Title: Evolution of embryo behavior: heterochrony of cued hatching mechanisms
Keywords: adaptive embryo behavior, phenotypic plasticity

Background: Adaptive phenotypic plasticity is mechanistically complex, requiring systems for sensing cues and producing different phenotypes, plus pathways that link information use to trait expression [9, 10]. Even within the egg, animals sense and respond to environmental conditions; environmentally cued hatching (ECH) is widespread and particularly well documented in amphibians [10,11]. Heterokairy is plasticity in rate, sequence, or timing of developmental events – an individual-level parallel to evolutionary heterochrony [5]. Both individual and evolutionary shifts in developmental timing of mechanisms enabling ECH might alter embryos' ability to use such plasticity to escape threats or exploit opportunities. The red-eyed treefrog (*Agalychnis callidryas*, Phyllomedusinae), is a well-studied case of ECH; embryos hatch early to escape snake attack, flooding, and other threats [4,6-8]. Recent work has revealed that these embryos hatch using two different types of hatching gland cells (HGCs), that develop at different stages, and rapid, regulated release of hatching enzymes [1, Cohen et al. unpublished]. One of *A. callidryas'* HGCs is similar to the single type described in other anurans, but neither multiple HGC types, nor regulated bulk enzyme release have been previously documented in amphibians. *A. callidryas'* flooding-induced, hypoxia-cued hatching response begins when the first HGC appear, but attack-induced, physical disturbance-cued hatching begins later, when inner ear mechanoreceptors begin to function (Warkentin, Jung, **Güell**, Kim, & Cohen, ms. in prep.; Jung et al. unpublished).

Comparative studies of phyllomedusine treefrogs found similar escape-hatching responses to hypoxia across species, but striking differences in responses to predators; most species tested show strong, effective embryo self-defense while others show minimal response to snakes [2,3]. However, the causes of these differences are unclear. **To elucidate how and why closely related species exhibit diverse phenotypic responses, I propose to conduct a comparative analysis of the ontogeny of mechanisms underlying adaptive plastic hatching in phyllomedusine treefrogs.** A mechanistic comparison of the well-studied *A. callidryas* with (a) two syntopic species (*A. spurrelli* & *A. saltator*), which both develop faster and have much worse escape success, and (b) a basally branching phyllomedusine (*Cruziohyla calcarifer*), may be particularly informative in assessing the evolution of mechanisms of hatching plasticity [2,3].

Proposed Research: For my Ph.D., I plan to work with Dr. Karen Warkentin at Boston University and conduct field research in Panama (Smithsonian Tropical Research Institute; STRI) and Costa Rica (La Selva Biological Station and Costa Rican Amphibian Research Center), focusing on four species of phyllomedusine treefrogs: *A. callidryas*, *A. spurrelli*, *A. saltator*, and *C. calcarifer* to address the following questions: **(1) What differences in mechanisms of cued hatching cause interspecific variation in escape success in snake attacks?** *Hypothesis: I predict that variation in escape success stems from differences in the timing of development of otic mechanoreceptors and HGCs.* Specifically, species with high escape success develop ear function soon after HGCs appear, long before spontaneous hatching; those with low success develop ear function later, near or after spontaneous hatching.

Methods: *Escape hatching function:* First, I will conduct mechanosensory and hypoxia-cued hatching experiments on developmental series of individual embryos to measure the natural pattern of onset of each response type in *A. spurrelli, A. saltator*, and *C. calcarifer*, to compare with similar data on *A. callidryas* embryos that I helped collect during my 2015 REU (Warkentin et al., ms. in prep.). *Otic mechanosensors and HGCs*: I will use scanning electron microscopy

(SEM) to examine the ontogeny of appearance of HGC types across species. I will measure the roll-induced vestibulo-ocular reflex (VOR) of embryos, in which eyes roll counter to body roll based on vestibular sensory input, to assess the development of otic mechanosensory ability across species. Based on my VOR results, I will use confocal fluorescence microscopy with phalloidin staining to visualize inner ear and mechanosensory cell development at key stages.

(2) How did ontogenetic changes in mechanisms enabling hatching plasticity evolve? *Hypothesis: I hypothesize that overall changes in development rate differentially affect the ontogenies of HGCs and mechanosensors.* If rate changes affect hatching ability more than ear development, accelerating development could, as a byproduct, increase periods when hatching-competent embryos cannot perceive mechanosensory cues. This could occur as intraspecific heterokairy (e.g. thermal effects on development) and interspecific heterochrony.

Methods: *Thermal manipulations of development rate*: To test if differences in timing of onset of hatching responses to different cue types can be increased or decreased by changing development rates, I will warm and cool embryos. *A. spurrelli* and *A. saltator* develop faster than syntopic *A. callidryas*. Thus, I will experimentally cool *A. spurrelli* and *A. saltator* and warm *A. callidryas* clutches to generate similar rates, or reversed rate differences, then assess their ontogeny of hatching responses to mechanosensory and hypoxia cues, compared with controls developing at typical temperatures.

Intellectual Merit: My research will elucidate the evolutionary changes in sensory and hatching mechanisms underlying functionally important interspecific differences in embryo behavior. This information will improve our understanding of the relationship between individual heterokairy and evolutionary heterochrony and test if desynchronized changes in development rate across traits explain large shifts in performance. It will highlight how multiple ontogenetic changes interact to influence the capacities of animals to respond adaptively to environmental conditions during highly vulnerable early life stages. Moreover, if functionally integrated performance traits can be temporally decoupled by thermally altered development rates, climate warming may impact ontogenetic adaptations and survival of early life stages.

Broader Impacts: Phyllomedusine treefrog embryos are charismatic and their self-defense mechanisms have great appeal for scientific training and public outreach. My REU work fostered strong relationships with researchers at Boston University (BU) and STRI, with whom I plan to work in existing and new outreach programs. I will disseminate my findings bilingually in open-access peer-reviewed journals and at English-language and Latin American scientific conferences to provide access for a broad group of STEM educators and fellow researchers. As a Latino, I have a strong desire to use my bilingualism and prior teaching experiences to formulate an authentic bicultural and bilingual outreach program for Hispanic K-12 students in Boston and Central America to stimulate interest in STEM fields using my work and experiences as examples to students. This NSF Graduate Research Fellowship will empower me to mentor STRI-REU students and other STRI-Interns, prioritizing underrepresented minorities from the U.S. and students from various Latin American countries during each of my field seasons. In addition, I intend to develop a cooperative online-outreach program with science-journalism students at BU to help increase the availability of accurate, engaging, and multilingual information about my study organisms using websites, video interviews, and public articles.

References: [1]Cohen et al. (2016) *J Exp Biol* [2]Gomez-Mestre et al. (2007) *Oecologia* [3]Gomez-Mestre et al. (2008) *Ecol Monogr* [4]Rogge & Warkentin (2008) *J Exp Biol* [5]Spicer et al. (2003) *Zoology* [6]Warkentin (1995) *Proc Natl Acad Sci USA* [7]Warkentin (2000) *Anim Behav* [8] Warkentin (2002) *Physiol Biochem Zool* [9]Warkentin (2007) *J Exp Biol* [10]Warkentin (2011) *Integr Comp Biol* [11]Warkentin (2011) *Integr Comp Biol*