

A Developmental Staging Series for the Lizard Genus *Anolis*: A New System for the Integration of Evolution, Development, and Ecology

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ABSTRACT Vertebrate developmental biologists typically rely on a limited number of model organisms to understand the evolutionary bases of morphological change. Unfortunately, a typical model system for squamates (lizards and snakes) has not yet been developed leaving many fundamental questions about morphological evolution unaddressed. New model systems would ideally include clades, rather than single species, that are amenable to both laboratory studies of development and field-based analyses of ecology and evolution. Combining an understanding of development with an understanding of ecology and evolution within and between closely related species has the potential to create a seamless understanding of how genetic variation underlies ecologically and evolutionarily relevant variation within populations and between species. Here we briefly introduce a new model system for the integration of development, evolution, and ecology, the lizard genus *Anolis*, a diverse group of lizards whose ecology and evolution is well understood, and whose genome has recently been sequenced. We present a developmental staging series for *Anolis* lizards that can act as a baseline for later comparative and experimental studies within this genus. *J. Morphol.* 269:129–137, 2008. © 2007 Wiley-Liss, Inc.

KEY WORDS: *Anolis*; lizard; evo-devo; genome; staging series

In recent years increasing attention has focused on devising a synthesis between evolutionary and developmental biology. This has led to significant advances in our understanding of the molecular bases of morphological evolution. However, as the field of “evolutionary developmental biology” has grown as a discipline, few have addressed the inextricable bonds between organismal development, population-based historical processes, and the external environment (with several notable exceptions, e.g., Colosimo et al., 2004; Beldade et al., 2005; Emlen et al., 2006; Hoekstra, 2006). This omission is in a large part based on the historic reliance of developmental biologists on the study of relatively few, evolutionarily distant “model” organisms that have been readily amenable to embryological and/or genetic manipulation, particularly within the vertebrate lineage; mainly mice, chickens, frogs, and zebrafish. While these organisms have been the focus for developmental biolo-

gists, they have few characteristics attractive to evolutionary biologists or ecologists who have traditionally focused on specious groups exhibiting great ecological and morphological diversity. Fortunately, technological advances have made modern molecular genetic techniques highly portable between species and greatly decreased our reliance on traditional model organisms. Likewise, new analytical advances in evolutionary biology and ecology now allow for more thorough analyses of the patterns and processes of evolutionary diversification. We believe that advances in these fields have made this the appropriate time for integrating population-based ecological analyses with evolutionary developmental biology. We have begun investigating the development of a classic vertebrate study system from evolutionary biology, the lizard genus *Anolis*, for which a large body of such data is already available.

Anolis comprises one of the largest vertebrate genera containing nearly 400 species. What is most striking about anoles is that they have convergently evolved nearly identical lizard communities on each of the major Greater Antillean islands: Hispaniola, Cuba, Jamaica, and Puerto Rico. Molecular phylogenetics has shown that adaptive radiations on each island have independently produced the same set of habitat specialists or “ecomorphs.” Habitat specialists tend to vary in traits such as limb length, girdle dimensions, number of sub-digital lamellae, and the dimensions of the skull, each of which is thought to be adaptive

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to the particular microhabitat the species most often inhabits (Losos and Sinervo, 1989; Losos, 1990a,b, 1994; Glossip and Losos, 1997; Irschick and Losos, 1998, 1999; Beuttell and Losos, 1999).

To date, surprisingly little is known about the developmental biology of *Anolis* lizards or of non-avian reptiles in general. Given the extensive ecological and evolutionary understanding of the anole radiations this is an excellent model system with which to study the developmental bases of evolutionary and morphological diversification. In addition, by exploiting a series of closely related species as a study system rather than relying solely on distantly related individual species, we can begin to dissect the relationship between the ultimate and proximate causes of morphological evolution within a phylogenetic context. As a first step in this direction, we present a generalized staging series for *Anolis* that will act as a baseline for later comparative and experimental studies within this genus.

MATERIALS AND METHODS

Study System

We studied the developmental ontogeny of eight species of *Anolis* from different islands and microhabitats and chose *A. sagrei* as the best developmental study system for this genus. This species breeds the most readily in captivity and can be collected in large numbers from introduced populations in Florida. Native to Cuba, the Bahamas, and elsewhere in the western Caribbean, *A. sagrei* primarily inhabits the bottom of tree trunks and the ground and thrives in disturbed areas such as hedgerows and flower gardens. The breeding season in southern Florida lasts from late March through mid-September, with a peak in late-May (Lee et al., 1989; TS. pers. obs.). Many *Anolis* species, including *A. sagrei* lay one egg from alternating oviducts every 1–4 weeks (Hamlett, 1952; Andrews and Rand, 1974; Andrews, 1985) and can store sperm for at least 7 months (Fox, 1963). Detailed descriptions of anole reproductive anatomy are available elsewhere and will therefore not be discussed here (Conner and Crews, 1980; Sever and Hamlett, 2002).

Specimen Collection

Nearly 400 embryos of *Anolis sagrei* and 600 embryos of *A. carolinensis*, *A. cristatellus*, *A. cybotes*, *A. evermanni*, *A. grahami*, *A. lineatopus*, and *A. valencienni* were examined for this study between 2002 and 2006. A breeding colony of *A. sagrei* was established from three populations in Southern Florida: one from Temple Terrace (2002, 2003), another from Coral Gables (2003, 2004), and a third near Fort Myers (2005, 2006). No gross variation was observed across this range in either the adult morphology or embryonic development. Detailed methods for the husbandry of *A. sagrei* and other *Anolis* species are described elsewhere (Sanger et al., submitted for publication). In brief, animals were held in captivity at Washington University in cages containing two to five females per male in rooms at ~27°C and 60–80% humidity, similar to the summer environment of south Florida. Eggs were collected daily and incubated at 27°C in a sealed Petri dish half filled with moist vermiculite (1:1 vermiculite to water by weight). To collect early stage pre-oviposition embryos, wild-caught and captive females were euthanized using 1% nembutal injected into the thoracic cavity, their eggs dissected from the oviduct, and embryos immediately dissected from these eggs.

Multiple embryos at each developmental stage were observed, although some stages were less frequently obtained than others. Eggs were dissected using watchmaker's forceps under a dissecting microscope while immersed in 1% phosphate-buffered-saline. Using the Hamburger and Hamilton (1951) chick embryo staging series as a guide, developmental stages were assigned as independently diagnosable units of development based on easily observable external morphological characteristics such as branchial arches, limb morphology, scale development, and body pigmentation. We avoided using quantitative measurements as a diagnostic character as is done in several other staging series (i.e., Hamburger and Hamilton, 1951; Dufaure and Hubart, 1961), until a more thorough investigation of developmental variation has been undertaken and to allow this series to be easily applied between anole species of different absolute size. We have used a simplified scheme of scale morphogenesis to avoid the need for histological staining or sectioning (Alibardi, 1996). Our simplified scheme includes the following stages: *epidermal papillae* where the epidermis takes on a wave-like appearance, *scale anlagen* where the scales appear as distinct discs, and *fully developed scales* where the scales are clearly raised from the surface and overlapping on the limbs, tail, and body.

Because early cleavage- or gastrula-stage embryos were never obtained, despite exhaustive examination of more than 60 pre-oviposition eggs, but might be in the future, we have simply numbered and named each stage with a brief description of its primary diagnostic features. We are aware that a developmental staging series represents an arbitrary division of developmental time into diagnosable units, but such a series is a necessary prerequisite for later comparisons in both experimental and descriptive studies. Variation between species has been carefully considered when characterizing the developmental stages, making this series applicable across the genus.

RESULTS

Nineteen developmental stages are described for *Anolis sagrei*. In the laboratory *Anolis* females lay eggs year round, although only embryos collected during the regular breeding season were included in this study. Following internal fertilization, early embryogenesis clearly proceeds within the oviduct in this species, and most eggs are laid at the "Early limb-bud" stage of development (Stage 4). Juvenile lizards hatch with a snout-to-vent length of ~1.6 cm, after 22–27 days of incubation. Developmental stages are illustrated in Figure 1 and described in detail below. Detailed drawings of the branchial arch region for Stages 1–6 are shown in Figure 2. The timing of each developmental stage, under the incubation condition described, is outlined in Figure 3.

Late Prelimb-Bud

Limbs: Not yet present.

Somites: 20–24; tail bud unsegmented and curving ventrad.

Branchial arches: Mandibular processes abut one another medially; maxillary processes not yet visible; branchial arches two to four present.

Eye: Lens and optic cup faint; choroid fissure open.

Brain: Slight expansion of the mesencephalon.

Fronto-nasal prominence: Blunt, extending rostral to anterior margin of the eye.

Otic vesicle: Visible, translucent.

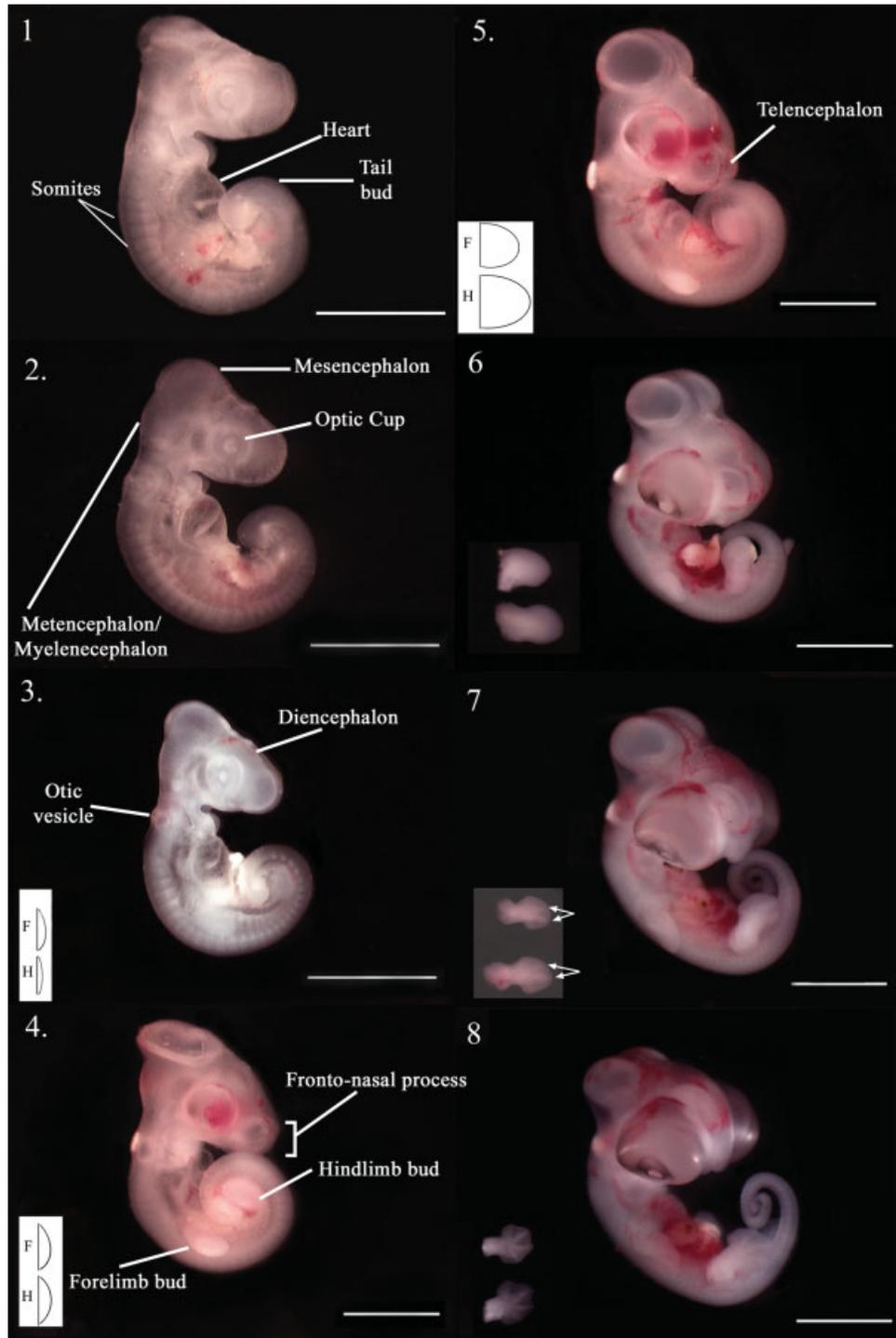


Fig. 1. Developmental staging series for *Anolis sagrei*. Stage numbers are located in the upper left of each image. Developing forelimb (F) and hindlimb (H) shown as insets in Stages 1 through 13. Scale bars = 2 mm.

Forelimb-Bud

Limbs: Forelimb-bud discernable; hindlimb-bud not yet visible except for slight thickening in flank mesoderm.

Somites: 24–28; do not extend beyond thickening of hindlimb-bud; tail bud still unsegmented.

Branchial arches: Mandibular processes about the second branchial arch but are not yet fused; mandibular processes fused medially.

Eye: Lens and optic cup more distinct; choroid fissure narrow.

Brain: Slight expansion of metencephalon/myelencephalon; first appearance of meso-metence-

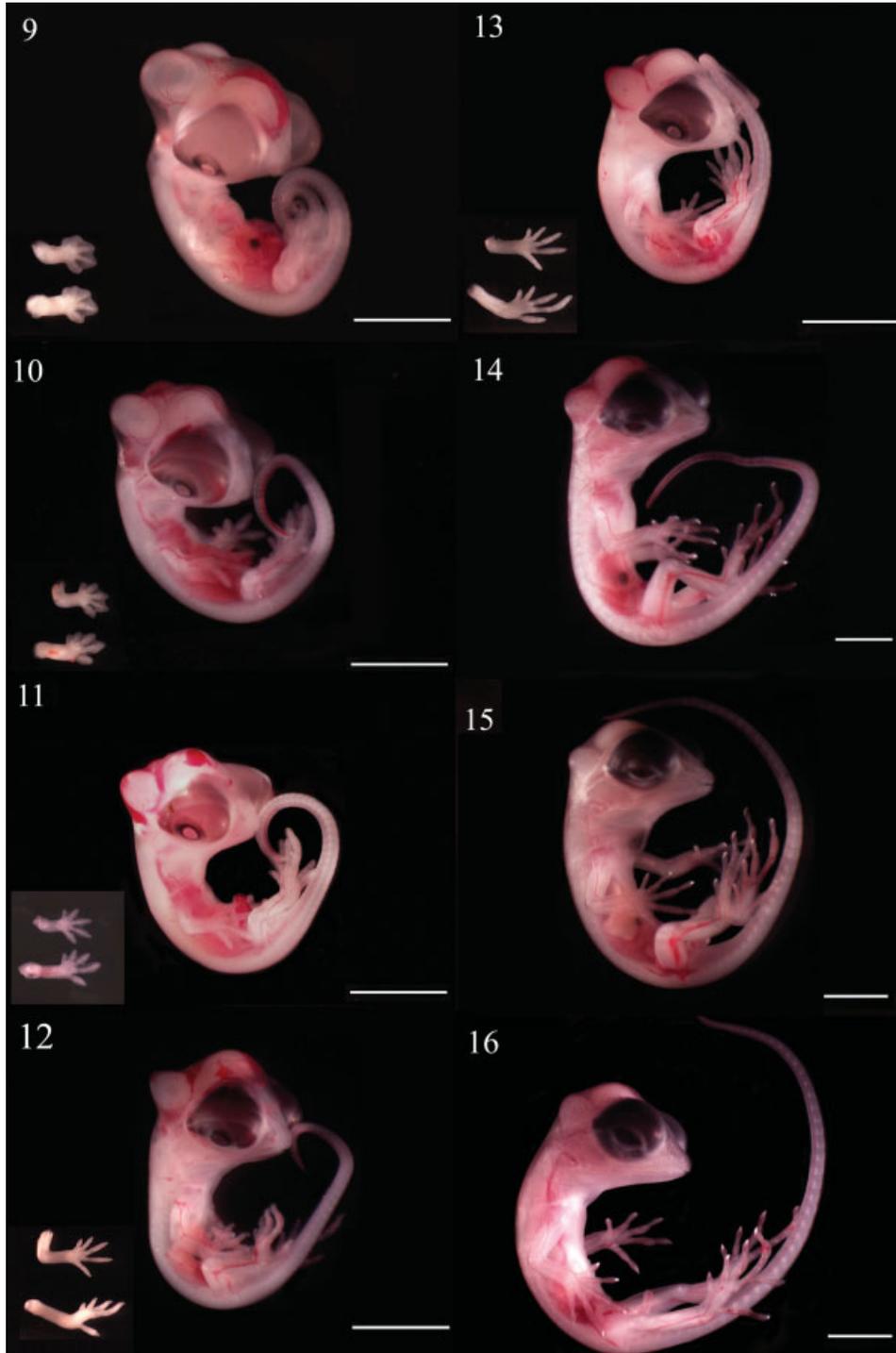


Figure 1. (Continued.)

phalic constriction; epiphysis (precursor to pineal organ) present.

Fronto-nasal prominence: Extends rostral to anterior margin of the eye.

Otic vesicle: Little change from previous stage.

Hindlimb-Bud

Limbs: Forelimb-bud slightly larger; hindlimb-bud present.

Somites: 27–30; tail bud unsegmented and curves back upon itself; somites extend beyond hindlimb.

Branchial Arches: Further medial fusion of mandibular processes; fusion of mandibular arch and branchial arch 2; maxillary process first visible.

Eye: Noticeably bulging from surface; choroid fissure closed but still visible.

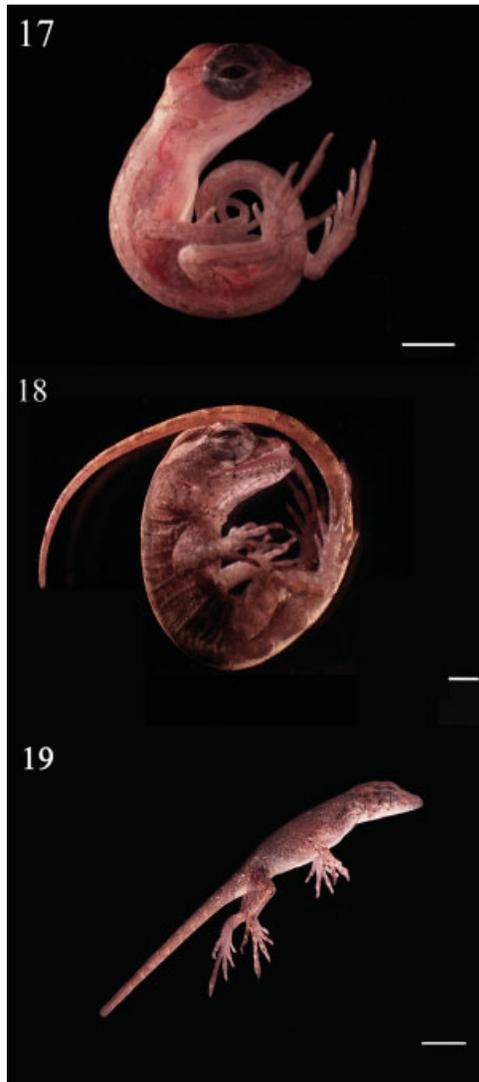


Figure 1. (Continued.)

Brain: Further enlargement of the mesencephalon, metencephalon/myelencephalon; diencephalon enlarged; telo-diencephalic, meso-diencephalic and meso-metencephalic constrictions obvious.

Fronto-nasal prominence: Slightly more elongate.

Otic vesicle: Little change from previous stage.

Early Limb-Bud

Limbs: Both limbs noticeably larger; forelimb and hindlimb-buds both about twice as long as they are wide and approximately the same size.

Somites: 29–32; tip of tail bud still unsegmented.

Branchial arches: Maxillary processes larger; medial fusion of posterior branchial arches and mandibulatory process complete; branchial arches 2, 3, and 4 partially fused.

Eye: Larger than previous stage; lens beginning to differentiate; diffuse pigment present in poste-

rior quadrant of retinal ectoderm; choroid fissure still visible.

Brain: Mesencephalon much larger than previous stage and appears hollow; telencephalic/diencephalic constriction more pronounced, metencephalon/myelencephalon slightly larger.

Fronto-nasal prominence: Little change from previous stage.

Otic vesicle: Fluid contents appear granular and white, reflective to light.

Late Limb-Bud

Limbs: Both limb-buds now wider than they are long; anterior and posterior margins of limb-buds nearly parallel; hindlimb-bud slightly larger than forelimb-bud.

Somites: More than 31; only extreme tip of tail bud unsegmented; tail curls multiple times.

Branchial arches: Maxillary process extends rostral to lens of eye and mandibulatory process; mandibulatory process extended rostrally; second and third branchial arches fused and nearly indistinguishable; fourth branchial arch not always visible.

Eye: Much larger than previous stage; retinal pigment more distinct posteriorly than anteriorly; choroid fissure faint; further differentiation of the lens.

Brain: Mesencephalon greatly enlarged; lateral telencephalic lobes first visible; epiphysis no longer visible.

Fronto-nasal prominence: Little change from previous stage.

Ear: Completely filled with granular white material.

Beyond this stage the number of somites becomes difficult to count due to the curvature of the tail and is no longer a useful diagnostic feature. Characterization of the following stages is based primarily on limb morphology, eye pigmentation, and branchial arch morphology.

Paddle-Shaped Bud

Limbs: Distal limb-bud paddle-shaped; hindlimb paddle more distinct than forelimb; forelimb flexes caudally.

Tail bud: One or two complete turns to tail; tail tip beginning to pinch off from proximal part of tail.

Branchial arches: Fourth arch rarely seen; maxillary processes clearly extend anterior to middle of eye; mandibulatory processes approximately level with center of eye.

Eye: Diffuse pigment across entire retinal surface, although noticeably concentrated at the dorso-ventral boundary of retina; early differentiation of iris; no eyelid visible; choroid fissure rarely visible.

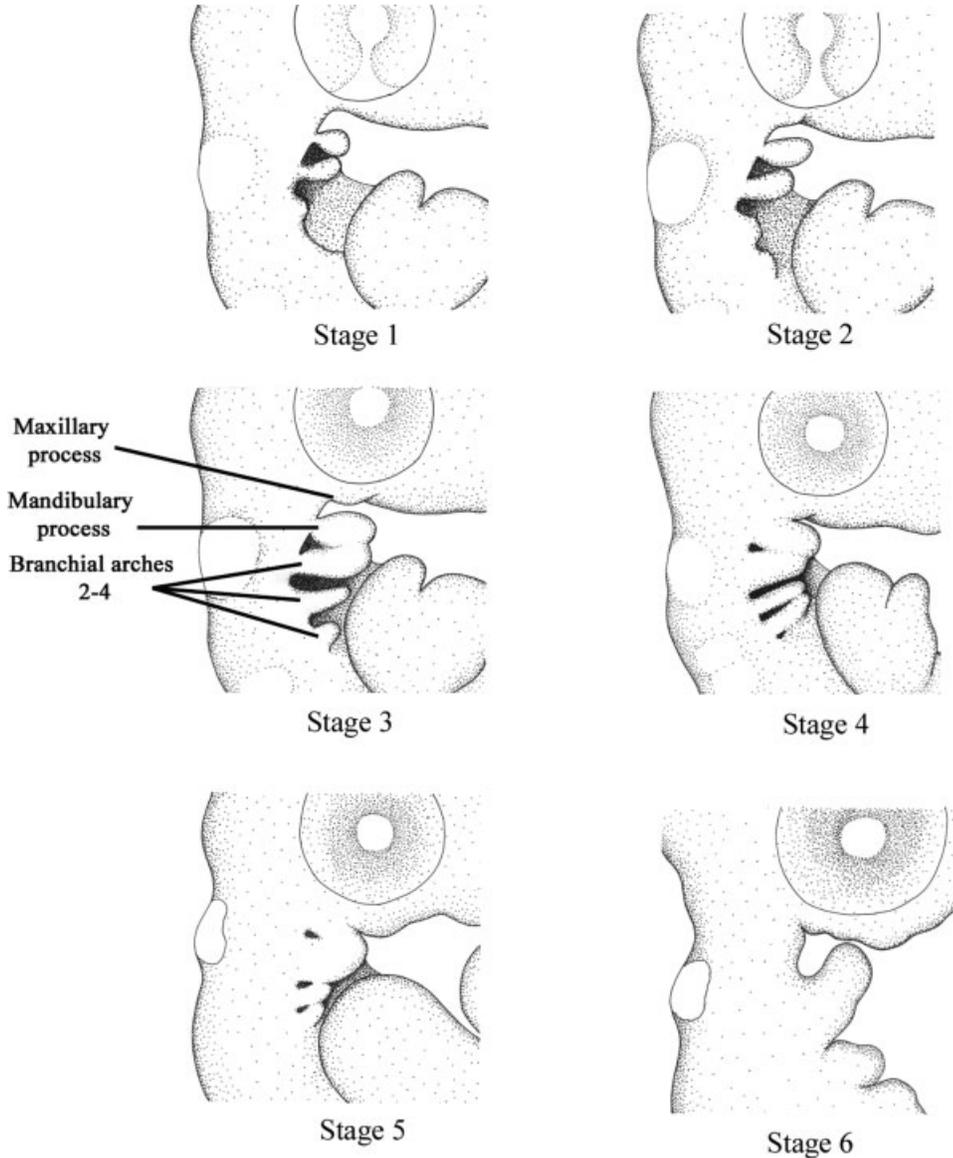


Fig. 2. Drawings of the branchial arch regions for Stages 1–6. See text for descriptions.

Brain: Noticeable expansion of telencephalon, lateral lobes distinct; further expansion of the mesencephalon.

Fronto-nasal prominence: Extends only slightly rostral to eye; medial nasal process directed caudally.

Digital Plate

Limbs: Paddles wider than previous stage; medial digit condensations visible; first recognizable proximodistal segmentation of hind limb.

Tail bud: One to three turns to tail.

Branchial arches: Maxillary processes extend to underside of medial nasal process; mandibular processes extend to anterior margin of eye; third branchial arch rarely visible; fourth branchial arch no longer visible.

Eye: Diffuse pigment across retina but more concentrated along the equator and in the iris; eyelid first visible at ventral margin.

Brain: Mesencephalon beginning to deflate; diencephalic processes larger.

Fronto-nasal prominence: Caudal to anterior margin of eye; medial nasal process points caudally.

Digital Condensations

Limbs: Long bone condensations clearly visible; condensations of all digit cartilages visible; slight thinning, but no regression, of interdigital webbing; limb joints more distinct.

Branchial arches: Maxillary processes beginning to fuse with medial nasal process; mandibular process extends rostral to anterior margin of eye

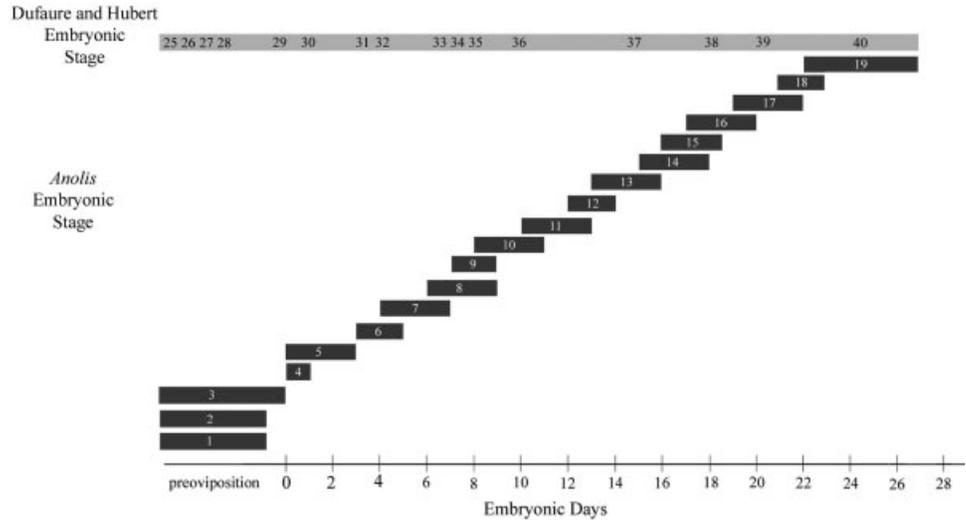


Fig. 3. Timing of developmental stages for *Anolis sagrei*.

but caudal to medial nasal process; branchial arches 2 and 3 rarely visible.

Eye: Increased pigmentation of the iris and retina.

Brain: Further regression of mesencephalon; mesencephalic lobes begin to separate laterally; diencephalic lobes bulge dorsal to level of eye.

Fronto-nasal prominence: Medial nasal process pointing anteriorly; extends to approximately anterior margin of eye.

Early Digital Web Reduction

Limbs: Distal tips of digits freed of digital webbing; digit 4 noticeably longer than other digits; limbs flexed 90° caudally at elbows; digit joints not yet obvious.

Branchial arches: Maxilla no longer distinct from medial nasal process; mandible not yet level with medial nasal process.

Eye: Increased pigmentation.

Brain: Mesencephalic lobes further deflated and separate; diencephalic lobes expanded.

Fronto-nasal prominence: Anterior tip ends in blunt point anterior to eye; nares sometimes visible.

Digital Webbing Partially Reduced

Limbs: Digital webbing receded approximately half the length of the digits; slight elongation of medial digits; digit joints sometimes present.

Jaws: Lower jaw equal in length to the upper jaw.

Brain: Further deflation of the mesencephalic lobes and diencephalon.

Eye: Eyelids covering approximately one quarter of eye.

It is at this time that the externally developing hemipeses become obvious in males.

Digital Webbing Completely Reduced

Limbs: Digital webbing fully regressed, occasionally a remnant of webbing remains between digits 2 and 3; digit joints visible; slight pinching at distal tip of digits where claws will form.

Brain: Mesencephalic lobes further separated and reduced in size relative to the rest of the head.

Eye: Darker retinal pigmentation; eyelids covering approximately half of eye.

Digital Pads

Limbs: Toe pads (precursors to lamellae) on digits, sometimes appearing on medial digits 3 and 4 first; occasional scale papillae visible; continued elongation of digits; further pinching at tip of digits, claws not yet refractive to light.

Brain: Little change from previous stage; pineal organ visible on dorsal surface.

Eye: Complete pigmentation of retina; eyelids covering three-quarters of eye.

Toe Lamellae

Limbs: Lamellae present on digital pads, sometimes appearing on medial digits 3 and 4 first; claws refractive to light.

Brain: Little change from previous stage.

Eye: Eyelid covering all of eye except iris and lens. Scales: Epidermal papillae often present on limbs; egg tooth visible.

From this point on much of organogenesis is complete and does not provide useful staging criteria. The remaining stages are diagnosed on the basis of regional scale formation and pigmentation.

Scale Anlagen

Scales: Lamellae more distinct; scale anlagen present on limbs; epidermal papillae on dorsal surface of body and head and along base of tail.

Pigmentation: Scattered melanophores around scales and scale anlagen, claws, distal tips of digits, and dorsal neck and back.

Brain: Further regression of mesencephalic lobes.

First Full Scales

Scales: Fully developed scales on limbs; scale anlagen on dorsal surface of body and head and along base of tail; occasional overlapping scales at base of tail; slight fold to eyelid.

Pigmentation: Diffuse melanophores across body and limb elements concentrated along margin of scales; no pattern discernable.

Brain: Little change from previous stage.

Fully Developed Scales

Scales: Fully developed scales on entire body; flat scales on eyelid and anterior snout discernable; increased fold to eyelid.

Pigmentation: First sign of discernible patterns on back under high magnification; melanophores on toe lamellae concentrated at distal margins.

Brain: Mesencephalic lobes reduced to small protuberances.

Pigmentation

Scales: Further development of scales, all body scales overlapping, scales of head more distinct.

Pigmentation: Patterns on back more discernible; increased density of melanophores, xanthophores and erythrophores create a reddish brown color; iridophores rare.

Brain: Little change from previous stage.

Near Hatchling

Active animal still within egg

Scales: All scales enlarged and overlapping.

Pigmentation: Expansion of pigmentation creates distinct patterns on back; iridophores more abundant creating an iridescent appearance to scales.

Brain: Mesencephalic lobes no longer visible.

Hatchling

Animal hatches from egg once yolk is fully consumed.

DISCUSSION

We have characterized 19 morphologically distinct developmental stages for *Anolis* embryos.

These stages have proven useful for standardizing comparisons between a number of diverse Caribbean *Anolis* species from a wide range of microhabitats. Although several developmental staging series have been published for squamates (snakes and lizards), ours is only the second to be published for an oviparous lizard, and the first for a species that lays single eggs rather than clutches. Series also exist for *Lacerta vivipara*, the most frequently cited series (Dufaure and Hubert, 1961; translated by Porter, 1972), *Agama impalearis* (Mouden et al., 2000) and *Calotes versicolor* (Muthukkaruppan et al., 1970). Our series approximately reflects Stages 26 through 40 of the *Lacerta* series, but more finely divides this period by paying greater attention to characters known to be important in anole biology. While there is not necessarily a one-to-one correlation between these two staging series we have attempted to relate their progression in Figure 3.

It is peculiar that we did not find embryos at stages prior to our pre limb-bud stage. Eggs were dissected from nearly 60 females collected randomly from the wild in late April of 2003. More than 80% of these females contained eggs at the early limb-bud stage of development (Stage 4). Approximately 95% of these females had at least one egg in their oviducts. An additional 10 females were dissected at 2, 3, or 4 days after their last egg with similar results. This is clearly an area in need of further detailed investigation.

Most embryonic variation in *Anolis sagrei* is similar to that found normally in adult lizards. For example, *A. sagrei* exhibits sexually dimorphic pigmentation patterns, whereby females tend to have a medial dorsal stripe and males generally have a mottled appearance. These differences become apparent around Stages 17 or 18 and fully reflect the adult patterns by the time of hatching. Variation between species as it pertains to this staging series is limited to slight differences in the rate and timing of events such as scale development or pigmentation. There exists, however, substantial variation in the ideal husbandry and incubation conditions for particular species (discussed by Sanger et al., submitted 2006).

CONCLUSION

Anolis as an Integrative Model System

We have presented a developmental staging series for *Anolis* that can act as a baseline for future comparative and experimental studies within this genus. As discussed above, *Anolis* represents a unique vertebrate genus that offers the rare opportunity to test many generalizations about evolutionary processes. Nearly 40 years of prior research have made the adaptive bases of *Anolis* evolution one of the best understood of any vertebrate genus. Overlaying detailed research on the

development of complex morphological traits onto this understanding of ecology and evolution has the potential to create a seamless understanding of how genetic variation at the organismal level is transferred into variation within populations and across species.

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