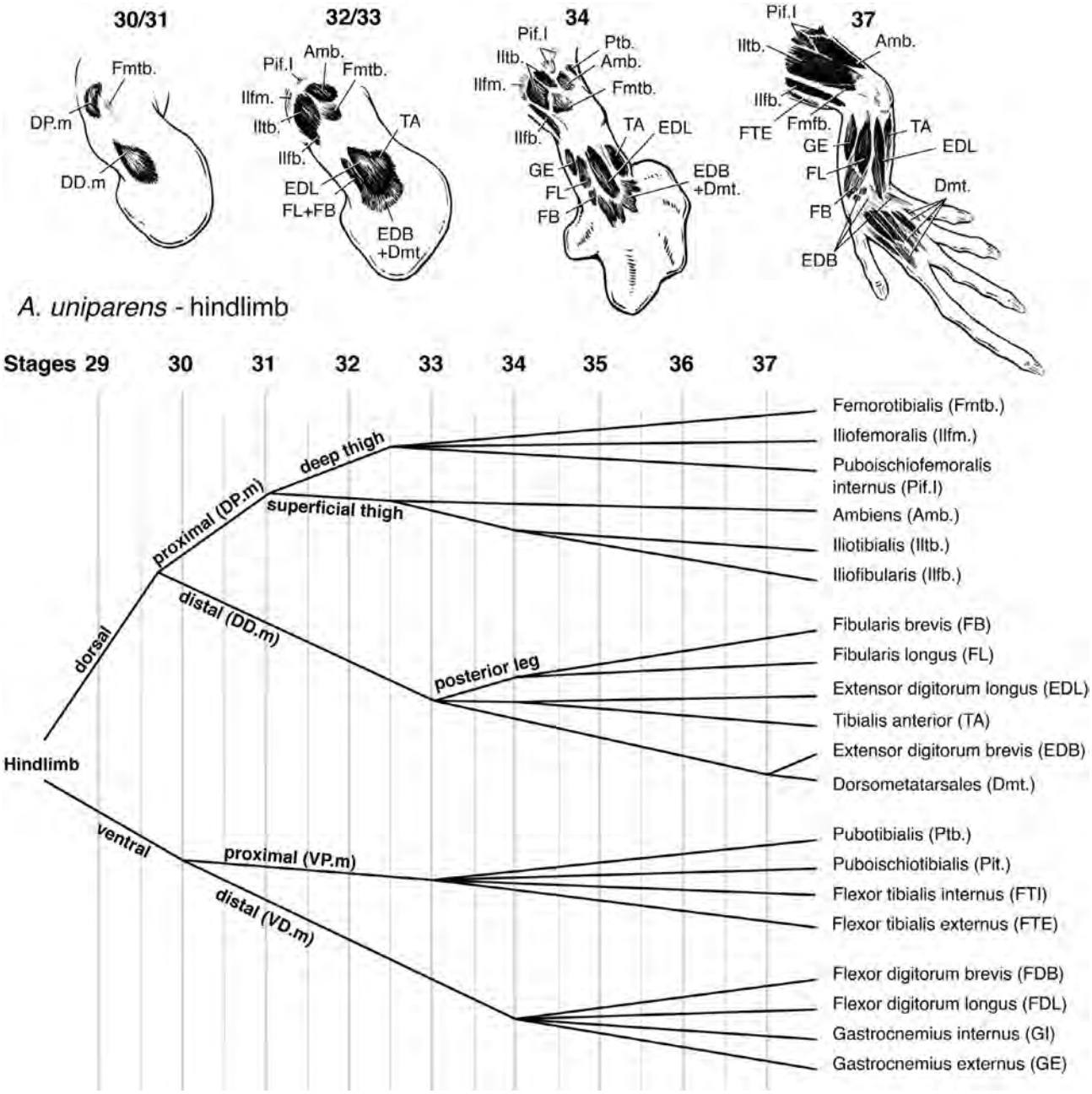

FIGURE 9 Branching diagram showing timing of appearance of hindlimb muscles of *Aspidoscelis uniparens*. At the top are schematic drawings of the hindlimb in dorsolateral view at stages 30/31, 32/33, 34, and 37. At stage 29, only dorsal and ventral muscle masses are present. At stage 30/31, the dorsal and ventral masses are divided into distinct proximal and distal portions and the femorotibialis is faintly visible in the proximal dorsal region. At stage 32/33, separations become visible between muscle groups in both the thigh and leg, including superficial-deep (eg, Amb. + Iltb. from Ilfm.) and mediolateral (FL + FB from EDL + TA). At stage 34, all of the thigh and leg muscles are differentiated. Between stages 34 and 37 the dorsal muscles of the pes become differentiated

superficial-deep one, where incipient femorotibialis becomes visible at an early stage (30/31) deep and medial to the rest of the dorsal proximal mass (Figure 3A). In contrast, at stage 30 [II] in the hindlimb of *P muralis*, the dorsal proximal mass splits anteroposteriorly into two divisions: "anterior" (femorotibialis, ambiens, puboischiofemoralis internus) and "posterior" (iliofibularis, iliofemoralis, iliotibialis).[16] We did not observe an anterior-posterior split of the dorsal proximal mass in *A uniparens*, *P vitticeps*, or *C calyptratus* until stages 32-33, when many of the muscles of Romer's anterior and posterior divisions had already differentiated. Notably, a similar pattern to that of *A uniparens* was described in the thigh of the chick, with early superficial-deep division of the dorsal thigh musculature and femorotibialis beginning to cleave from the deep layer at stages 30-31 [II/28].[58,64] However, in the other lizards, femorotibialis is not seen until later (33 in *P vitticeps* and *C calyptratus* and 31-32 [III] in *P muralis*). At stage 34 in *A uniparens* and *P vitticeps*, the dorsal thigh muscles are completely differentiated (Figures 3D and 5C), like the condition at stage 33 [IV] in *P muralis*. In *C calyptratus*, however, this differentiation is not complete until stages 35-37 (Figure 7D).

We were unable to visualize the deep, proximal muscles of the ventral thigh in our samples, but we did observe a split at stage 33 in *P vitticeps* between the deeper pubotibialis and the more superficial puboischiotibialis (Figure 5F), consistent with an early split between superficial and deep divisions. Similarly, at stage 30 [II] in *P muralis*, the ventral proximal mass splits into three divisions: flexor (puboischiotibialis, flexor tibialis internus, and externus), caudal (caudofemoralis longus and brevis, ischiotrochantericus—we could not visualize these muscles), and deep (puboischiofemoralis externus, adductor femoris, pubotibialis).[16] We did not observe differentiation of the "flexor" group in our specimens until stages 34-37, but the proximal ventral muscles were very difficult to see and it is likely that these events happened earlier, as in *P muralis* (at stage 31 [III] in *P muralis* almost all of the ventral thigh muscles were differentiated). In chicks, the arrangement of muscle groups is similar, but the order of division and cleavage is somewhat different (eg, muscles in the deep group separate from each other before the deep group divides from the flexor group[64,66]).

As in the forelimb, the distal portion of the hindlimb of the three lizards we studied showed anterior-posterior and proximal-distal divisions at stages 32-34 (Figure 9). In addition, the ventral crus showed a superficial-deep division at stage 34 (33 in *C calyptratus*), with the flexor digitorum longus appearing deep to the two heads of gastrocnemius (Figures 3I, 5G, and 7F). At this stage in *P vitticeps*, two muscle bellies appear on the proximolateral part of the ventral crus (Figure 5G), most likely representing parts of the gastrocnemius externus which will fuse at a later stage. Intrinsic pedal muscles in *C calyptratus* differentiate slightly earlier than those of the manus, yet they are delayed relative to the other lizards (Figure 7C,G).

3.2 | Comparison with other tetrapods

Comparative development of muscles of the shoulder and/or thigh region has been studied in frogs, turtles, salamanders, birds, and mammals, and, to a lesser extent, in squamates, as noted in the Introduction. All tetrapods studied so far share a common pattern of early muscle differentiation: first, muscle precursors segregate into dorsal and ventral masses, and, second, they separate at the future elbow and knee into proximal and distal masses. Moreover, in both the forelimb and hindlimb, muscles in the proximal segment (brachium/thigh) tend to differentiate before those in the distal segments (antebrachium/crus and manus/pes), and dorsal muscle groups tend to differentiate before ventral ones.[64]

We hypothesized that differences among our study taxa would arise in the patterns and order of muscle cleavage, with the most pronounced differences arising between more distantly related and more specialized taxa. For example, the wing and pes of chickens are specialized for flying and perching, and more developmental differences from lizards are present in those regions than in the thigh.[84] Muscles particular to one taxonomic group (eg, supraspinatus and infraspinatus in mammals; anterior and posterior parts of latissimus dorsi in birds[61,62] tend to form later in development by cleavage of an ancestral muscle. For these reasons, we expected to find very similar developmental patterns in *Aspidoscelis uniparens* and *P vitticeps* to previous descriptions of the wall lizard *Podarcis* ("*Lacerta*") *muralis*[16,17] and, to a lesser extent, the painted turtle *Chrysemys picta marginata*.[60] In addition, distal limb muscles may be more variable among taxa because they are further separated from the "phylotypic stage" (develop later[85]). Moreover, distal limb muscles are the ones in contact with the substrate, so they may acquire more specializations related to locomotion. Therefore, we expected to find the most differences from the generalized lizard condition in the distal limb regions of *C calyptratus*, which are highly specialized for a unique form of arboreal locomotion. This prediction was borne out: *C calyptratus* exhibited delayed differentiation of the muscles of the manus and pes compared to the other lizards, paralleling a similar delay described in the manus of chickens,[62] and the proximal

muscles of the limbs are much more similar between chameleons and the other lizards studied than are the distal ones. While the more proximal limbs have a more conserved set of muscles between lizards, their appearance shows some heterochrony. At stage 34, chameleon thigh and crus muscles (Figure 7C,G) show less cleaving into the adult muscle complement when compared to the whiptail (Figure 3D,I), with chameleons showing a delayed separation of the Amb, Fmtb, and Iltb. Thus, there appears to be a delay in muscle development when examining the chameleon hindlimb relative to a generalized lizard (with forelimb muscles very similar between the chameleon and whiptail at stage 34; Figures 2I and 6G).

Comparisons with mammals have remained challenging. While the domestic mouse (*Mus musculus*) and Norwegian rat (*Rattus norvegicus*) remain as the primary mammalian models in the laboratory for understanding developmental processes, studies lack where both forelimbs and hindlimbs are examined solely to describe limb morphogenesis. Recent (yet short lived) attempts to present a digital database for E14.5 mouse limb muscles (http://www.nimr.mrc.ac.uk/3dlimb/) were a first step in this direction.[86] Most studies in the mouse developmental literature examine specific regions of the limb or muscles relative to a mutant phenotype, while generally focusing on dorsal (extensor) musculature at the expense of ventral descriptions. Further complexities arise from differences in muscle markers visualized, stages, mouse lines used along with image orientations and muscle nomenclature.[18,55,63,70,74,86-106] Rafipay et al[94] recently examined tissue-specific marker expression in developing mouse forelimb and hindlimb; while useful images were provided, they do not allow for clear identification of muscles and attempts at labeling muscles were not provided. Recent work on human embryos has provided excellent images and developmental sequences though these focus solely on the distal limbs[69,70] or only the forelimb.[89] Confocal imaging and new computational methods for segmenting muscles will help fill in this gap in our resources for comparative muscle development.[69,74,89,107]

3.2.1 | Comparative development of forelimb musculature

Even in highly specialized forms such as wings and phylogenetically distant taxa such as salamanders, divisions of muscle masses and cleavage of muscles follow much the same pattern.[19,37] After segregation of muscle precursors into dorsal and ventral masses and proximal and distal divisions, the proximal dorsal mass forms four divisions (deltoid, subscapular, latissimus dorsi, and triceps), while the proximal ventral mass forms three divisions (pectoral, supracoracoid, and ventral brachial).[62] The same condition was observed at stages 32-33 in the lizards we studied, although we were not able to visualize some of the deep proximal groups due to being obscured by the more superficial muscles in wholemount (while we cannot exclude the possibility that insufficient antibody penetrance to deeper muscles may have played a factor as well; Figures 2H, 4J, and 6E,F). The triceps and ventral brachial groups are among the last to fully differentiate, and differences in the timing of cleavage of muscles/muscle heads within these groups are present both within lizards and among tetrapod clades. After the formation of these divisions, muscle cleavage then proceeds proximodistally via longitudinal cleavage of first the antebrachial muscles (radial, ulnar, and intermediate divisions) and finally the manual muscles (divisions associated with each digit).

The main differences in timing and order of developmental events occur in regions of morphological specialization, including the shoulder girdle of turtles and salamanders and the manus of chicks and chameleons. The process of muscle cleavage in the forelimb of turtles (*Chrysemys picta marginata*) is similar to that of *P. muralis*,[60] but some differences appear in the shoulder region (most likely related to derived condition within turtles in which the ribs migrate dorsal to the girdles). For example, the chelonian "coracobrachialis magnus"—thought to be homologous with the lepidosaurian coracobrachialis longus—forms a distinct proximal division at an early stage of development (stage II, roughly equivalent to our stage 30).[60] Later (stage 33 [IV]), the chelonian muscle "teres major" (not homologous with the mammalian muscle of that name[37]) cleaves from the latissimus dorsi.[60] Although the musculoskeletal structure of the chick forelimb is highly modified from the generalized tetrapod condition, shoulder and brachial development proceeds in a similar pattern to that of lizards early on, whereas avian specializations, such as the differentiation of distinct anterior and posterior portions of latissimus dorsi, arise at relatively late stages (stage 33 [29, 30]).[62] Similarly, mammals such as opossums (*Didelphis virginiana*) possess additional limb muscles (eg, supraspinatus, infraspinatus, and cleidoacromialis) that mainly arise late in development via cleavage from an ancestral tetrapod muscle (in this case from the SPC).[61,62] Despite the phylogenetic distance between the two taxa, forelimb muscles in salamanders (*Necturus maculosus*) develop similarly to those of the lizards, although, as in turtles, there are some differences in timing and order of differentiation of the dorsal shoulder and brachial muscles.[59] For example, in the salamander triceps coracoideus appears relatively early (stage 32 [17.5-19 mm]) and subcoracoscapularis appears later

(stage 34 [21 mm+]).[59,61] These differences may relate the specialization of the shoulder girdle in salamanders, in which the clavicle is lost and the pro-coracoid cartilage is gained.[17]

3.2.2 | Comparative development of hindlimb musculature

Hindlimb muscle development across tetrapods has not received as much attention as the forelimb, but it has been described in taxa such as salamanders (*Ambystoma mexicanum*[67]; *Necturus maculosus*[59]), frogs (*Eleutherodactylus coqui*[68]), mice (*Mus musculus*[63]) and humans[69,70] as noted earlier, and also in the domestic chicken *Gallus gallus*. Unlike in the shoulder, patterns of early muscle development in the thigh are notably different between lizards and chicks (to aid in comparison, we describe avian development using the names of homologous muscles in lepidosaurs[108]). Romer[58] described a pattern of thigh development in *G gallus* very similar to that observed in lizards, interpreted as evidence that muscle development in reptiles is highly conserved. However, redescriptions of thigh development utilizing histology and wholemount staining to provide data which can be more directly compared to our own have revealed a more complex picture, with less conserved patterns in the ventral thigh than in the dorsal thigh.[64,66] Overall patterns of division and cleavage were similar among the three lizards we studied and also similar to those described for *P muralis*[58] except for the first division of the dorsal thigh muscles (superficial-deep in *P muralis* and anterior-posterior in the other lizards). In addition, *C calyptratus* exhibited delays relative to the other lizards in differentiation of the dorsal thigh muscles as well as in separation of the pedal muscles from the crural division, whereas the superficial-deep division in the ventral crus occurred slightly earlier.

The distal hindlimb in chicks is specialized for perching, and its muscle development differs from that of the lizards in several ways. The chick exhibits earlier differentiation of intrinsic pedal musculature and an early anterior-posterior separation between anlagen of the long flexors of medial and lateral digits.[66] In contrast, the development of crural musculature is very similar, with early proximodistal separations between the thigh, crus, and pes (stages 30-33 [26–29]). However, this early stage includes the first step toward specialization of the pedal musculature in *G gallus*: a split in the ventral shank division between the medial and lateral parts of the future "flexor digitorum longus (FDL)," which comprises eight separate muscles in adult chickens.[108] In addition, this mass undergoes a superficial-deep cleavage between the future FDL homologs and the future gastrocnemius muscles,[66] a separation that was visible much later in the lizards. At stage 32/33 [29] in *G gallus* the dorsal and ventral crural divisions cleave antero-posteriorly, very similar to the equivalent stage in the lizards (Figure 9) despite the different number of adult crural muscles in chicks and lizards.[108] At this stage (32/33 [29]), the intrinsic pedal muscles are fully separate from the crural muscles, an event that occurs even later in the lizards (stages 34-37). Muscle cleavage in *G gallus* is complete by stage 37 [35] and the last muscles to differentiate are the intrinsic pedal muscles and fibularis longus from fibularis brevis,[66] similar to the case in lizards (Figure 9).

3.3 | Adaptation for arboreal locomotion in chameleons

The adult forelimb musculature of chameleons is highly modified for their specialized form of arboreal locomotion, particularly in the shoulder and autopodium.[72,77] Therefore, it is not surprising that muscle development differs more between *C calyptratus* and the nonchameleon lizards than it differs among the non-chameleon lizards. These differences are apparent in the arrangement and shape of early muscle masses, the timing of differentiation of muscles relative to limb morphogenesis, and the developmental delay in formation of distal muscles.

In addition to increased mobility of the girdle on the body wall, the chameleon shoulder has several modifications thought to increase anterior–posterior range of motion, including loss of the clavicle, loss of the coracoid head of triceps, and more proximal areas of muscle insertion on the humerus.[57,77] It is possible that the persistent connection at stage 32 between the deltoideus and triceps anlagen (Figure 6E) is related to the loss of triceps coracoideus. The anlagen of these three muscles form a Y-shaped mass at this stage, whereas in *A uniparens*, the triceps and deltoideus anlagen appear as two separate condensations from the earliest stage (30/31; Figure 2F). (Stages 31-32 were not observed in *P vitticeps*.) In addition, the SPC in adult chameleons has a more dorsal origin and more vertical fiber direction, thought to facilitate shoulder protraction.[77] This difference is evident very early in development at stage 32, at which point in *C calyptratus* this muscle has already migrated to the dorsal side of the shoulder (Figure 6E), similar to stage 33 in the other two lizards (Figures 2H and 4F,J). Several additional differences appear at the final stage in our series (37), including the cleavage of the chameleon muscle triceps internus from triceps humeralis medialis and the cleavage of brachioradialis (supinator longus) from extensor carpi radialis (Figure 6G,K).

Fewer locomotor adaptations are obvious in the thigh and crus of chameleons, but there are some differences in the adult musculature.[72] For example, chameleons have more complex dorsal thigh musculature than non-chameleon lizards, with iliotibialis and iliofemoralis each consisting of two parts rather than one; this complexity may relate to the later differentiation of dorsal thigh muscles relative to other lizards (stage 37, vs 34 in other lizards). Alternatively, the central role of the forelimb in slow arboreal quadrupedalism (providing propulsion and body support) may produce a relative delay in hindlimb muscle development in chameleons. In comparison, hindlimb development is more advanced than forelimb development at stage 32 in leopard geckoes.[82] Finally, chameleons lack a separate fibularis brevis, which is one of the last hindlimb muscles to differentiate in non-chameleon lizards (stages 34-37).

The intrinsic autopodial muscles of chameleons have several adaptations that appear to enhance grasping ability. In particular, the superficial portion of the flexores digitorum breves in the manus and pes exhibit more transverse fiber orientations, increased cross sectional area, and direct insertion on the phalanges, which likely increase their ability to adduct the digits.[72] In addition, the extensores digitorum breves in the manus originate more proximally (partially from the radius, ulna), possibly reflected in the relatively later separation and differentiation of the intrinsic manus musculature (stage 34, vs 32-33 in other lizards).

One of the most distinctive synapomorphic characters in chameleons is the cleft manus and pes phenotype, which allows for grasping of narrow substrates in their environments. Diaz and Trainor[23] examined the embryonic patterning and development of the chameleon autopodia and concluded that programmed cell death was only one of the mechanisms that must be in place for the cleft to form between digits III and IV in the manus and II and III in the pes.

Recent work by Francisco Botelho et al[109] presents a fascinating system in which the zygodactylous nature of the avian pes (a posterior shift of digit I) is driven by the activity of embryonic limb muscles *in ovo* and leads to twisting of metatarsal I and retroversion of the hallux, resulting in the unique and opposable pes morphology found in avians known as zygodactyly (functionally analogous but structurally and mechanistically different from the formation of the chameleon autopodium termed as "chamaeleodactyly" [pers. Comm.: D. Naish, C. Anderson, M. Scherz]). Paralysis of embryonic muscles prior to the rotation of metatarsal I in avians (HH 34[66,109,110]) prevents digit rotation, a stage equivalent to between our stages 34 and 37, at which point the majority of distal limb muscles have differentiated and separated from earlier masses and significantly later than the stage when clefting is clearly visible (our stage 33, similar to the avian limb at HH29/30). While functionally similar, an underlying mechanism of formation appears to not be shared and the force driving the bifurcation of chameleon limbs is not yet understood. Based on the muscle data obtained in our previous study of adult chameleons[72] and the present study of muscle limb development of chameleons and other lizards, we plan to address this fascinating topic in future studies.

4 | CONCLUSIONS

Romer[58] asserted that muscle development in tetrapods is extremely conserved even among groups with divergent adult morphologies, a view that is still widely accepted[23,28,111-117]). Our results showing a conserved pattern of early muscle development among lizards with different adult morphologies and ecologies support this view. Differences among taxa were concentrated in regions of the body with specialized bone morphology, such as the autopodia of chameleons, and they tended to arise late in development. Based on evolutionary and developmental studies, it has been proposed that muscle morphology is more conserved than bone morphology.[118] Additional examples of surprising evolutionary conservation of muscles can be found in adult chameleons,[72] limb reduced lizards,[118] and birds.[119]

The relative conservation of muscle anatomy bears on the question of evolution of extreme phenotypes, such as in chameleons. While muscle precursors differentiate into muscle cells through intrinsic factors,[56] their ultimate fate with respect to the type of muscle, spatial arrangement and splitting patterns are determined by extrinsic signals from ectoderm, skeletal tissue and connective tissue forming the muscle fascia, tendons, and ligaments.[52,55,104,120-125] Because later muscle bundle patterning is directed by signals from connective tissue, it is considered an "exploratory process" that facilitates evolution of complex structures by increasing the capacity to produce viable outcomes.[126] By this process, muscles can accommodate to evolutionary changes in bone morphology without any additional genetic changes.[126] Thus, the robustness and adaptability of muscle patterning facilitates evolution of extreme musculoskeletal phenotypes (eg, Goldschmidt's[127] "hopeful monsters"[128-131]). In chameleons, major developmental changes like the bifurcation of the autopodia change the internal skeleton, leading to the minor but functional changes of the muscles described in the present study, ultimately leading to a different (but still functional) phenotype. The present study paves the way for future studies on the

developmental differences between chameleons and other lizards to discover the mechanisms that drive them, and ultimately to understand how the phenotype of chameleons came to be.

5 | EXPERIMENTAL PROCEDURES

5.1 | Embryo collection

Veiled chameleon (*Chamaeleo calyptratus*; Chamaeleonidae) and desert grassland whiptail lizard (*Aspidoscelis uniparens*; Teiidae) embryos were harvested at the Stowers Institute for Medical Research (SIMR IACUC Proposal 2010-0065) through the Reptiles and Aquatics Facility, while bearded dragon (*P vitticeps*; Agamidae) embryos were harvested at Southeastern Louisiana University (SELU IACUC Proposal 0046) through the Diaz Lab in accordance with respective Institutional Animal Care and Use Committee approved protocols. Two to three embryos were collected from the same clutch at the designated time point and were used for antibody staining. Eggs were incubated in moist vermiculite at 28°C and were collected at stages prior to and through limb development based on known tables (see Reference 132) for veiled chameleon and whiptail, and Ollonen et al[133] for the bearded dragon). Eggs were cleaned of vermiculite and placed in ice cold 1× phosphate-buffered saline (PBS). Prior to cutting, eggs were candled to identify location of embryo and egg was cut circumferentially (avoiding embryo) while immersed in 1× PBS. Only the eggshell was cut and removed, leaving the embryo enveloped in extraembryonic membranes.[13] Embryos were euthanized with MS-222[134] prior to being fixed in 4% paraformaldehyde (PFA) overnight at 4°C on a nutator and were subsequently washed in cold 1× PBS and gradually dehydrated into absolute methanol for storage at −20°C.[23] Images were taken through a Leica MZ FLIII stereoscope equipped with a Carl Zeiss color AxioCam HRc utilizing the AxioCam Software. Adobe Photoshop 2021 and Adobe Illustrator 2021 within the Adobe Creative Cloud suite were used for image cleaning and creation of figures, respectively.

5.2 | Wholemount immunohistochemistry

Methods for wholemount immunohistochemistry have been described previously.[13,135,136] Muscle myosin heavy chain was targeted with a MF20 primary antibody (Mouse IgG; Developmental Studies Hybridoma Bank, diluted 1:20 in blocking buffer; see below) and visualized with a Goat anti-Mouse IgG (H + L) secondary antibody (Horseradish peroxidase conjugated; Invitrogen #62-6520, diluted 1:500 in blocking buffer) in combination with Diaminobenzidine (DAB) Substrate Kit (Roche: 11718096001). DAB based precipitate revelation was

TABLE 2 Equivalent developmental stages in different tetrapod embryos

Wise Stage Leopard Gecko[a]	Ollonen Stage Bearded Dragon[b]	Hamburger-Hamilton Chick[c]	Post coitus staging Mouse[d]	Romer Sand Lizard,[e] Turtle,[f] Opossum[g]	Chen Necturus[h]
29	4 dpo	HH22/23	E10.5	I	
30	8 dpo	HH25	E11	II	
31	12 dpo	HH28	E11.5	III	17.5 mm
32	15 dpo	~HH29/30	E12	III-IV	17.5-19 mm
33	~15 dpo	~HH29/30	E12.5	IV	19-21 mm
34	18 dpo	HH30/31	E13.5	V	21 mm+
37	24-28 dpo	HH35/36	E15.5		
40	~40 dpo	?	?		
41	~48dpo	?	?		

Note: The current manuscript uses wise stages for the Leopard Gecko.
[a]*Eublepharis macularius*.[82]
[b]*Pogona vitticeps*.[133]
[c]*Gallus gallus*.[142]
[d]*Mus musculus*.[143,144]
[e]*Podarcis muralis*.[16,17]
[f]*Chrysemys picta marginata*.[60]
[g]*Didelphis virginiana*.[61]
[h]*Necturus maculosus*.[59]
Abbreviations: ?, equivalent stage unclear.

TABLE 3 Comparative development of the hindlimb

Region	*Aspidoscelis uniparens*	*Pogona vitticeps*	*Chamaeleo calyptratus*
Stages 30–31			
Dorsal HL: proximal	Three masses along proximal-distal axis; large, proximal, bilobed mass (DP.m) w/smaller anterior lobe and proximodistally elongated posterior lobe; smaller, deeper MMP population between DP.m and DD.m	(Stage 30) two larger populations of MMPs visible proximally (DP.m) and distally (VD.m), connected by a continuous band of MMPs joining the anterior margins of DP.m and VD.m	NV
Dorsal HL: distal	Proximally tapering tear-drop shaped dstal mass (DD.m); distal autopodial region free of MMPs		
Ventral HL	Two MMP populations, proximal (VP.m) and distal (VD.m)	NV	
Stages 31-32			
Dorsal HL: proximal Dorsal HL: distal	Two main MMP populations still visible; proximal mass unevenly bilobed as described above; middle MMP population expanded proximodistally between DP.m and DD.m and remains deeper and more anterior within incipient thigh region.	NV	(Stage 32): MMP population differentiating into proximal (DP.m) and distal (DD.m) masses w/few scattered MMPs in between
Ventral HL: proximal	Damaged during limb excision		Similar to the dorsum, MMP populations separated into distinct distal (VD.m) and proximal (VD.m) masses
Ventral HL: distal	Diffuse distal MMP population visible (VD.m)		
Stages 32–33			
Dorsal HL: proximal	Two portions: anterior (anlagen of Amb. superficially and Pif.I deep) and posterior (Iltb. superficially and Ilfb. laterally); anlagen of Ilfm. and Fmfb. appear	(Stage 33): two masses: superficial (Amb. and Iltb.) and deep (Pif.I and Fmtb., which is derived from MMP band that connected DP.m and DD.m) portions; anlage of Ilfb. appears	(Stage 33): DP.m cleaves proximodistally and anteroposteriorly; anlagen of Amb. (superficially and anteriorly), Iltb. (superficially), Fmtb. (deeper and distal) appear; Ilfb. appears
Dorsal HL: distal	Two superimposed sheets; superficial (anlagen of TA and EDL, still attached), and deep (EDB distally and FL + FB laterally)	Three masses: midline (anlagen of EDL, DMT, and EDB), anterior (TA), and posterior (FL)	DD.m expands and becomes squarish; no distinct muscles visible
Ventral HL: proximal	Damaged during limb excision	Two masses: superficial and midline (anlage of Pit.) and geep (Ptb.)	Damaged during limb excision
Ventral HL: distal	Sheet-like mass with no distinct muscles	NV	Two domains: anterior (anlagen of GI + GE) and posterior (FDL)
Stage 34			
Dorsal HL: proximal		Ilfm. appears; Ilfb. cleaves from Iltb.; Fmtb. enlarges and moves superficially	Muscles enlarge

TABLE 3 (Continued)

Region	*Aspidoscelis uniparens*	*Pogona vitticeps*	*Chamaeleo calyptratus*
	Disk-shaped ambiens (Amb.) fully separates and expands posteriorly; two heads of PIF.i appear; Ilfb. cleaves from Iltb.		
Dorsal HL: distal	Anterior, superficial sheet cleaves anterioposteriorly into TA and EDL; FB cleaves from FL; distal sheet cleaves anterioposteriorly into EDBs of digits II-V	FB cleaves from FL; autopodial portion separates into EDBs (deep and proximal) and DMTs (superficial and distal)	DD.m expands into fan-shaped mass (anlagen of EDB + DMT); anlage of FL appears
Ventral HL: proximal	NV	NV	NV
Ventral HL: distal	Muscles differentiate: GI + GE (lateral), FDL (midline); FDB anlage appears in autopodial region	Crural portion cleaves anteroposteriorly into FDL, GI, GE (with proximal superficial and distal deep portions), and separate "medial head" of GE; FDB begins to cleave into individual muscles	GE cleaves from GI; anlagen of FDB appear; FDL enlarges
Stage 37			
Dorsal HL: proximal	Muscles larger and deeper staining	Muscles larger, fibers more visible	Muscles larger, fibers more visible
Dorsal HL: distal	Muscles larger and more fully differentiated; Abd. D1 appears; Dmt's separate from EDBs	Dmt's increase in number and expand disally	FL and FB remain undivided; Dmt's now associated w/individual digits.
Ventral HL: proximal	Muscles larger and deeper staining; FTI visible	Fibers more clearly visible; Fmtb. extends toward knee region; Pit. and FTI appear	FTE and FTI2 appear
Ventral HL: distal	GE and GI expand; FDB cleaves anteroposteriorly into separate muscles	FDL and FDB expand distally	No change

Note: NV indicates a stage/region that was not available/visible. See Table 4 for muscle abbreviations.
Abbreviations: HL, hindlimb; MMPs, migratory muscle precursors.

TABLE 4 Forelimb muscle abbreviations, synonyms, and attachments

Abbr.	Muscle and synonyms	Adult anatomy in *Aspidoscelis uniparens*
DD.m	**Dorsal distal muscle mass**	N/A
DP.m	**Dorsal proximal muscle mass**	N/A
VD.m	**Ventral distal muscle mass**	N/A
VP.m	**Ventral proximal muscle mass**	N/A
Abd.D1	**Abductor pollicis longus** (part 2 of extensor ossis metacarpi pollicis[83])	From ulna and ulnare to MC1
Abd.D5	**Abductor digiti minimi** (flexor brevis minimi digiti[83]; abductor digiti quinti[57])	From pisiform to MC5
BB	**Biceps brachii**	From anterolateral coracoid to radius and ulna
Br.	**Brachialis** (brachialis inferior[17])	From anterolateral humerus distal to deltopectoral crest; inserts on proximal ulna with biceps brachii
Brd.	**Brachioradialis** (supinator longus[83])	Not present as a separate muscle?
CL	**Coracobrachialis longus** (coracobrachialis part 1[83])	From posterior inferior corner of coracoid to distal humerus just above its medial condyle
DC	**Deltoideus acromialis et clavicularis** (clavodeltoideus[57,77]; deltoid 1 and 2[83]; coracohumeralis anterior and sternohumeralis anterior in chameleons[57])	Single head arising mainly from clavicle but also from scapula/coracoid and inserting on the deltopectoral crest
Dmc.	**Dorsometacarpales** (extensores phalangorum[83]; extensores digitores breves profundi[57])	Five muscles, from MC1-5 to the distal phalanges of respective digits
DS	**Deltoideus scapularis** (scapulodeltoideus[57]; suprascapularis 1 and 2[83] scapulodeltoideus and suprascapularis[77])	From suprascapula and scapula to proximal humerus
EA	**Epitrochleoanconeus** (part of flexor carpi ulnaris[83])	From humeral medial condyle to proximal ¼ of posterior ulna
EACU	**Extensor antebrachii et carpi ulnaris**	From humerus to proximal MC5, partially fused with flexor carpi ulnaris
ECR	**Extensor carpi radialis** (part 1 of extensor ossis metacarpi pollicis[83])	From distal humerus to lateral MC1; blends distally with abductor pollicis longus
ED	**Extensor digitorum** (extensor carpi radialis longior and brevoir[83])	From humeral lateral epicondyle and shaft of radius to MC2-5
EDB	**Extensores digitorum breves** (extensores III-IX[83]; extensores digitores breves superficiales[57])	Five muscles from carpal region to the proximal phalanx of each digit
FBP	**Flexores breves profundi** (part of interossei[83]; interossei ventrales and lumbricales[57])	Five muscles from MC1-5 to proximal phalanfes of the respective digits
FBS	**Flexores breves superficiales** (flexor brevis digitorum and adductor digiti tertii and adductor digiti quarti[83]; flexores digitores breves[57])	Ten muscles that insert on the proximal phalanx of D1-5 (2 muscles for each digit), and then fuse with tendons of flexor digitorum longus, thus inserting also on the middle and distal phalanges
FCR	**Flexor carpi radialis**	From humeral medial epicondyle to MC1
FCU	**Flexor carpi ulnaris** (part of flexor carpi ulnaris[83])	From humeral medial epicondyle to pisiform
FDL	**Flexor digitorum longus** (flexor pollicis longus + flexor profundus digitorum[83])	Two bellies form a common tendon that splits to insert on the distal phalanges of D1-5; deep head from carpals to D2 tendon
LD	**Latissimus dorsi**	From fascia of erector spinae muscles to latissimus dorsi process on medial aspect of head of humerus
P	**Pectoralis**	From sternum, fascia of external oblique muscles, and sternal ribs 1–3 to deltopectoral crest of humerus
PT	**Pronator teres**	From humeral medial epicondyle to distal 2/3 of radius
Spc.	**Supracoracoideus** (subclavius[83])	From anterolateral coracoid and part of scapula to proximal humerus

TABLE 4 (Continued)

Abbr.	Muscle and synonyms	Adult anatomy in *Aspidoscelis uniparens*
T	**Trapezius**	From neural spines of approximately the third cervical through fourth thoracic vertebra to acromion and anterodorsal clavicle
TH	**Triceps humeralis lateralis** and **medialis** (triceps part 2 and part 3, respectively[83])	Via two heads from shaft of humerus; unite with triceps scapularis and insert on olecranon of ulna
TS	**Triceps scapularis** (triceps part 1[83] and scapulotriceps[77])	From posterior scapula just above glenoid and scapulohumeral ligament to olecranon of ulna

Source: Modified from Reference 72.

TABLE 5 Hindlimb muscle abbreviations, synonyms, and attachments

Abbr.	Muscles and synonyms	Adult anatomy in *Aspidoscelis uniparens*
Abd.D1	**Abductor hallucis longus** (extensores I and II[83]; adductor et extensor hallucis et indicus[57])	From distal end of fibula to MT1
Abd.D5	**Abductor digiti minimi** (flexor brevis minimi digiti[83] and abductor digiti quinti[57])	From plantar ossicle to the proximal phalanx of D5
Amb.	**Ambiens** (rectus femoris[83]; sartorius[84])	From ventral margin of acetabulum to common extensor tendon
Dmt.	**Dorsometatarsales** (extensores phalangorum[83])	Five muscles from distal portion of MT1-5 via 2 heads to the distal phalanges of D1-5
EDB	**Extensores digitorum breves** (extensors III-IX[57])	Five muscles from carpal region to distal phalanges of D1-5 (with dorsometatarsales)
EDL	**Extensor digitorum longus**	Via two heads, from anterior distal femur to MT2-3
FB	**Fibularis brevis** (part of peroneus[83])	From proximal fibula to MT5
FBP	**Flexores breves profundi** (part of interossei[83])	Five muscles from MT1-5 to proximal phalanges of respective digits
FBS	**Flexores breves superficiales** (flexor brevis digitorum, adductor digiti secondi, and adductor digiti tertii[83])	From aponeurosis of the deep head of the gastrocnemius externus to D1-4 and possibly also D5
FDL	**Flexor digitorum longus** (flexor digitorum longus, flexor hallucis longus, and flexor tertius digiti[83])	From femur, fibula and fibulare + intermedium to D1-5 with a large sesamoid bone proximal to tendon split; plantar heads from MT4 to D2-5
FL	**Fibularis longus** (part of peroneus[83])	Via two heads, from distal femur to MT4-5
Fmtb.	**Femorotibialis** (vastus externus, vastus internus, and crureus[83])	Via three heads, from medial femur and lateral femur to patella (with other extensors)
FTE	**Flexor tibialis externus** (biceps[83]); includes flexor cruris et tarsi tibialis	From tendinous arch to fibula with iliofibularis and, via long tendon, to plantar ossicle
FTI	**Flexor tibialis internus** (semitendinosus and semimembranosus[83])	Part 1 from ilio-ischiatic ligament to posterior knee joint and proximal tibia with pubotibialis; part 2 via tendon from internal ischium to proximal tibia
GE	**Gastrocnemius externus** (femoral gastrocnemius[57])	From proximal tibia to base of MT5 and plantar aponeurosis (with flexor tibialis externus tendon)
GI	**Gastrocnemius internus** (femorotibial gastrocnemius[57])	From femur and proximal half of posterior tibia to plantar aponeurosis
Ilfb.	**Iliofibularis** (ilioperoneal[83]; tenuissimus[84])	From external ilium to middle portion of lateral fibular shaft
Ilfem.	**Iliofemoralis** (gluteus primus, secundus, and tertius and pectineus[83])	From ilium to femur

(Continues)

TABLE 5 (Continued)

Abbr.	Muscles and synonyms	Adult anatomy in *Aspidoscelis uniparens*
Iltib.	**Iliotibialis** (rectus femoris 2 and 3[83])	From anterior ilium to cnemial crest of tibia via common extensor tendon
Pif.E	**Puboischiofemoralis externus** (quadratus femoris and obturator externus[83]; includes iliacus, pectineus, obturator internus, obturator externus and adductor brevis of various authors[57])	Part 1 from ischium to head of femur; part 2 from ischio-pubic symphysis to greater trochanter
Pif.I	**Puboischiofemoralis internus** (iliacus[83])	Three parts, from anterior internal pubis to proximal femur
Pit.	**Puboischiotibialis** (gracilis[84])	From pubo-ischiadic ligament to proximal medial tibia
TA	**Tibialis anterior** (tibialis anticus[83])	From anteromedial tibia to MT1

Source: Modified from Reference 72.

carried out at 4°C for 5-10 minutes with gentle manual rocking via nutator (embryos were either in six-well petri dish or 20 mL vial). Excess staining was allowed to diffuse out of embryos by placing in 4% paraformaldehyde (PFA) or 1× PBS for 2 days at 4°C.

Embryos for all stages and species were processed for sarcomeric (mature) muscle myosin heavy chain immunoreactivity using the MF20 monoclonal antibody (DSHB[137]). The MF20 monoclonal antibody recognizes the light meromyosin portion of the heavy chain of myosin II (all isoforms) and was used to check for interspecies differences in mature muscle fiber development in limbs at equivalent stages of limb morphogenesis. Cells known to be positive for MF20 are mature myocytes in the somites and primary myotubes of developing skeletal muscle as well as muscle cells of the myocardium.[52,53,55,137-141] MF20 reactivity at stage 29 was not found in the limb buds while being highly expressed in the myocytes of somites (Figure 1A-C). Skeletal muscle precursor cells are expected to be present in the limb at sage 29 but do not stain for sarcomeric myosin at this stage.[55] MF20 antibody staining parallels specificity when compared to published studies of limb muscle development in mouse[55] and is sufficient for our examination of limb muscle mass formation, growth, and ontogenetic cleavage into distinct muscles.

5.3 | Anatomical staging and terminology

Given our focus on limb muscles, staging of embryos utilized limb criteria presented in the literature[82] for the leopard gecko (*Eublepharis macularius*); equivalent limb stages to other taxa are presented in Table 2. These equivalent stages are used throughout the sections 2 and 3, with the stages reported in the original manuscripts shown in brackets throughout this paper. Because we have more developmental timepoints (stages) for *A uniparens*, the descriptions for muscle development in the limbs are based on this species with comments on differences in muscle morphogenesis in *P vitticeps* and *C calyptratus* (Note: not all stages were exact, so descriptions within *A uniparens* are based on closest stage for other taxa). Muscle names follow the terminology of Molnar et al[72] and Diogo et al[84] (see our Tables 3 and 4 for synonyms and abbreviations).

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