Chapter 19

Model Clades Versus Model Species: *Anolis* Lizards as an Integrative Model of Anatomical Evolution

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Abstract

Anolis lizards, known for their replicated patterns of morphological diversification, are widely studied in the fields of evolution and ecology. As a textbook example of adaptive radiation, this genus has supported decades of intense study in natural history, behavior, morphological evolution, and systematics. Following the publication of the *A. carolinensis* genome, research on *Anolis* lizards has expanded into new areas, toward obtaining an understanding the developmental and genetic bases of anole diversity. Here, we discuss recent progress in these areas and the burgeoning methodological toolkit that has been used to elucidate the genetic mechanisms underlying anatomical variation in this group. We also highlight the growing number of studies that have used *A. carolinensis* as the representative squamate in large-scale comparison of amniote evolution and development. Finally, we address one of the largest technical challenges biologists are facing in making *Anolis* a model for integrative studies of ecology, evolution, development, and genetics, the development of ex-ovo culturing techniques that have broad utility. Ultimately, with the power to ask questions across all biological scales in this diverse genus full, anoles are rapidly becoming a uniquely integrative and powerful biological system.

Key words Eco-evo-devo, Evolution, Macroevolution, Dimorphism

1 Introduction

Deep in El Yunque, the expansive Puerto Rican rainforest, a green lizard sits perched on a broad, green leaf around eye level to a human. The lizard's body proportions, such as its limb length, dimensions of its adhesive toe pads, and tail length, have become optimized over the course of its evolutionary history for living in this particular part of the forest canopy. Higher up, another lizard species four times the first lizard's size moves slowly through the branches searching for fruits and insects to consume, its body proportions optimized for this part of the arboreal canopy. Another species occupies the base of the tree, another the small twigs and vines, and another still is found within the grasses along the forest's edge. In each case the species' body proportions are distinct from

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those living in other parts of the forest, each well adapted to its unique place of the habitat (Fig. 1). Similar scenarios to this play out across the islands of the Greater Antilles: Jamaica, Hispaniola, Cuba in addition to Puerto Rico. Strikingly, all of the lizards in the scene described above are the members of the genus *Anolis*, or anoles as they are commonly referred. Examination of the evolutionary history and ecology of the lizards on each island and comparisons of the species between islands have fascinated biologists for decades [1]. But, more recently, a new community of researchers has also began investigating the developmental, genetic, and genomic bases of anole diversity. Rather than a single anole species becoming molded into another clichéd "model species," this community is using anoles as a model clade, capable of testing evolutionary hypotheses in a rigorous comparative phylogenetic framework. The community has developed the ability to move across evolutionary scales, from comparisons within species, to comparisons among sister groups on different islands, to comparisons among distantly related clades. Below we highlight the way that this phylogenetic approach has advanced the study of anatomical evolution and several of the technical challenges that our community needs to overcome to continue moving forward with the development of *Anolis* as an integrative model clade of morphological evolution.

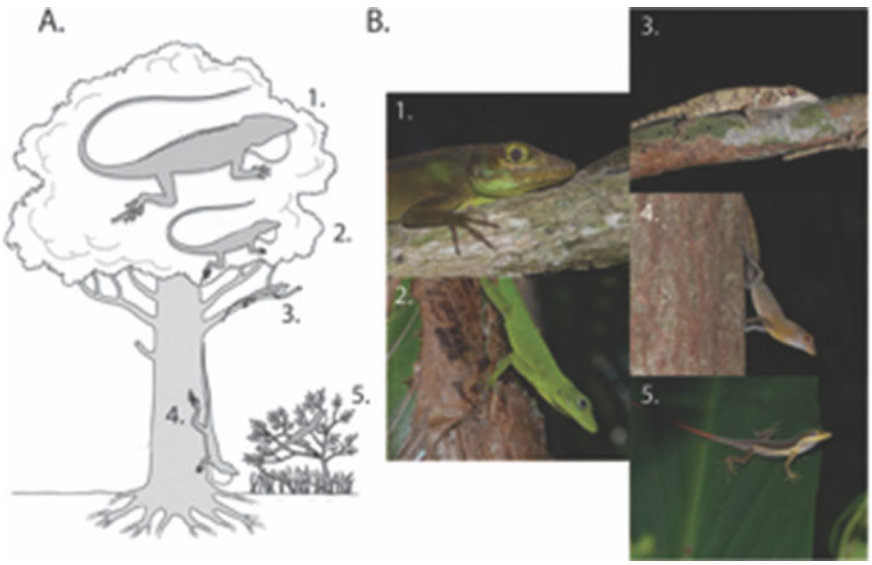

Fig. 1 (**a**) A schematic of the Puerto Rican anole community. Note the differences in body size and the relative position of species across the forest canopy. Tree modified from Losos [1]. (**b**) Representatives of the Puerto Rican habitat specialists: (*1*) *A. cuvieri*, crown-giant; (*2*) *A. evermanni*, trunk-crown; (*3*) *A. occultus,* twig; (*4*) *A. gundlachi*, trunk-ground; (*5*) *A. krugi*, grass-bush. Habitat specialists are named for the microhabitat most often inhabitiated by the species

1.1 Anolis Lizard Diversity and Evolution

Anolis is a group of approximately 400 species distributed throughout the Caribbean, Central and South America, and the southeastern United States, but it is the anoles of the Greater Antilles that have received the most attention by biologists [1]. On each of the four islands a suite of distinct habitat specialists independently evolved by dividing the arboreal habitat based on perch height and perch width. The near perfect convergence in body proportion, color, body size, and behavior among species living in similar microhabitats on different islands is remarkable. Phylogenetic analysis of these species has repeatedly confirmed that this similarity is due to convergence and not common ancestry [2–4]. The evolutionary history of this group has, therefore, created a natural experiment with which to test the predictability of evolution at different biological levels.

Many fields in biology are driven forward by new technologies that can advance research areas or open up entirely new areas of investigation. The creation of these resources often requires great financial, intellectual, and personnel investment in a single species, such as *Danio rerio* "the" zebrafish, *Mus musculus* "the" mouse, or *Drosophilla melanogaster* "the" fly. Although other vertebrate lineages have well-established model organisms, squamates (i.e., lizards and snakes) have never had a representative species become a model for developmental or genetic studies (although there was a wealth of descriptive embryology performed during the mid-twentieth century [5–7]). Following the publication of the *A. carolinensis*, the green anole, genome [4], it can be argued that this species became the first squamate species with the potential of being developed for experimental embryology and functional genomics (i.e., knock-down or overexpression studies). The genome sequence opened up the possibility for investigators to more readily clone genes for expression analysis [8, 9], analyze coding and noncoding DNA sequences [8, 10, 11], and provide the needed out-group to polarize genomic comparisons across amniotes [4, 8, 12, 13]. A number of trait-specific descriptive developmental studies [14–16] of the green anole and its relative *A. sagrei,* the brown anole, have also followed since this the publication. With the advent of new tissue and ex-ovo embryo culturing techniques [8, 17, 18] (*see* below), the anole community is poised to make the next step toward establishing the ability to functionally test gene function, not just examine gene expression.

Following from the previous decades of research, one of the greatest intellectual strengths of *Anolis* lizard is providing a framework for testing evolutionary hypotheses at different phylogenetic scales: within species, among closely related anole species, and as a representative squamate for comparisons among distantly related amniotes. We have organized our discussions to emphasize this evolutionary hierarchy.

1.2 Variation Within Species: The Evolution of Sexual Dimorphism

One of the most striking features of anoles is the variable level of sexual dimorphism observed throughout the genus. In many species males are larger in body size, possess relatively longer limbs and larger head dimensions, and have an extensible, colorful throat fan called a dewlap that is used in communication [19–22]. In other species males and females are nearly indistinguishable without examining the genitalia. Thus, one of the discrete strengths of using the genus *Anolis* to examine the developmental bases of sexual dimorphism is the ability to drill deep into developmental bases of dimorphism in the green anole model and then compare these findings among closely related species with variable levels of dimorphism (a similar approach has also been applied to studies of neuromuscular reproductive physiology [23, 24]).

A general belief that transcends biological disciplines is that male-biased dimorphism—whether in size, shape, color, or behavior—is associated with sexual differences in testosterone. Within anoles there appears to be a correlation between male body size and levels of circulating testosterone [25]. Testosterone stimulates male growth and, in turn, increases the degree of dimorphism observed. Conversely, in squamate species with no dimorphism or female-biased dimorphism, testosterone may inhibit male growth allowing for the exaggeration of female size. The flexible role of testosterone on organismal growth has been termed the "bipotential regulation hypothesis" [25].

The developmental basis of tissue-specific shape dimorphism in anoles does not readily fit within this paradigm. While males and females of many anoles species have subtle differences in facial length, two lineages have reached extreme levels of facial length dimorphism, surpassing two standard deviations from the average value [22]. One of these lineages reaches extreme levels of dimorphism through modification to a widespread, likely ancestral, mechanism whereby male and females diverge early in growth and maintain those differences throughout life (Fig. 2). The other lineage has evolved a novel mechanism not observed elsewhere in the genus. In this novel strategy, males and females do not diverge until sexual maturity and continue to diverge throughout their reproductive life. Rather than changes in androgen signaling, this novel growth mechanism appears to be associated with changes in estrogen signaling, particularly at the level of estrogen receptor beta [9]. There were no differences in the expression of androgen receptor or its complimentary molecules in the diverging facial tissues. Ultimately, use of the comparative developmental analyses of dimorphic characters in anoles has the potential to add greater resolution to the mechanisms by which mosaic patterns of dimorphism arise and evolve. Examination of males and females of a single species or of a single sex among many species will never offer the same explanatory power.

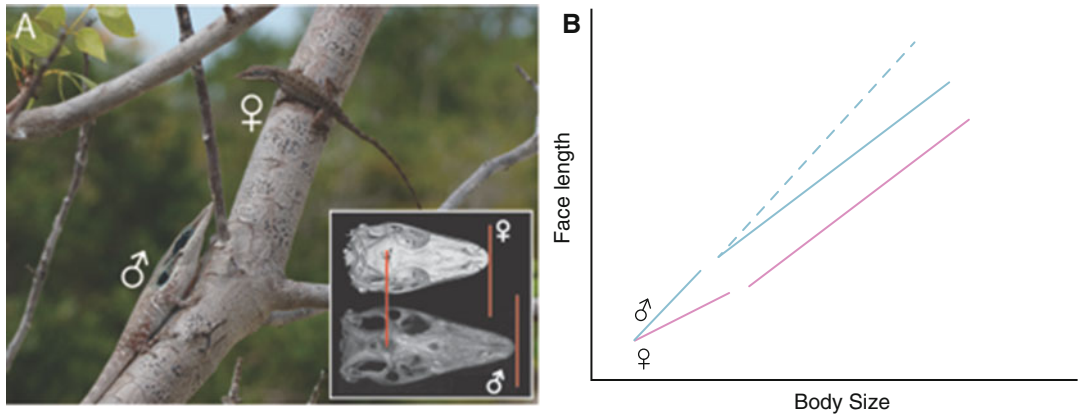

Fig. 2 (**a**) Male *A. brunneus* have much longer faces than their female conspecifics. (**b**) Differences in face length arise early in development in many anole species (*solid lines*). However, extreme levels of facial length dimorphism in the *carolinensis* clade are reached by a novel mechanism observed only in this lineage, late divergence of males and females (*dashed line*)

1.3 Variation Among Anolis Species: The Developmental Bases of Anole Diversity

One of the objectives of twenty-first century evolutionary biology is to understand the evolutionary processes that coordinately shape genomic, developmental, and phenotypic variation [26]. The phenotypic convergence of lizards living in similar habitats across the Greater Antilles provides a powerful model to assess the relative predictability and contingency of evolution at different levels of the biological hierarchy (tissues, cells, gene expression, nucleotide, etc.). Investigators have yet to uncover the genes responsible for the anatomical diversification of *Anolis* lizards, presumably because of the lack of molecular technologies and because distantly related species are not readily amenable to genetic mapping crosses. Despite those caveats, progress has been made toward understanding the developmental *processes* contributing to morphological divergence and convergence for several traits.

Relative limb length (limb length proportional to body size) is one of the most important morphological traits for conferring anole species their habitat-specific performance capabilities [1]. Despite the many ways limb length variation could be generated—through patterning, differential pre-hatching growth, or differential post-hatching growth—Sanger et al. [27] found that the divergence in limb length on each island consistently occurs through developmental modification to limb bud patterning, before the formation of the cartilaginous anlagen. This deep conservation of the processes generating evolutionarily relevant variation is a similar pattern as described for the widespread, potentially ancestral mechanism that underlies craniofacial dimorphism in *Anolis* [22].

Without formal phylogenetic analysis one might conclude that the same developmental processes have been independently

recruited on each island to generate the variation observed among anoles. Although this remains a formal possibility, the alternative hypothesis is that the same generative mechanisms underlie the production of limb length variation across the radiation of anoles because they were inherited from their common ancestor. Under this scenario, the successive speciation events never erased that ancestral signature of *variation*, the actual fodder of natural selection. Repeated selection on limb length, therefore, recruited that same developmental mechanisms because of this ancestral signature, not because of those developmental processes offered something unique to selection relative to its other options. This pattern is consistent with Vavilov's Law of Homologous Series, which explained that homologous traits will exhibit "parallel variability" among closely related species [28]. In other words, closely related species tend to vary along similar dimensions not by chance, but because of their shared history. The challenge for the next round of biologists to investigate these traits will be to accurately relate changes at the genotypic level to the previously described patterns at the developmental and phenotypic levels. These researchers must sample deeply enough to tease apart the effects of lineage-specific mutations, molecular convergence across islands, and latent genetic polymorphisms inherited from their common ancestor.

1.4 Variation Among Amniotes: Anole as a Representative Squamate

Anolis lizards have provided an important benchmark for researchers interested in the evolution of amniotes. For later part of the twentieth century researchers drew conclusions about the developmental evolution of amniotes based on comparisons between the chick and mouse model systems, not allowing for objective determination of the amniote ancestral condition. As the utility of anoles has grown, they have been used to polarize comparisons among distantly related amniote lineages in studies of heart development [14, 29], tail regeneration [15, 30, 31], longitudinal body axis formation [12, 13], and external genital (i.e., phallus) development [8, 10, 32]. Reviewing the findings across this diversity in structures is beyond the scope of this chapter, but we briefly highlight the significance of anoles in our understanding of external genital evolution because of the collaboration of one author (TJS) with several studies in this area.

Among amniotes there is a tremendous degree of variation in the adult anatomy of external genitalia, which has confounded the accurate interpretation of its evolutionary history [32, 33]. Whether a phallus evolved once at the origin of amniotes or several times independently in distinct amniote lineages remained an open question. The answer to this lingering question came not through technical advances, but through detailed comparative embryology. By close examination of squamate (including *Anolis*), alligator, avian, and mammalian phallus embryology, it was found that in spite of the confounding variation in adult anatomy, all amniote

phalluses have a common embryological origin as paired swellings flanking the cloaca [16, 34–38]. Even species that have secondarily lost their phallus maintain this embryological signature [33, 38], suggesting that the phallus evolved once and was later modified within each of the amniote radiations.

The developing limb buds and genital tubercle, the developmental precursor to the phallus, express many of the same genes [8, 39]. Recent developmental studies comparing gene expression and gene regulation across amniotes have raised questions about whether these shared expression profiles arise because of a shared embryological (i.e., cellular) origin of limbs and external genitalia or because the limbs and phallus share a common cis-regulatory landscape associated with appendage development [8, 10]. Use of the green anole and its genome have also been critical for polarizing the comparisons of transcriptomic and regulatory profiles between the limbs and external genitalia among evolutionarily distant lineages of birds, mammals, and reptiles.

Since the publication of the green anole genome several other squamate genomes have been completed, although they are not all annotated at this time [40–43]. These additional sequences will improve our power to dissect amniote genome evolution and to more precisely determine the conservation and flexibility of gene regulatory elements. Evolutionary-developmental biology has, however, moved beyond correlative studies at the sequence and expression levels. The field has emphasized the need to experimentally validate those differences. Reaching this level of experimental rigor is likely the largest hurdle facing the anole community in coming years.

1.5 Raising the Bar: Can Functional Genomics Be Performed on Anolis Lizards?

Gene function can evolve. Genes can also have multiple functions in one organ or distinct functions in different organs in the same species. Therefore, differential expression analysis is not adequate as the only source of information to define the underlying causes of anatomical evolution. Likewise, sequence analysis cannot alone determine when and where genes are expressed as spatiotemporal differences in gene expression are often due to changes in gene regulation, not changes in protein-coding regions [44]. One of the hallmarks of the most successful studies in evolutionary-developmental biology has been the functional validation of expression differences.

Functional validation can be performed using a range of experimental methods. In genetic systems such as *Mus*, *Danio*, and *Drosophila* functional analysis has been done with true knock-out and knock-in experiments where segments of the genome are removed, added, or edited. The opportunities for these experiments have significantly expanded with the recent advances in CRISPR-Cas9 genome-editing technology in both model and non-model species [45, 46]. In species that are not amendable to

genetics, knock-down and up-regulation experiments are possible using pathway-specific small molecule inhibitors, electroporation of expression vectors [17, 47], or through the implantation of protein-soaked beads. In each of these cases, however, the critical hurdle to overcome is access to the developing embryo. In chicks, for example, the egg can be windowed and the embryo can be cultured in its natural shell until hatching. Squamates possess leathery eggs with high turgor pressure limiting our ability to window the egg without damaging the embryo. Although the utility of these experimental techniques could be extended to anole embryos in principle, the anole community's first hurdle will be to find reliable culturing techniques that allow long-term access to embryonic tissues.

Several published attempts have been made regarding culturing protocols for anole embryos and their cells (with varying degrees of experimental detail). Transfection of expression constructs into micromass cultures may prove to be a powerful tool to examine the conservation gene regulatory networks in anoles [17]. Park et al. [17] described micromass culturing for cells derived from early limb buds. For these experiments cells from the limb buds were disaggregated using trypsin, concentrated by centrifugation, and plated in a DMEM/F12 culture media. Cultured cells were incubated for up to 24 days in 5% atmospheric carbon dioxide conditions. After 2 weeks in culture differentiation of cartilage nodules were readily visible, which was slower than chicken cultures raised under the same conditions. These cultures were successfully electroporated with expression vectors driving GFP under the control of the CMV promoter and an experimental regulatory element of Pitx1. Together these results show great promise toward testing whether tissue-specific gene regulatory networks known in other more widely studied taxa are conserved in *Anolis* lizards.

Micromass cultures are ideal for testing regulatory and expression hypotheses, but cannot readily advance our understanding of anatomical morphogenesis where maintaining three-dimensional context is critical. Nomura et al. [47] described a protocol for windowing Madagascar ground gecko, *Paroedura pictus*, eggs for electroporation, but this has not been successful with *Anolis* eggs that are much smaller and under greater turgor pressure. Tschopp et al. [8] infected green anole embryos at the early stages of morphogenesis with a GFP cell-lineage tracing lentivirus using an ex-ovo culturing technique (Fig. 3a). In this experiment, partially shelled eggs were dissected from gravid females. The opaque layers of the shells were dissected away, leaving the internal membranes intact. The yolk mass and embryo were then transferred to an egg-shaped impression in dish of Nobel agar dissolved in media. The cultures were maintained in a humidified chamber at 28 °C for up to 12 days. The number of embryos that survived this incubation period was not reported, but our attempts to replicate this length of

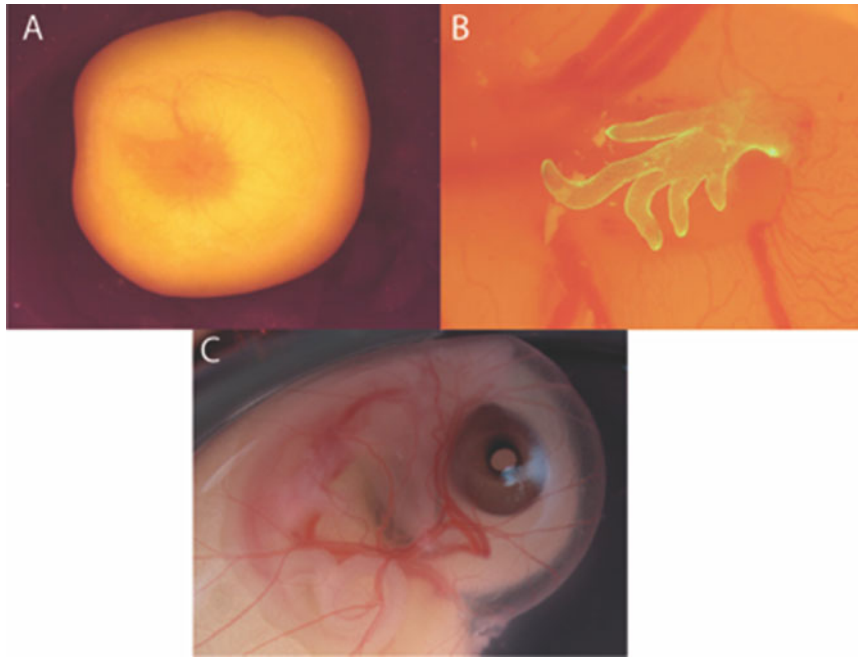

Fig. 3 (**a**) Early stage *A. sagrei* ex-ovo culture following the protocol of Tschopp et al. [8] alive after 24 h incubation. (**b**) Stage 12 *A. sagrei* limb stained with Vybrant cell labeling solution explanted in a chicken embryo after 24 h. (**c**) *A. sagrei* embryo at the air-liquid interface, incubated following the ex ovo protocol by Diaz and Trainer [18]. Embryo was incubated 12 days prior to shell removal and incubated for 24 h. Note the fully intact membranes surrounding the embryo

time have not been successful. We would also like to develop more sustainable protocols that will not require the euthanasia of gravid females. However, the use of this ubiquitously expressed GFP lentivirus is the first proof-of-concept that viral transgenesis may be a viable mechanism to manipulate the genome of anole embryos.

2 Materials

We have tested two additional ex-ovo culturing techniques that we feel have potential for advancing the experimental repertoire of anole biologists. The reagents required for the techniques described below are as follows.

- 5% Clorox bleach.
- 1× Phosphate-buffered saline (PBS).
- 2× Phosphate-buffered saline (PBS).
- Vybrant cell labeling solution (Life Technologies).
- Fertilized chicken eggs (UConn Poultry Farm, CT or Sunnyside Farms, WI).

– 1:1 DMEM/F12 (Life Technologies) with 10% calf serum, 2× pen/strep, 20 mM HEPES, 50 μM ascorbic acid.

Additional equipment needed for the protocols listed below include 12-well culture dishes, incubator, sharp #5 forceps, spring scissors with 3–4 mm cutting surface, and a Geuder perforated keratoplasty spatula (or perforated spoon depending on the manufacturer). Protocols for *Anolis* care and husbandry are described elsewhere [48].

3 Methods

3.1 Embryonic Tissue Explant

We have successfully explanted developing limbs of *Anolis* embryos onto the chicken embryo host (Fig. 3b). Before you begin with the *Anolis* egg dissection, window a chicken egg with Hamburger Hamilton stage 17–20 embryo. The protocol is as follows:

1. Sterilize anole eggs with two washes of 5% Clorox bleach (5 min in bleach solution followed by 5 min rinse, 5 min in bleach solution followed by 5 min rinse).
2. Dissect the embryo from its shell and extra-embryonic membranes while submerged in sterile PBS using #5 forceps and spring scissors. Transfer embryo to clean PBS using the spatula.
3. *Optional:* Because anole limbs are significantly smaller than chicken embryos and may be difficult to visualize, stain the anole tissue with Vybrant cell labeling solution. Dilute Vybrant following manufacturer's instructions. Incubate whole embryo for 30 min at 30 °C while rocking. Rinse twice with PBS.
4. Wound the blood vessels near the posterior of the chicken embryo using forceps or needle. Transfer the stained explant to the wounded area. The natural healing processes of the embryonic tissues lead to the establishment of a blood supply toward the anole tissue within 24 h when incubated at 30 °C (intermediate between the standard incubation temperatures of anole and chicken embryos).

3.2 Ex-Ovo Embryo Culture

We have also successfully cultured whole brown anole embryos with minor modification of Diaz and Trainor's [18] protocol used for chameleon embryos (Fig. 3c).

1. Sterilize in two washes of 5% Clorox bleach as above.
2. Dissect the opaque outer shell away from the yolk mass and embryo while submerged in sterile PBS using #5 forceps and spring scissors (*see* **Note 1**).
3. Transfer the embryo and yolk mass to a 12-well dish with DMEM/F12 growth media. Position the embryo at the air-liquid interface.

4. Incubate in normal air at 28 °C. Perform a full media change daily. We have observed a steady heartbeat for up to 10 days of incubation (*see* **Notes 2** and **3**).

4 Notes

1. From our experience, the largest hurdle for successful ex-ovo culturing protocols appears to be dissecting the opaque shell away from the yolk mass without damaging the underlying blood vessels. The success of this procedure can be very stage-specific. The inner and outer membranes appear to be most easily dissected before oviposition and after 5–7 days of incubation. At oviposition the membranes are partially adhered to one another making removal of the outer shell extremely difficult. Soaking the eggs for 2 min in 2× PBS may help to separate the inner and outer membranes, but is not always necessary.
2. The embryos readily move within their membranes. The embryos appear hardy when remaining enclosed within their membranes, but we have observed membranes rupture several times.
3. The embryos develop at a pace slightly slower than a normal.

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