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The Timing and Pattern of Myogenesis in Hymenochirus boettgeri

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kJ/g (SE = 0.263, N = 4 groups of six fruit and one of five).

The energy content of the figs is similar to the 22.6 kJ/g recorded for insects (Golley, 1961). In terms of energy, one single fig must therefore be the equivalent of many black flies (*Simulium* spp.), which is the most common prey of *P. broadleyi*. Since the lizards do not digest seeds, our estimate of the digestible energy content is liberal, but the fig may represent a substantial reward to the lizard. Another benefit to eating figs in this arid environment is their high water content (87.2% of the pulp of *F. burtt-davyi*; Compton et al., 1996). It therefore appears as if both the lizards and the fig trees gain from this feeding-dispersal relationship, although a rigorous test of a mutualistic relationship awaits further study.

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The Timing and Pattern of Myogenesis in *Hymenochirus boettgeri*

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Differences in the relative timing of homologous developmental events among closely related species, known as heterochronies, may provide valuable clues in understanding evolutionary relationships (McKinney, 1988; McNamara, 1995). Examining the timing of myogenic events is a relatively easy and effective method for finding heterochronic events. For example, whether muscle proteins and myofibrils appear before or after multinucleation can be determined through histological techniques (Kielbowna, 1981). Simple observations of live specimens can pinpoint functional landmarks such as first twitch (spontaneous or due to external stimuli) and first heartbeat.

Heterochronies are known to exist in amphibian myogenesis, particularly in the formation of axial muscles. A common pattern of muscle development, as seen in the common Eurasian spadefoot toad (*Pelobates fuscus*), begins in the myotome with the appearance of mononucleated myotomal myoblasts, which then fuse to form elongated, multinucleated muscle cells (Kielbowna, 1981). The muscle fiber then synthesizes myofibrils, which is followed by first twitch (see Radice et al., 1989 for review). *Xenopus lae-*

vis and *Bombina variegata* differ from this myogenic pattern by exhibiting early muscle function. Developing muscle in *X. laevis* displays a remarkably early expression of myosin and actin fibers and becomes functional prior to becoming multinucleated (Muntz, 1975; Kielbowna, 1981; Gurdon et al., 1985; Boudjelida and Muntz, 1987). First twitch in *X. laevis* can be observed at about 24 h post-fertilization, preceding the first heartbeat by about a day and the multinucleation of the myotome by about three days (Nieuwkoop and Faber, 1975). The similarity in myogenic pattern between *X. laevis* and *B. variegata* may suggest that early myogenesis is an ancestral myogenic condition for anurans since these taxa represent basal lineages within Anura (Ford and Cannatella, 1993). However, additional members among basal anurans must be examined to test this hypothesis.

We have studied another pipid species. *Hymenochirus boettgeri*, to determine whether the pattern of myogenesis seen in *X. laevis* is unique to that species, or represents a pattern specific to the pipid lineage.

A total of 86 specimens of *Hymenochirus boettgeri* were examined. Larvae were preserved in Dent fixative, consisting of four parts methanol to one part dimethyl sulfoxide (Dent et al., 1989), and staged according to the Nieuwkoop and Faber (1975) normal table of development for *X. laevis* (NF stages). The specimens ranged from NF 24–50. Larvae were raised from "naturally induced" (non-hormone injected) clutches obtained in the laboratory from three females and three males. Adults were bred in 40-l aquaria and fed with commercial fish food. Eggs were removed from the aquaria and stored in sterilized disposable culture dishes at room temperature. At stage 42, the larvae were transferred to a 40-l aquarium and fed brine shrimp, *Daphnia* copepods, and commercial fish food. Experiments were conducted in accord with approved Institutional Animal Care and Use guidelines.

Nieuwkoop and Faber's (1975) normal table of development could not be used to stage *H. boettgeri* specimens between stages 43–45. Changes in intestinal structure are used to distinguish among these stages, but intestinal development in *H. boettgeri* differs significantly from that in *X. laevis*. The next reliable stage marker common to both species is the first appearance of hind limb buds, which identifies stage 46/47. A normal staging table for *H. boettgeri* is in preparation (Olson, 1997).

Determinations of first twitch (18 specimens), first heartbeat (12 specimens), and immunohistochemical staining (33 specimens), were made by using a Nikon dissecting microscope. Observations of axial myotome multinucleation (23 specimens) were made with a Nikon Optiphot microscope.

To observe muscle cell nuclei, fixed specimens were embedded in glycol methacrylate. Axial muscles were sectioned longitudinally at 2 μm using glass knives, transferred to a slide, and stained for 10–15 sec with 0.1% toluidine blue in 1% sodium tetraborate (Dawes, 1979).

Whole-mount immunohistochemical staining was performed on *H. boettgeri* specimens from stages 24–32 to identify the initial presence of muscle protein. For comparison, immunohistochemical staining was also performed on *X. laevis* specimens from stages 17–25. The procedure was adapted from Hanken et al. (1992, 1997) using monoclonal antibody 12/101, a muscle-specific antibody that recognizes an antigen in amphibian skeletal muscle (Kintner and Brockes, 1984). Antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. The primary antibody was visualized using the Vectastain Universal Kit (biotin-avidin complex) and diaminobenzidine (DAB). Stained embryos were cleared with benzyl alcohol:benyzl benzoate (1:2). Embryos and histological sections were photographed with Kodak Technical Pan film.

Immunohistochemical staining of the *H. boettgeri* specimens detected the initial presence of muscle protein in axial muscle at NF stage 25, approximately 24 h post-fertilization (Fig. 1A). At this stage, only the most anterior axial myotome was visible. Muscle protein was not detected at NF stage 24, as indicated by the absence of DAB staining in the dorso-medial part of the embryo (Fig. 1A). In contrast, muscle protein was first detected in *X. laevis* at NF stage 20 (Fig 1B).

First twitch of axial muscle, stimulated by poking live specimens with a metal probe, was observed no later than NF stage 27/28, approximately 5 h after the earliest detected presence of muscle protein. Spontaneous mid-body flexing was observed by stage 30/31. For comparison, *X. laevis* shows stimulated twitching at stage 22/23, and spontaneous flexing at stage 25 (Nieuwkoop and Faber, 1975).

Observation of methacrylate sections revealed the presence of mononucleated myotome cells from stage 24 to stage 42 (Fig. 1C). Because of the difficulties of staging *H. boettgeri* between NF stages 43–45, it was not possible to determine whether muscle cells became multinucleated during these stages. However, the myotome fibers clearly were multinucleated by stage 46/47 (Figure 1D), which is distinguished by the first appearance of hind limb buds. First heart beat was not observed in *H. boettgeri* until stage 36/37. It occurs at stage 33 in *X. laevis* (Nieuwkoop and Faber, 1975).

It is possible that patterns of myogenesis are lineage specific within anurans. If so, then other pipids should display myogenic patterns similar to *X. laevis*. We have found that three landmarks of skeletal muscle myogenesis—muscle protein synthesis, first twitch, and multinucleation—indeed occur in the same sequence in *H. boettgeri* and *X. laevis*. Because these myogenic landmarks are relatively easy to assess, additional phylogenetic comparisons will be possible if live, early stages of additional species can be obtained.

Although the sequence of these myogenic events is the same in both pipid species studied, the timing of these events relative to other developmental markers is delayed in *H. boettgeri* compared with their timing in *X. laevis*. The timing of these events is summarized in Fig 2. The earliest stage at which muscle-specific antigens can be detected is NF stage 20 in *X. laevis* and stage 25 in *H. boettgeri*. Antigen expression in *H. boettgeri* is later in absolute time as well as relative developmental age since both species reach NF stage 25 at the same time—about 24 h post-fertilization at 22 C. First stimulated twitch is also delayed in *H. boettgeri* (stage 27/28, about 29 h) compared with *X. laevis* (stage 22, about 22 h). First spontaneous twitch is correspondingly later, occurring as late as stage 31 in *H. boettgeri* versus stage 25 in *X. laevis*. Although

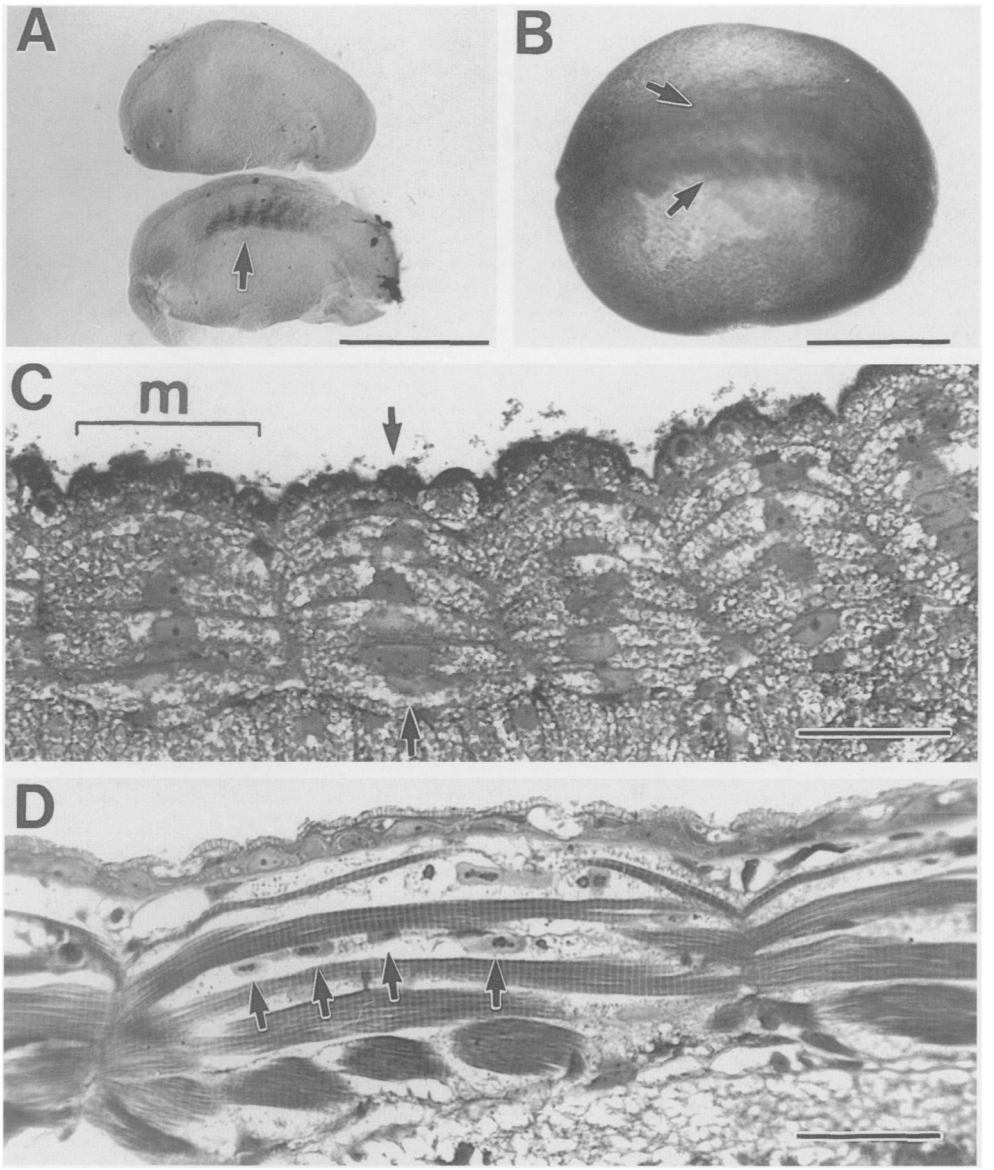

FIG. 1. First appearance of muscle specific antigens in embryos of *Hymenochirus boettgeri* and *Xenopus laevis*. A. Lateral view of *H. boettgeri* whole mount. Anterior is to the left, dorsal is toward the top. The upper embryo is at stage 24, the lower embryo is at stage 25. Muscle-specific staining is seen in axial myotomes (arrow) beginning at stage 25. Bar, 0.5 mm. B. Dorso-lateral view of *X. laevis* whole mount at stage 20. Anterior is to the left. Staining of the lateral rows of myotomal muscles is seen at arrows. Bar, 0.5 mm. C. Appearance of mononucleate axial muscle cells in *H. boettgeri*. Frontal and slightly oblique section of a stage 31 tadpole. Each muscle cell spans the width of a myotome (m) and has a single nucleus at its center. An example is seen between the arrows where nuclei from five cells line up in a single column. Bar, 50 μm. D. Appearance of multinucleate muscle cells in *H. boettgeri*. Frontal section of stage 46/47 embryo. At this stage, striated myofibrils can be seen extending the length of each muscle cell. Four nuclei in a single cell are seen at arrows. Bar, 50 μm.

relatively late, first twitch still precedes first heartbeat in *H. boettgeri*, which occurs at approximately stage 36 compared with stage 32 in *X. laevis*. The time and stage of multinucleation were more difficult to compare because the morphological characters used to stage *X. laevis* at stage 43–45 are not present in *H. boettgeri*. Nevertheless, it is safe to conclude that time of multinucleation is not delayed in *H. boettgeri*. *Xenopus laevis* becomes multinucleated at stage 46, when hind limb buds first appear. Axial muscle is also multinucleated in *H. boettgeri* at stage 46. Thus, the early events of both skeletal and cardiac myogenesis seem

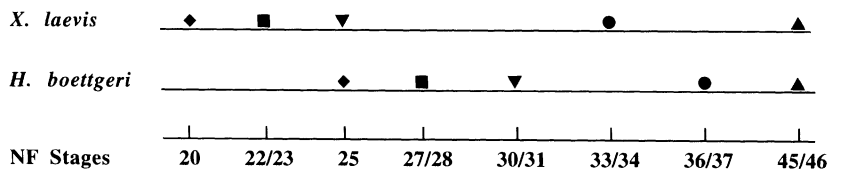

FIG. 2. Five myogenic events were examined in *Xenopus laevis* and *Hymenochirus boettgeri*: 1) the initial presence of a muscle protein in axial muscle (♦), 2) first stimulated twitch of axial muscle (■), 3) first spontaneous twitch of axial muscle (▼), 4) first heartbeat (●), and 5) multinucleation of axial myotome (▲). These developmental events occur between Nieuwkoop and Faber (1975) stages 20–46 (NF Stages). The time between stages is not linear. Early muscle development in *H. boettgeri* appears to be delayed at early stages. However, axial muscle multinucleation occurs at stage 46 in both species.

to be heterochronically delayed as measured by both absolute time and development stage in *H. boettgeri* compared with *X. laevis*, but multinucleated fibers appear at about the same developmental stage.

Muscle development is somewhat delayed in *H. boettgeri* compared with *X. laevis*, but both species have unusually early myogenesis compared with other anurans (Radice et al., 1989). One explanation proposed for early muscle function in *X. laevis* is that it is an adaptation for fast development to a free-swimming tadpole (Blackshaw and Warner, 1976; Forman and Slack, 1980). Alternatively, the pattern may arise from historical constraints in the pipid lineage. The present study shows that myogenesis in *H. boettgeri*, though occurring slightly later than in *X. laevis*, still occurs much earlier in development than in other anurans studied (Radice et al., 1989), consistent with an evolutionary conservation of myogenic timing. Examining additional pipids, and other families with faster or slower developmental rates, as well as direct and indirect developing anurans, will be necessary for a more complete understanding of the constraints on myogenic patterns.

In addition, it will be important to extend the comparison to events preceding myogenesis, including mesoderm formation and somitogenesis. Minsuk and Keller (1996) compared the cellular mechanics of mesoderm formation in *H. boettgeri* and *X. laevis* and found major differences in the origin and migration of axial and paraxial mesoderm, which includes the precursors to skeletal myoblasts. In contrast, we have found that the later sequence of myogenesis is largely the same in the two species. It will be interesting, and valuable, to compare patterns of cellular rearrangements during the intermediate steps of somitogenesis.

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Ontogenetic Shifts in Carrion Attractiveness to Brown Tree Snakes (*Boiga irregularis*)

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The brown tree snake (*Boiga irregularis*) is a nocturnal, primarily arboreal, rear-fanged colubrid native to parts of Australasia (Savidge, 1987; Greene, 1989). Throughout their range, brown tree snakes eat a variety of prey, including lizards, rats, and birds (Greene, 1989; Shine, 1991; Rodda, 1992; Rodda et al., *in press*). Brown tree snakes on Guam have a wide diet consisting mainly of lizards and lizard eggs, but a variety of other items were found in snake stomachs, including odd items such as cooked spareribs (Savidge, 1988). Savidge (1988) noted an ontogenetic shift in Guam brown tree snake diets; small snakes consumed lizards and lizard eggs and larger snakes consumed birds, bird eggs, and mammals.

Brown tree snakes were introduced to Guam in the late 1940s or early 1950s as a passive stowaway in cargo (Savidge, 1987; Rodda et al., 1992). Since the brown tree snake's introduction on Guam, its population has irrupted: population densities may occasionally reach 50–100 snakes/ha (Rodda et al., 1992). The snake has virtually extirpated the island's avifauna (Savidge, 1987), and concern that the snake will invade elsewhere has spawned intensive trapping programs (U.S. Dep. Agric., 1996).

Managers use live mouse lures in brown tree snake traps. The desire to avoid using mice has given rise to a quest for inanimate attractants for brown tree snakes (Fritts et al., 1989; Shivik and Clark, *in press*). Substances such as blood and saliva have shown promise in laboratory studies (Chiszar et al., 1992, 1993, 1997, in press), but have proven ineffective in the field (Rodda et al., 1997). Therefore, it is important to validate laboratory methods with field tests. Furthermore, previous lures based on odors associated with live mice were relatively ineffective in field trials because live prey odors require a simultaneous visual cue to attract brown tree snakes into traps (Shivik, 1998). Carrion lures produce capture rates similar to live mice lures; however, carrion does not need to be coupled with a visual cue in order to attract brown tree snakes (Shivik and Clark, 1997).

It is important to investigate thoroughly the use of carrion-based odor as an inanimate attractant prior to incorporating this technique into a management strategy. Here, we hypothesized that lure type, specifically a live or dead lure, could attract different size classes and sexes of brown tree snakes. The objective of this study was to test brown tree snakes on Guam for an ontogenetic shift in the attractiveness of carrion.

Snakes were collected during two studies on Guam. For both studies, we used wire mesh minnow traps fitted with one-way doors, and placed traps 20 m apart (Linnell et al., *in press*). Trap lines were established in forest edge along roads and trails. In traps, we enclosed lures within hardware cloth boxes ($7 \times 7 \times 20$ cm boxes of 6 mm mesh) to prevent snakes from eating lures. To minimize extraneous biological odors, we cleaned traps with a high-pressure water spray, soaked them in a 1:60 bleach: water solution for ≥two hours, and sun-dried them before placement. We ran each trap-line for two nights and each line contained 10 traps/treatment type (ordered randomly). Traps were checked every morning, and snakes were brought to a laboratory for measuring and sexing (probing hemipenes).

In the first study, we set 90 traps containing live mice, quartered dead mice, or empty control traps (10 traps per lure type in three traplines). Traps were set during April, 1997 adjacent to Tarague Beach, Guam (Shivik and Clark, 1997). For dead-mice traps, commercially purchased frozen mice were defrosted early in the day and allowed to rot in traps for two nights.

Because previous work showed that the importance of a visual cue was dependent upon whether lures were live or dead mice, we replicated an earlier study (Shivik and Clark, 1997) and collected sex and length data on captured snakes. We hypothesized that different size classes of snakes may be attracted differentially to live or dead prey (as examined in Study 1), or to visually apparent or visually obscured prey. Traps contained live mice, dead mice, live mice obscured, or dead mice obscured. Lures were obscured by wrapping their holders in black felt. Traps in the second study were set adjacent to Tarague Beach and Haputo Beach, Guam. We set 160 traps (10 traps per lure type in four trap lines) in March and 200 traps (five trap lines) during August 1997.

We examined differences in snake snout–vent length (SVL) using analysis of variance (ANOVA). In Study 1, we performed a one-way ANOVA to determine if snake size varied by lure type. Also, we used a log-likelihood chi-square to determine if captures differed by lure type and sex. In Study 2, we performed a two-way ANOVA examining the effects of a live or dead lure and a visual and odor or an odor only lure. We used a Mantel-Haenszel chi square (Kirby, 1993; Ott, 1993) to determine if male and female snakes showed differential attraction to trap lures.

In the first study, we captured 22 snakes using live mice, 14 snakes using dead mice, and two snakes in

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