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Matthew T. Smetanick

Rafael O. de Sá
University of Richmond, rdesa@richmond.edu

Gary P. Radice

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Do Timing and Pattern of Myogenesis Correlate with Life History Mode in Anurans?

MATTHEW T. SMETANICK, RAFAEL O. DE SÁ, AND GARY P. RADICE, *Department of Biology, University of Richmond, Richmond, Virginia 23173, USA. E-mail: rdesa@richmond.edu*

The timing and pattern of myogenesis varies among anurans that have been studied and the different patterns may provide useful phylogenetic information. Specific myogenic markers have been described (Muntz, 1975; Kielbowna, 1981; Boudjelida & Muntz, 1987; Radice et al., 1989) and they can provide information on evolutionary changes for closely related lineages within a clade. For example, we previously compared first appearance of a muscle-specific protein, first twitch of axial muscle, onset of multinucleation within axial myotome, and first heartbeat in two pipid genera (Smetanick et al., 1999). We found that although the timing of myogenesis differed, the sequence of events was the same for these two pipids. The similarities we saw in the two pipids could be due their common lineage, or alternatively, be a result of sharing a life history mode. For example, appearance of muscle twitch prior to multinucleation could be an adaptation for rapid development, an advantage in frogs with free-swimming tadpoles. If so, it might occur in other lineages with free-swimming tadpoles regardless of phylogenetic distance.

Herein, we analyze myogenesis among seven species in four anuran families. Species selected for this study also differ in their life histories and general modes of reproduction. We sought to determine whether the timing or sequence of myogenesis, or both, correlated with phylogenetic group or with life history mode.

We grouped species in three categories based on reproductive mode. The non-direct developers, i.e.,

TABLE 1. Gosner (1960) stages correlated with Nieuwkoop and Faber (1975) (NF) and Townsend and Stewart (1985) (TS) stages. * Dashes indicate an inability to directly correlate a NF or TS stage with a particular Gosner stage. ** Gosner correlations of stages 26–27 to NF stages 46–47 from Just *et al* 1981.

NF stage	Gosner stage	TS stage
18	15	3
19–21	16	3
22–23	17	4
24	18	4
25–32	—*	—
33–34	19	5
35–39	—	5
40	20	5
41	21–23	6
42	24	6
43–45	25	6
46**	26	6
47**	27	6

Hymenochirus boettgeri, Rana sylvatica, R. utricularia, Xenopus laevis, and *X. tropicalis,* lay large clutches of eggs in lentic water. Embryos hatch into free-swimming tadpoles before developing into juvenile frogs. *Xenopus* and *Hymenochirus* are pipids, but differ in their diet. *Xenopus* tadpoles are filter feeders whereas *Hymenochirus* tadpoles are carnivorous and eat mainly copepods, mollusc larvae, and in the laboratory, brine shrimp. *Rana* tadpoles were chosen as detritivors or grazers, grazing on algae by scratching rocks with their horny beaks, and, because they are commonly studied, were readily available. The direct developer, *Eleutherodactylus coqui,* lays arboreal eggs that lack the intermediate free-swimming tadpole stage. Instead, embryos are positioned on top of a yolk sac from which they obtain nourishment throughout development and hatch directly into froglets. The "intermediate developer," *Agalychnis callidryas,* has a reproductive mode intermediate between those previously described. Eggs are arboreal, and intracapsular development is extended beyond the embryo stage into what are, morphologically, early tadpole stages. Upon hatching, they drop into the water below and become free-swimming tadpoles. Nourishment during intracapsular development is provided by yolk reserves, whereas the free-swimming tadpoles are filter feeders.

A total of 454 specimens were examined (27 *Agalychnis callydrias,* 36 *Eleutherodactylus coqui,* 86 *Hymenochirus boettgeri,* 60 *Rana sylvatica,* 35 *R. utricularia,* 120 *Xenopus laevis,* and 90 *X. tropicalis*). Specimens were preserved in either Dent fixative (1 part dimethyl sulfoxide: 4 parts methanol; Dent et al., 1989) or 4% neutral-buffered formaldehyde. Species were staged following, or correlating with, the Gosner (1960) table of normal development. Staging correlations are discussed below and a summary given in Table 1.

The National Aquarium in Baltimore provided fertilized *Agalychnis* eggs. Adult *Eleutherodactylus* were collected in the wild in Puerto Rico and bred in the laboratory (non-hormone induced). *Agalychnis* and *Eleutherodactylus* embryos were raised at 22 C in sterilized culture dishes on sterile filter paper (moistened with 10% HEPES-buffered Steinberg solution, Peng, 1991). *Hymenochirus* adults were purchased from Blue Lobster Farm, Madeira, CA; embryos were raised from clutches obtained in laboratory aquaria (non-hormone induced breeding). Eggs were removed and transferred to sterilized disposable culture dishes, and embryos raised at 22–24 C. *Rana sylvatica* eggs and embryos were collected at Fort AP Hill, VA. Fertilized eggs of *Rana utricularia* were purchased from Charles D. Sullivan Co. (Nashville, TN). Both *Rana* species were raised in bowls of dechlorinated tap water at 22–24 C. *Xenopus* embryos were obtained from hormonally-induced breeding. Clutches were raised in 10% Steinberg solution at 22–24 C in sterile dishes. Experiments were conducted in accordance with approved Institutional Animal Care and Use guidelines (98-2).

Observations of first twitch and immunohistochemical staining were made using a Nikon stereomicroscope. First stimulated twitch of muscle was examined by poking live specimens with metal probes. Observations of multinucleation in axial myotomes were made using a Nikon Optiphot microscope. In order to observe muscle cell nuclei, specimens were embedded in glycol methacrylate (JB4+ embedding kit, Polysciences, Inc). Axial muscle was sectioned longitudinally at 2 μm using glass knives, transferred to a slide, and stained for 10 sec with 0.1% toluidine blue in 1% sodium tetraborate (Dawes, 1979).

Whole-mount immunohistochemical staining was performed to identify the initial presence of muscle protein. All embryos were preserved in Dent fixative prior to staining. The procedure was adapted from Hanken et al. (1992, 1997) and used monoclonal antibody 12/101, a muscle-specific antibody that recognizes a specific antigen in amphibian skeletal muscle (Kintner and Brockes, 1984). Antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. The 12/101 primary antibody was visualized using the Vectastain Universal Kit (biotin-avidin complex) and diaminobenzidine (DAB) substrate. Stained embryos were cleared with one part benzyl alcohol: two parts benzyl benzoate. Whole embryos and histological sections were photographed with Kodak Technical Pan film.

Correlation of Developmental Tables.—Our analysis compares the stages during development at which skeletal muscle myogenic markers appear. Unfortunately, at least three different tables of normal development are commonly used to gauge developmental age in the species we studied. We therefore needed to correlate the different normal tables to a standard, which we arbitrarily chose to be the Gosner (1960) stages. To do this we accepted previous partial stage correlations by Just et al. (1981) and Townsend and Stuart (1985). We then extended these and filled in gaps during the stages of skeletal muscle myogenesis by normalizing to the appearance of developmental characters that we presume are independent of skeletal muscle development, including neural tube formation, first heartbeat, gill circulation and gill expansion, and limb development. Our complete correlation is summarized in Table 1.

Townsend and Stewart (1985) provided correlations of their *Eleutherodactylus coqui* stages from Gosner Stages 1–18. We extended the correlation for TS Stages 5 to Gosner Stages 19/20 based on initial heart beat

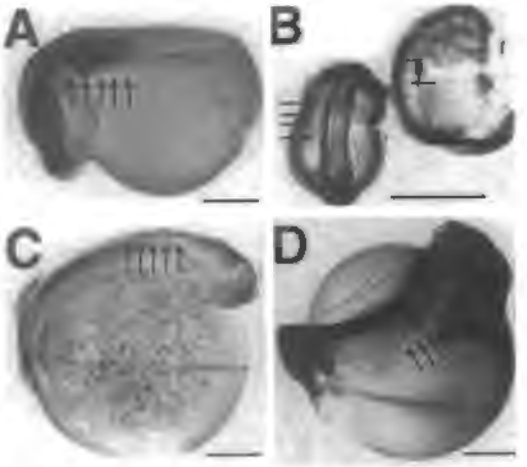

FIG. 1. Immunohistochemical detection of muscle-specific antigen expression. Whole embyros were reacted with anti-muscle antibody 12/101. Binding was detected with a peroxidase-linked secondary antibody and detected with diaminobenzidine. In some embryos this procedure stained the central nervous system in addition to skeletal muscle. Arrows indicate areas of specific muscle staining. Scale bars, 0.5 mm. (A.) Lateral view of *Rana utricularia* at Gosner stage 17. Anterior is to the left. Antigen is detected in anterior myotome but not posterior unsegmented mesoderm. The staining pattern of *Rana sylvatica* is similar (not shown) (B.) *Xenopus tropicalis*, Nieuwkoop and Faber stage 20 (dorsal view, left) and 22 (lateral view, right). Anterior is toward the top. Note that embryos of this species are much smaller than the other anurans shown. (C.) Ventro-lateral view of *Eleutherodactylus coqui*, Gosner stage 5. Staining is just detectable above background in anterior myotome. (D.) *Agalychnis callidryas*, Gosner stage 5. Arrows indicate myotome staining.

and beginning of gill circulation. TS stage 7 is distinguished by the first appearance of foot paddles, which can be directly correlated with Gosner stage 31. Consequently, Gosner stages 21–30 were correlated with TS Stage 6, which mainly involves elongation of the limbs (Townsend and Stewart, 1985).

12/101 Protein (Fig. 1 and Table 2).—The initial presence of axial muscle protein was detected in somites of both *Xenopus* species by Stage 16. It was observed slightly later, at early Stage 17, in both *Rana* species, and at Stage 18/19, in *Hymenochirus*. The protein was detected at Stage 19/20 in *Eleutherodactylus*, whereas in *Agalychnis* it was found at Stage 17/18. We did not section the specimens to confirm that staining occurred in muscle. However, the antibody is known to recognize a muscle specific protein (Kintner and Brockes, 1984) and we could easily visualize entire somites stained in the cleared specimens. Since the bulk of amphibians somites is myotome, with only small contributions from sclerotome and dermatome (see Keller, 1999, for review), we are confident that the staining we observed represents expression in myoblasts or muscle cells. Note also that our results for the stage of first expression depend on the sensitivity of whole mount immunocytology. More sensitive methods could detect earlier expression. Hence they should be considered the latest stages at which muscle protein first appears.

Stimulated Twitch (Table 2).—First stimulated twitch of axial muscle occurred by Stage 17 in *X. laevis* and slightly later in *X. tropicalis*, Stage 17/18. It was observed at Stage 18 in *Rana* species, Stage 18/19 in *Hymenochirus*, Stage 19/20 in *Eleutherodactylus*, and at Stage 18/19 in *Agalychnis*.

Spontaneous Twitch (Table 2).—The first spontaneous twitch of axial muscle was observed at Stage 18/19 in *Xenopus* species, Stage 18 in *Rana* species, Stage 18/19 in *Hymenochirus*, between Stages 21–30 in *Eleutherodactylus*, and at Stage 18/19 in *Agalychnis*.

Multinucleation (Table 2).—Multinucleated axial myotome was initially detected at Stage 26/27 in *X. tropicalis* and at Stage 26 in *X. laevis*. It was observed at Stage 17 in *Rana* species. Similar to the other pipids, *Hymenochirus* multinucleation was present by Stage 26. Multinucleation was observed at Stage 19/20 in *Eleutherodactylus* and at Stage 18 in *Agalychnis*.

Hymenochirus boettgeri, *Xenopus laevis*, and *X. tropicalis* were originally staged according to the Nieuwkoop and Faber (1975) normal table of development for *X. laevis*. Just et al. (1981) and Trueb and Hanken (1992) provided a correlation for NF stages 46–47 with Gosner stages 26–27. We determined the correlation between NF stages 18–45 and Gosner stages 15–25. NF stage 18 was correlated with Gosner Stage 15 based on neural groove formation. Neural tube development linked NF Stages 19–21 with Gosner Stage 16. NF Stages 22–23 were correlated with Gosner Stage

TABLE 2. Comparative stages of myogenesis of several anuran species.

	Gosner stage when first detected			
Species	12/101 protein expression	Stimulated twitch	Spontaneous twitch	Multinucleation
E. coqui	19–20	19–20	21–30	19–20
H. boettgeri	18/19	18/19	18/19	26
X. tropicalis	16	17/18	18/19	26/27
X. laevis	16	17	18/19	26
R. sylvatica	16/17	18	18	17
R. utricularia	17	18	18	17
A. callidryas	17–18	18–19	18/19	18

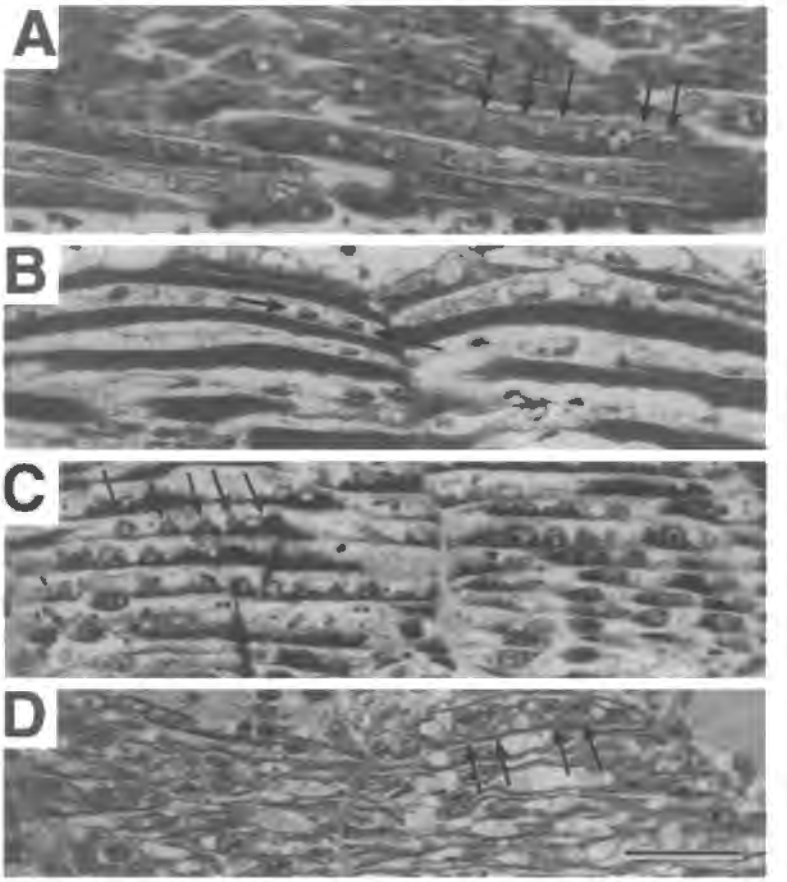

FIG. 2. Myofiber multinucleation in several anurans. A–D represent frontal sections through the myotome, oriented so that the medial surface (facing the notochord) is towards the bottom of the panel and the lateral aspect toward the top. Portions of two myotomes are shown in each panel. Individual myofibers extend the length of a myotome. Examples of multiple nuclei within a single myofiber are marked arrows. (A) *Rana sylvatica*, Gosner Stage 17; (B) *Xenopus tropicalis* Gosner St. 26/27; (C) *Agalychnis callidryas* Gosner St 18; (D) *Eleutherodactylus coqui* Gosner St.21-30. A–D were photographed at the same magnification. Bar, 50 μm.

17; these stages involve the early formation and appearance of the tail bud. NF stage 33/34 is characterized by first heartbeat, which is observed at Gosner Stage 19. We found no direct correlation between NF stages 25–32 and any given Gosner stage. Consequently, NF Stages 25–32 would correspond to the transitional period between Gosner Stages 18 and 19. Gill circulation links NF Stage 40 and Gosner Stage 20; thus, NF Stages 35–39 correspond to the period between Gosner Stages 19 and 20. NF Stage 41, at which the gills become "broader and flatter" (Nieuwkoop and Faber, 1975) corresponds to Gosner Stages 21–23 which involve further development of the gills. NF Stage 42 correlates with Gosner Stage 24 based on the initial development of the operculum. Further operculum development linked NF Stages 43–45 with Gosner Stage 25 just before the initial presence of the hindlimb bud. Initial limb bud development directly correlates NF Stage 46 with Gosner stage 26.

Having correlated normal stages we then compared the stage and sequence in which common skeletal muscle myogenic events occurred (Table 2). Then we determined whether a particular pattern of myogenesis corresponded to a life history mode. We selected *Rana*, with free-swimming and completely aquatic tadpoles, as the standard reproductive mode to compare myogenic events among species because this genus has been widely studied and was readily available. The initial presence of muscle protein, as judged by whole-mount immunocytology, was detected at Gosner Stage 17 in *Rana*. The appearance of muscle protein in other non-direct developing larvae examined differed. It was detected earlier, Stage 16, in *Xenopus laevis* and *X. tropicalis*, whereas it was not found until later in *Hymenochirus*, Stage 18/19. In *Eleutherodactylus* synthesis of muscle protein appears delayed and was first detected at least two stages later than in *Rana*. Multinucleation precedes the first twitch of axial muscle in *Rana*; however, *Hymenochirus* and *Xenopus* exhibit functional, but mononucleated, axial myotome at a point remarkably earlier than the onset of multinucleation. In *Agalychnis* and *Eleutherodactylus* multinucleation is also slightly delayed, but it occurs at about

the same time as the activation of axial muscle (first twitch).

Overall, with the exception of *Hymenochirus*, the non-direct developing taxa have an earlier expression of muscle protein and muscle function than species representing other reproductive modes. The myogenic pattern of *Agalychnis* is delayed in its entirety relative to *Rana*, but it exhibits muscle protein expression, muscle function, and multinucleation earlier than in *Eleutherodactylus*. Initially, we ranked *Agalychnis* as an intermediate developer, based on its extended intracapsular development. The present analysis shows that the myogenic pattern of *Agalychnis* is intermediate between that of non-direct and direct developing anurans. Myogenesis in *Agalychnis* occurs faster than in *Eleutherodactylus*, but it is generally slower relative to *Rana* and pipids.

The present study suggests that myogenic events vary with reproductive modes. Furthermore, it suggests a progressive delay of myogenesis associated with a delay in hatching, i.e., extended intracapsular development. If this is correct, we would predict that myogenic events in other anuran taxa such as *Dendrobates* and centrolenids would resemble the pattern described for *Agalychnis* whereas myogenic events in *Cophixalus*, *Ceratobratrachus*, and some *Gastrotheca*, (all direct developers) would resemble the pattern of *Eleutherodactylus*.

Although life history may correlate with patterns of myogenesis, some myogenic events may be better understood in the light of evolutionary relationships. For example, among the anurans with free swimming tadpole, the pipid taxa studied showed delayed multinucleation of axial muscle, delayed further than it is in *Eleutherodactylus*. However, within pipids *Hymenochirus* differs from *X. laevis* and *X. tropicalis* in its delayed expression of muscle protein and first twitch. The departure of *Hymenochirus* from the pattern of myogenesis found in other pipids is not surprising since tadpoles of this taxon have been shown to differ in the characteristics and development of other musculoskeletal structures (e.g., Sokol, 1959, 1962, 1977; de Sá and Swart, 1999). Moreover, multinucleation occurs in pipids after first twitch, whereas in the other species multinucleation precedes or coincides with muscle function. Hence part of the myogenic sequence is altered in pipids relative to other anurans studied thus far. Thus, multinucleation following function may be a synapomorphy for the pipidae. If so, then it should also occur in the genus *Pipa*. The genus *Pipa* will be particularly interesting to examine because there are both free swimming tadpole (e.g., *P. carvalhoi*) and direct developing (*P. pipa*) members of the genus. This could allow one to determine more directly whether myogenic patterns are related to life history rather than historical events.

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